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Odontogenic differentiation of dental pulp stem cells by glycogen synthase kinase-3β inhibitory peptides

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Abstract

Background To investigate the effects of peptide-based substrate competitive inhibitors of GSK-3β (GSK-3βi) on promoting odontogenic differentiation of human dental pulp stem cells (hDPSCs).

Methods The biocompatibility and proliferation of hDPSCs treated with GSK-3βi peptides (pS9, LRP 6a, L803, and L803-mts) were evaluated using the tetrazolium reduction assay and cell counting kit-8 assay, respectively. The differentiation of hDPSCs following peptide treatment was determined using the alkaline phosphatase assay (ALP), calcium mineralization (alizarin red staining), and quantification of mRNA expression of differentiation markers via quantitative real-time polymerase chain reaction. The accumulation of β-catenin in the nucleus of GSK3-βi-treated hDPSCs was determined using immunofluorescence staining. The effect of peptide treatment on hDPSC migration was characterized using the transwell assay.

Results All tested concentrations of the peptides were found to be biocompatible with the hDPSCs, with no significant difference compared to the control ($p > 0.05$). The peptides had no effect on the proliferation of hDPSCs compared to the control ($p > 0.05$). However, all the tested peptides significantly increased ALP activity and calcium deposition in a dose-dependent manner ($p < 0.05$). Specifically, L803-mts showed significantly greater ALP activity and mineralization compared to the other peptides and the controls ($p < 0.05$). Additionally, L803-mts showed a significant increase ($p < 0.05$) in the expression of DSPP, DMP-1, Runx-2, along with increased protein expression of DSPP and DMP-1 compared to the control. Furthermore, it enhanced the nuclear translocation of β-catenin and increased the chemotactic migratory potential of hDPSCs.

Conclusions L803-mts, a peptide-based substrate competitive inhibitor of GSK-3β, enhanced the odontogenic differentiation of hDPSCs by activating the Wnt signaling pathway.

Keywords Dental pulp stem cells, GSK inhibitors, Wnt signaling, Peptides

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Background

Dentine, a composite of organic and inorganic constituents, serves as a protective barrier for the pulp [1]. Prolonged insults, such as dental caries, can compromise dentin integrity, leading to pulpal injury characterized by tissue damage and pulpal inflammation resulting from microbial invasion that overwhelms the innate reparative capacities of the pulp [2, 3]. To maintain the vitality of the dental pulp, Vital Pulp Therapy (VPT) is a commonly employed clinical procedure where biocompatible and bioactive materials are used to "seal" the exposed pulp. Traditionally, calcium silicate-based materials, such as mineral trioxide aggregate (MTA) were utilized for this purpose [4, 5]. However, there are several disadvantages associated with the use of MTA, such as long setting time, difficult handling characteristics, discoloration, and high cost. Furthermore, the use of synthetic materials poses challenges in achieving restoration of a complete and regular dentine barrier [6, 7].

As a result of microbial invasion compromising the dentin barrier, the vitality of the pulp is impaired, prompting the activation and differentiation of stem cells in the dental pulp into odontoblasts, which subsequently secrete tertiary or reparative dentin [8, 9]. To achieve dentin repair and regeneration, novel approaches have explored biological therapies that augment the intrinsic reparative process of the dental pulp [10]. The Wnt signaling pathway, a crucial molecular pathway involved in tissue repair, including the pulp, plays a significant role in this regard [1, 6, 7]. Within this pathway, the cytoplasmic regulator, Glycogen Synthase Kinase (GSK)-3β enzyme, serves as a key determinant, controlling the activation or suppression of the Wnt pathway based on the binding of Wnt ligand to its receptor [10]. When the Wnt ligand is absent, GSK-3β phosphorylates β-catenin complexed with Axin-2, leading to its ubiquitination and subsequent degradation. Conversely, in the presence of the Wnt ligand, GSK-3β activity is inhibited, enabling β-catenin to translocate into the nucleus and initiate the expression of target genes responsible for cellular differentiation and repair [6, 7].

Small molecule Wnt agonists, Tideglusib (4-Benzyl-2-(naphthalen-1-yl)- [1, 2, 4]thiadiazolidine-3,5-dione), BIO (2′Z,3′E)-6-Bromoindirubin-3′-oxime), and CHIR99021(6-[[2-[[4-(2,4-Dichlorophenyl)-5-(5-methyl-1 H-imidazol-2-yl)-2pyrimidinyl]amino]ethyl] amino]-3-pyridinecarbonitrile), have shown promising results in inhibiting the GSK-3β enzyme and promoting the development of a robust tertiary dentin barrier in a rat model [7]. Tideglusib, originally developed for Alzheimer's disease, has demonstrated favorable outcomes in dentin regeneration, with Wnt agonists-treated exposed pulps exhibiting greater thickness and mineralization compared to traditional pulp capping materials like MTA [6, 7]. The exploration of these notable GSK3 small molecule inhibitors has paved the way for further advancements in dentistry, providing opportunities to investigate a variety of compounds with similar functions but possessing favorable physicochemical properties [11–13].

Peptide-based substrate competitive inhibitors are another class of GSK-3β inhibitors that act as pseudo-substrates and target the GSK-3β enzyme. These inhibitors exhibit high water solubility, demonstrate high selectivity towards the GSK3β enzyme, and possess the added benefit of facile functionalization and modification surpassing the limitations posed by the small molecule-based inhibitors [14, 15]. A key benefit of peptides is their ability to elicit site-specific and time-dependent responses. This is crucial considering the temporal and dosage-sensitive role of Wnt signaling in regulating tissue equilibrium and cellular functions. In contrast, small molecule inhibitors of GSK-3β induce irreversible enzyme inhibition, potentially leading to prolonged and unintended side effects. A thorough review of literature identified four peptides which acts as a pseudo substrate to the GSK-3β enzyme: pS9, mimics the N-terminal end of GSK-3β [16, 17], LRP 6 a-motif peptide (LRP 6a), mimics the LRP6, a wnt recognition ligand on the cell wall [18], L803 and L803-mts, mimics heat shock factor-1 (HSF-1), one of the substrates of GSK-3β [14, 15]. Despite being studied for their liver and neural regenerative effects, the application of these peptide on dental tissue regeneration remains largely unexplored [19, 20]. Therefore, the aim of this study was to investigate the effect of GSK-3βi peptides on the proliferation, mineralization potential, odontogenic differentiation, and migration of human dental pulp stem cells (hDPSCs) by the activation of Wnt signaling. The null hypothesis was that the peptides have no effect on the proliferation, mineralization, odontogenic differentiation, and migration of hDPSCs.

Methods

Cell culture and characterisation

The protocol for isolation of dental pulp stem cells from human dental pulp tissue was approved by the Institutional Review Board (20221026-003-000). Caries-free maxillary third molars ($n = 5$) were collected from male/female patients aged 25–30 years old at the Oral and Maxillofacial Surgery Clinic. The human dental pulp stem cells (hDPSCs) were extracted from the pulp tissue and validated as previously described [21]. The hDPSCs were cultured in α-minimum essential medium (MEM) (Gibco, Life Technologies, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma Aldrich St. Louis, MO, USA) and 1% Penicillin (100 units/mL)/Streptomycin (100 µg/mL) solution. Cells between passages 3 and 5 were used in all the experiments. The hDPSCs were

then characterised using surface-specific markers of mesenchymal stem cells (MSC). The isolated hDPSCs were incubated with antibodies against CD73, CD90, CD105, and CD45 (Abcam, Cambridge, UK), followed by analysis with fluorescence-activated cell sorter (FACS) [22] to characterise the hDPSCs and determine the expression of the stem cell markers.

For adipogenic differentiation, 1×10^5 DPSC cells were seeded in a 12-well plate and allowed to form a monolayer. The cells were induced with adipogenic differentiation medium containing 0.1 mg/mL of insulin, 1µmol/L dexamethasone, 0.2 mmol/L of indomethacin, and 1 mmol/L of IBMX (Sigma Aldrich St. Louis, MO, USA) for 16 days. The lipid droplet accumulation was examined using oil red O staining.

For osteogenic differentiation, the hDPSCs were incubated in α-MEM supplemented with 10% FBS; 50 µg/mL Ascorbic acid; 10 mM β-glycerol phosphate, and 10nM Dexamethasone (OM, odontogenic differentiation media). The cells (1×10^5 cells/well) were seeded on 12-well plates in OM and incubated for 14 days. The mineralization of the DPSCs was observed by staining with Alizarin red, using the method mentioned below.

Biocompatibility and cell proliferation

The peptides investigated in this study, pS9, LRP 6a, L803, and L803-mts, were obtained from GL Biochem, Shanghai, China. The purity of the peptides was assessed using high-performance liquid chromatography, and the molecular weight of the peptide was determined using mass spectrometry. The amino acid sequence and the special features of each peptide have been tabulated (Additional file 1). The biocompatibility and proliferation of hDPSCs treated with the peptides were assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) metabolic activity assay and cell counting kit-8 (CCK-8), respectively. Briefly, hDPSCs (1×10^4/well) were seeded on sterile tissue culture-treated 96-well plates containing α-MEM and incubated at 37℃, 5% CO_2 for 24 h to form a monolayer. The cells were then treated with varying concentrations of the peptides for 24 h at 37℃ under 5% CO_2. To assess the biocompatibility, adherent cells on the well plate were incubated with 0.5 mg/mL of MTT solution for 4 h to form formazan crystals. The formazan crystals were solubilized with dimethyl sulfoxide (DMSO) and the absorbance was measured at 570 nm using a microplate reader. The obtained OD values of the peptide-treated hDPSCs were normalized to the control. Cell proliferation assays were performed on days 1, 3 and 7 using the CCK-8 kit (Sigma Aldrich St. Louis, MO, USA). The cells were incubated with CCK-8 for 3 h at 37℃, 5% CO_2, followed by measurement of the absorbance at 450 nm using a microplate reader. The experiments were performed in triplicates on three independent occasions.

Alkaline phosphatase (ALP) activity assay

The hDPSCs were incubated in α-MEM supplemented with 10% FBS; 50 µg/mL Ascorbic acid; 10 mM β-glycerol phosphate and 10nM Dexamethasone (OM, odontogenic differentiation media). The cells (1×10^5 cells/well) were seeded on 12-well plates in OM. Peptides were added to the plates at concentrations ranging from 25 to 100 µM. ALP activity was measured on day 14 using the ALP assay kit (Abcam, Boston, MA, USA) following the manufacturer's instructions. Cells incubated only with OM served as the negative control, while OM supplemented with Tideglusib (100 nM) served as the positive control (PC). The cells were washed twice with ice-cold PBS, and 50 µL of the cell lysate was mixed with assay buffer containing p-nitrophenyl phosphate substrate (pNPP). The plate was incubated in 25℃ for 60 min. The ALP activity was determined by measuring the absorbance at 450 nm. The ALP activity was then normalised to the total protein content, which was determined by the Bicinchoninic acid assay (BCA) [23]. The experiments were performed in triplicates on three independent occasions. The amount of ALP produced from the samples was determined using a standard curve analysis and the values were normalised to the total protein content from their respective samples.

Alizarin red S (ARS) staining

The cells (1×10^5 cells/well) were seeded on 12-well plates and treated with the peptides or tideglusib (positive control) as described above. Untreated cells served as the control. After 21 days, the cells were washed with phosphate-buffered saline (PBS) and 1 mL of 40 mM Alizarin Red S dye was added to each well. The plate was then incubated at room temperature for 30 min with gentle shaking. The dye was removed, and the wells were washed twice with PBS. The dye was then dissolved with 10% acetic acid and the absorbance was measured at 405 nm to quantify matrix mineralization. Photographs were obtained with a digital camera and the imaging for mineralisation was obtained using an inverted microscope (Eclipse Ti, Nikon Instruments Inc., Tokyo, Japan). The experiments were conducted in duplicates on three independent occasions. The percentage of calcium deposited was calculated by normalising it to the control.

Gene expression analysis

Temporal expression of DMP-1 (Dentine matrix protein-1), DSPP (Dentine sialophosphoprotein), ALP (Alkaline phosphatase), OCN (Osteocalcin), Runx-2 and OPN (osteopontin) was determined using qRT-PCR. Briefly 1×10^6 cells/well were seeded on 12-well plates and treated with the peptides. At 14 and 21 days, the attached

cells were harvested, and total RNA was extracted using RNeasy Plus Kits (QIAGEN, Hilden, Germany) following the manufacturer's protocol. The extracted RNA was then converted to cDNA using a reverse transcription kit (Applied Biosystems, Waltham, MA, USA). The primer sequences have been presented (Additional file 1). The gene expression profiles were normalised to the housekeeping gene GAPDH. Data output was expressed as relative fold change using the $2^{-\Delta\Delta Ct}$ method [24].

Western blot analysis

The odontogenic differentiation of hDPSCs treated with peptides was assessed via western blotting. hDPSCs were seeded at a density of 1×10^6 cells per well of the 6-well plate and cultured for 21 days with and without peptides as described above. After treatment, the hDPSCs monolayer was washed with ice-cold PBS and lysed using Pierce™ RIPA buffer: Halt protease & phosphatase inhibitor cocktail (Thermo Scientific, Waltham, MA, USA) at a ratio of 100:1. After scraping and centrifugation, the supernatant was collected and the protein concentration was quantified using the Pierce™ BCA protein assay kits (Thermo Scientific). An equal amount of protein lysate from each sample was separated using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

The proteins were then transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% skim milk in TBST (Tris-buffered saline and Tween 20 medium) and incubated with primary antibodies for the following proteins: β-catenin (2 µg/mL), DSPP (1:1000) DMP-1 (1:1000), and using GAPDH (1:500) (Invitrogen, Waltham, MA, USA) as an internal control at 4℃ overnight. Samples were then incubated with HRP-conjugated secondary antibody (1:10,000) and visualised using Pierce™ ECL western blotting substrate (Thermo Scientific), according to the manufacturer's protocol. Images were captured using iBright 1500 imaging system (Invitrogen) and quantified using ImageJ software.

Nuclear localisation of β-Catenin

hDPSCs (1×10^5 cells/well) were seeded in a 35 mm confocal dish (SPL life sciences, Pocheon-si, Gyeonggi-do, Korea), and incubated for 42 h to allow its attachment to the culture dish. Then, the cells were treated with 100 µM of L803-mts and incubated at 37℃, 5% CO_2 for 24 h. The L803-mts-treated cells were then fixed with 4% paraformaldehyde at room temperature for 15 min. The cells were then washed with PBS and permeabilized with Triton®-X100 in PBS for 10 min. Bovine serum albumin (3%) was used as a blocking agent to limit non-specific binding. The cells were then incubated with 1:1000 dilution β-catenin primary antibody (Invitrogen) overnight at 4℃. Following this, the cells were stained with a mixture of goat anti-mouse IgG conjugated with DyLight™ 488 (1 µg/mL) and Phalloidin iFluor 594 conjugate (1:1000) for 2 h [25, 26]. The dishes were then washed twice with PBS to remove the excess stain. The nuclei were counterstained with Hoescht stain (1:1000). The protein expression and localization of β-catenin were visualized using a Confocal Laser Scanning Microscope (Zeiss LSM 900, Oberkochen, Germany).

Migration assay

The migration assay was performed with 8 µM aperture transwell chamber plates (Corning Inc., Corning, NY, USA). Briefly, 5×10^5 hDPSCs with 100 µM of L803-mts were seeded on the membrane of the upper chamber of transwell plates in serum-free α-MEM media. The lower chamber contained α-MEM with 10% of FBS. The cells were then incubated at 37℃ for 24 h to allow the migration of hDPSCs. The cells that migrated to the bottom well were fixed with 4% paraformaldehyde for 15 min and stained with Crystal Violet. The wells were then photographed under an inverted microscope [27]. The number of migrated cells was then quantified using ImageJ software.

Statistical analysis

The data are presented as the mean ± standard deviation from at least three independent biological replicates. Data sets acquired from each assay were evaluated for their normality (Shapiro–Wilk test) and homoscedasticity assumptions (modified Levene test). If those assumptions appeared to be violated, the data sets were nonlinearly-transformed to satisfy the assumptions prior to the use of parametric statistical methods. Statistical analysis was performed using GraphPad Prism 9.1.1 software. One-way ANOVA, followed by Tukey's multiple comparisons test, was used to assess the significant difference between the control and the treated groups in terms of biocompatibility. Two-way ANOVA and Tukey's multiple comparison tests were employed to assess the significance for cell proliferation, ALP expression, calcium nodule formation and gene expression, as well as for the analysis of DMP-1 and DSPP expression through western blotting. For the migration assay and protein expression of β-catenin, two-tailed t-tests were conducted to determine the statistical significance between control and treated groups.

Results

Characterisation of isolated hDPSCs

The isolated human dental pulp stem cells (hDPSCs) exhibited a characteristic spindle-shaped fibroblast-like morphology. Flow cytometric analysis further confirmed that these cells expressed surface markers typical

of mesenchymal stem cells (Fig. 1A). Specifically, they showed positive surface expression of CD73 (100%), CD90 (100%), and CD105 (72.2%), while lacking expression of CD45, a hematopoietic cell marker [21, 22]. These results confirmed that the isolated hDPSCs have the typical characteristics of mesenchymal stem cells. To further analyse the stem cell properties of the isolated DPSCs, their ability to differentiate into at least two mesodermal lineages, namely adipogenic and osteogenic lineages, was examined. The study confirmed the potential of the isolated hDPSCs to undergo osteogenic differentiation by observing mineral nodule formation using alizarin red S staining. Additionally, the potential for adipogenic differentiation was demonstrated by observing intracellular lipid accumulation using oil red O staining in response to the appropriate induction mediums.

The GSK3βi peptides exhibit biocompatibility with hDPSCs and do not influence cell proliferation

The viability of hDPSCs was evaluated after treating them with GSK3βi peptides (pS9, LRP 6a, L803, and L803-mts) using the MTT assay. Different concentrations of the peptides (ranging from 3.13 to 200 μM) were tested. The percentage biocompatibility of the cells was determined by normalizing the optical density (OD) values with their respective controls at each time point. All the GSK3βi peptides showed high biocompatibility, as the cell viability did not significantly differ from the control group ($p > 0.05$) (Fig. 2). This demonstrates that even at a concentration as high as 200 μM, the peptides remained biocompatible.

Additionally, the proliferation of hDPSCs was assessed using the CCK-8 assay on days 1, 3, and 7 (Fig. 3). The results of the 2-way ANOVA revealed that the factor "time" had a significant effect ($p < 0.05$) on cell proliferation; while the factor "peptide concentrations" did not have a significant effect ($p > 0.05$). Furthermore, the interaction of the two factors was also not significant ($p > 0.05$). The four tested peptides exhibited similar effects on hDPSC proliferation, with no significant differences observed among them ($p > 0.05$). From day 3 to day 7, the number of hDPSCs continued to increase without any significant differences ($p > 0.05$) between the control group and the peptide groups.

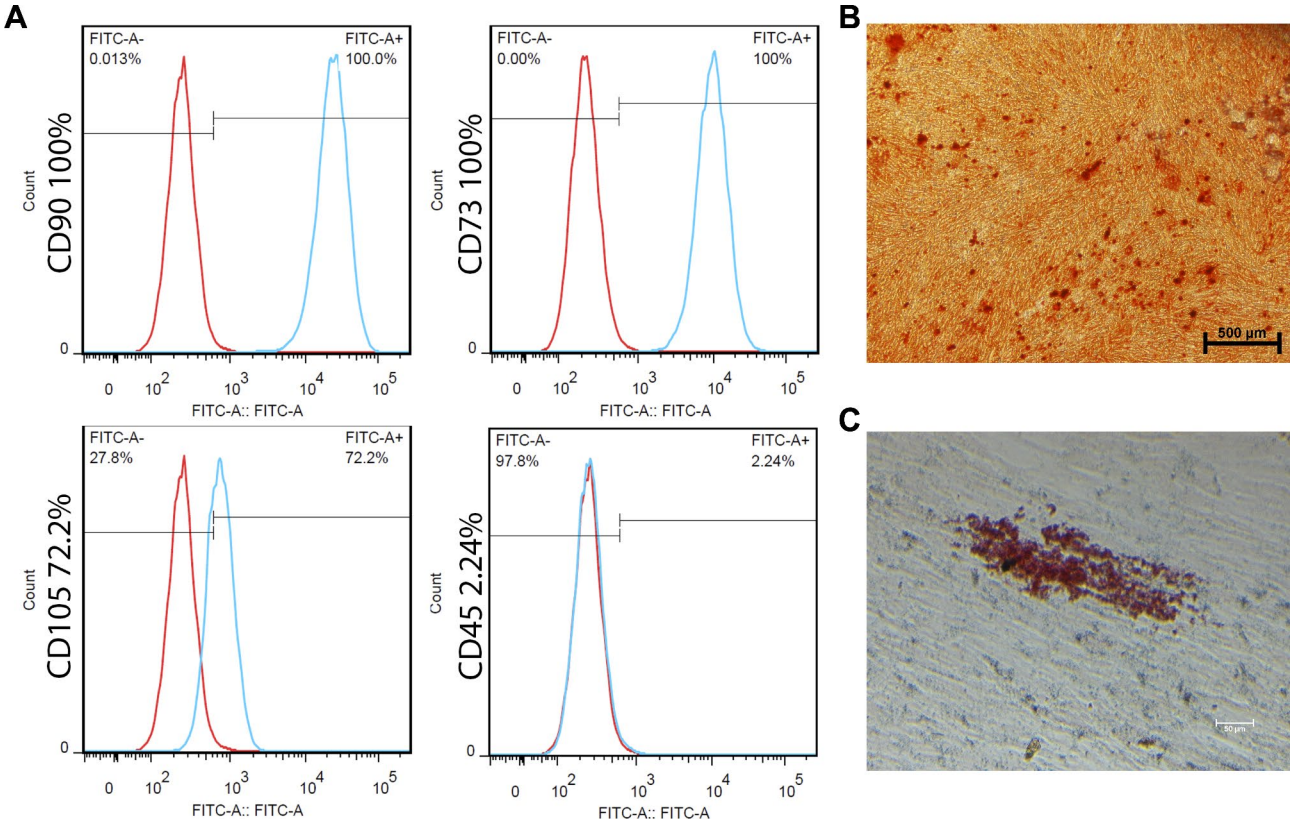

Fig. 1 Characterisation of mesenchymal stem cells isolated from dental pulp tissues. The stem cell surface markers were evaluated using flow cytometry. (**A**) CD90, a stem cell positive marker showing 100% surface expression. CD73, a stem cell positive marker showing 100% increase in the surface expression of DPSC. CD105, stem cell positive marker showing 72.2% expression. CD45, a negative marker for stem cells showing only 2.24% expression. FITC A- indicates the percentage of cells with low or negative for FITC fluorescence and FITC A + indicates the percentage of cells positive for FITC fluorescence. (**B**) and (**C**) represents the osteogenic and adipogenic differentiation properties of the DPSCs, respectively

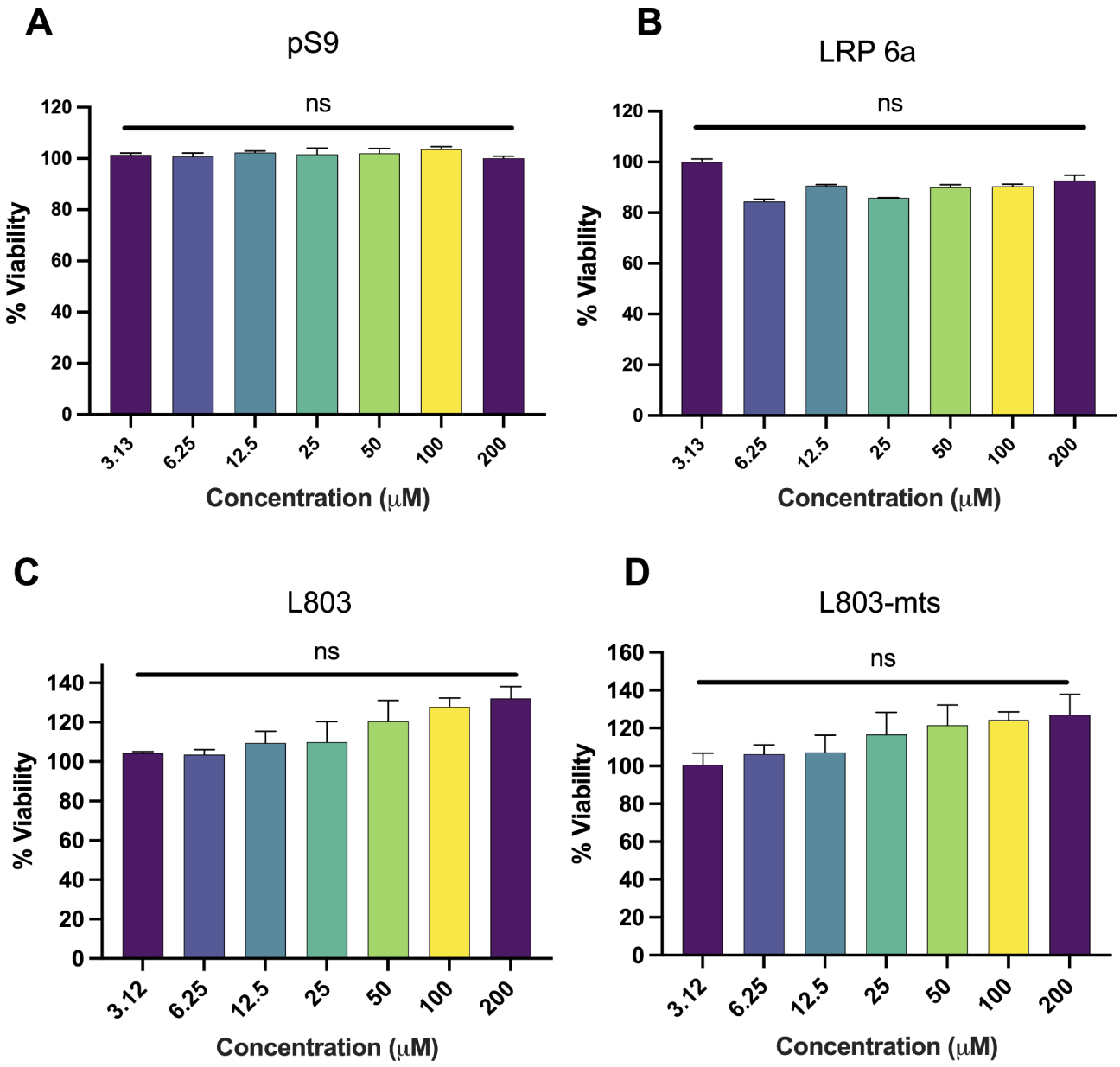

Fig. 2 Effect of GSK-3βi peptides on the viability of hDPSCs. (**A-D**) represent the viability of cells treated with pS9, LRP 6a, L803 and L803-mts, respectively. The values were normalised to the control. Data are expressed as mean ± SD, assays were repeated three times, ns indicates no significant difference compared to the control ($P > 0.05$)

L803-mts markedly increases ALP and calcium deposition

The expression of alkaline phosphatase (ALP) and calcium deposition, indicators of odontogenic differentiation, were evaluated on day 14 and 21, respectively. The results of the 2-way ANOVA revealed that both factors, "peptides" and "concentrations", had significant effects ($p < 0.05$) on ALP expression and calcium deposition. Additionally, interaction of the two factors was also significant, suggesting that the peptides have varying effects on ALP expression and calcium deposition based on the tested concentrations. Treatment with 100 μM of L803, L803-mts, pS9, and LRP 6a resulted in a significant increase ($p < 0.05$) in ALP expression compared to the negative control. Among the tested peptides, L803-mts exhibited the most prominent effect, showing a marked increase in ALP expression at 100 μM (14.9 nmol of pNPP/μg of total protein), significantly higher than the positive control (10.6 nmol pNPP/μg of total protein) ($p < 0.05$). The other peptides did not show significant differences compared to the positive control ($p > 0.05$).

To confirm the differentiation properties of hDPSCs, the peptides were administered for 21 days to allow for the formation of calcium nodules or mineralization. Except for 12.5 μM of L803-mts, pS9, and LRP

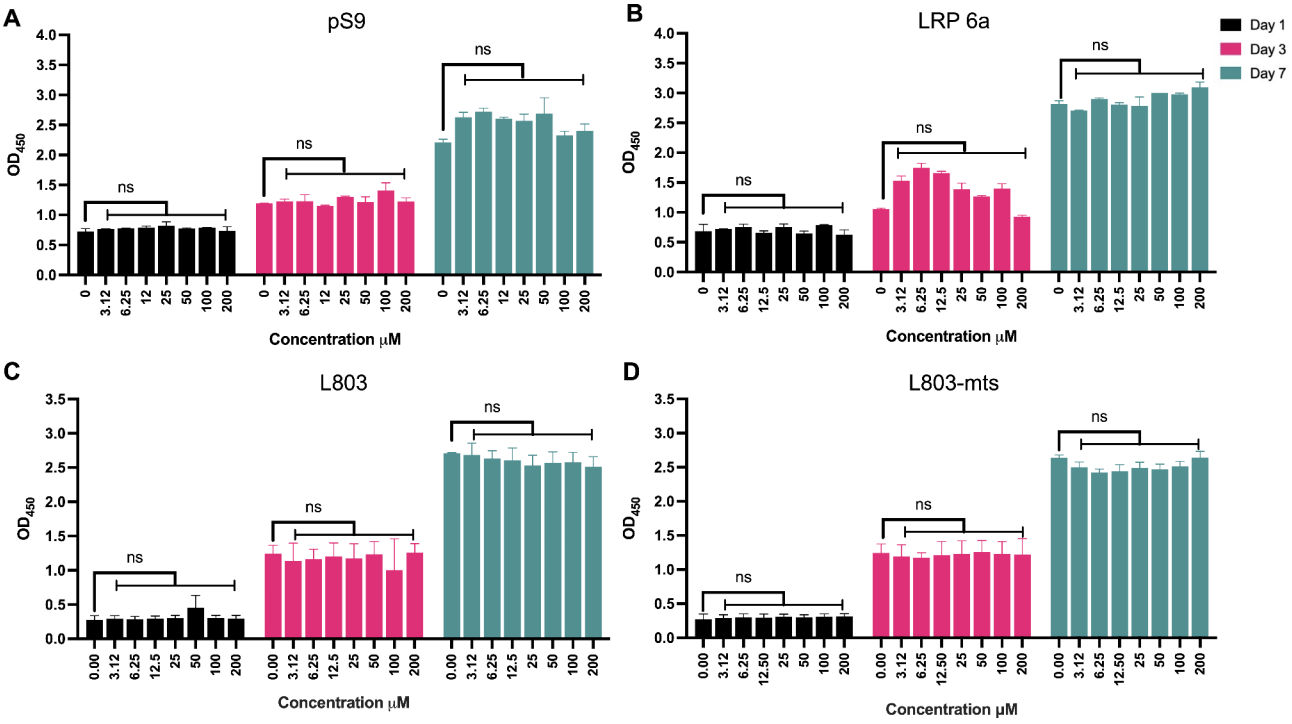

Fig. 3 Effect of the peptides on hDPSCs proliferation. (**A-D**) represents the proliferation of HDPSCs on day 1, 3 and 7, when treated with pS9, LRP 6a, L803 and L803-mts, respectively. HDPSCs without any treatment served as the control. Data are expressed as mean ± SD, assays were repeated three times, ns indicates no significant difference compared to the control ($P > 0.05$)

6a peptides, all tested peptides significantly increased the mineralization properties of hDPSCs compared to the negative control ($p < 0.05$) (Fig. 4B). Notably, treatment with 100 μM of L803-mts resulted in a substantial increase in mineralization compared to both the positive and negative controls ($p < 0.05$). This observation was further confirmed by microscopic images (Fig. 4C).

Overall, among the tested peptides, L803-mts at 100 μM showed the greatest increase in ALP expression and calcium deposition. Therefore, the mRNA expression of odontogenic markers was evaluated for the L803-mts (100 μM)-treated hDPSCs on day 14 and 21 (Fig. 5A). The results of the two-way ANOVA revealed that both factors "L803-mts" and "time" had significant effects ($p < 0.05$) on gene expression. Additionally, the interaction between these two factors was also significant ($p < 0.05$). On day 14, ALP gene expression significantly increased ($p < 0.05$) in the L803-mts group, while other gene expressions did not show a significant increase. However, on day 21, ALP gene expression declined, while the expressions of other odontogenic markers, such as DSPP, DMP-1, Runx-2, and OPN were significantly increased ($p < 0.05$) compared to the control. These findings were further confirmed by protein expression analysis using western blotting, which showed a significant increase in DMP-1 ($p < 0.05$) expression after 21 days in hDPSCs treated with 100 μM L803-mts (Fig. 5B and C; Additional file 2) compared to the control. However, the expression of other marker DSPP showed a moderate increase with no significant difference ($p > 0.05$) from the control (Fig. 5B and C; Additional file 2).

L803-mts activates wnt signaling in hDPSCs and facilitates cell migration

To confirm that L803-mts activates the Wnt signaling pathway, the subcellular and nuclear localisation of β-catenin was observed via immunofluorescence and western blotting (Fig. 6). Treatment with L803-mts (100 μM) activated the Wnt signaling pathway in hDPSCs. Immunofluorescence imaging (Fig. 6A) showed a prominent increase in the expression of β-catenin inside the nucleus, and western blotting confirmed a significant increase in cytoplasmic and nuclear localisation of β-catenin in the L803-mts-treated group compared to the control (Fig. 6B and C; Additional file 2). Furthermore, hDPSCs treated with 100 μM L803-mts showed a significant increase in cell migration compared to the control group (Fig. 7; $p < 0.05$). These findings indicate that the activation of the Wnt signaling significantly enhances the migration of DPSCs towards serum media.

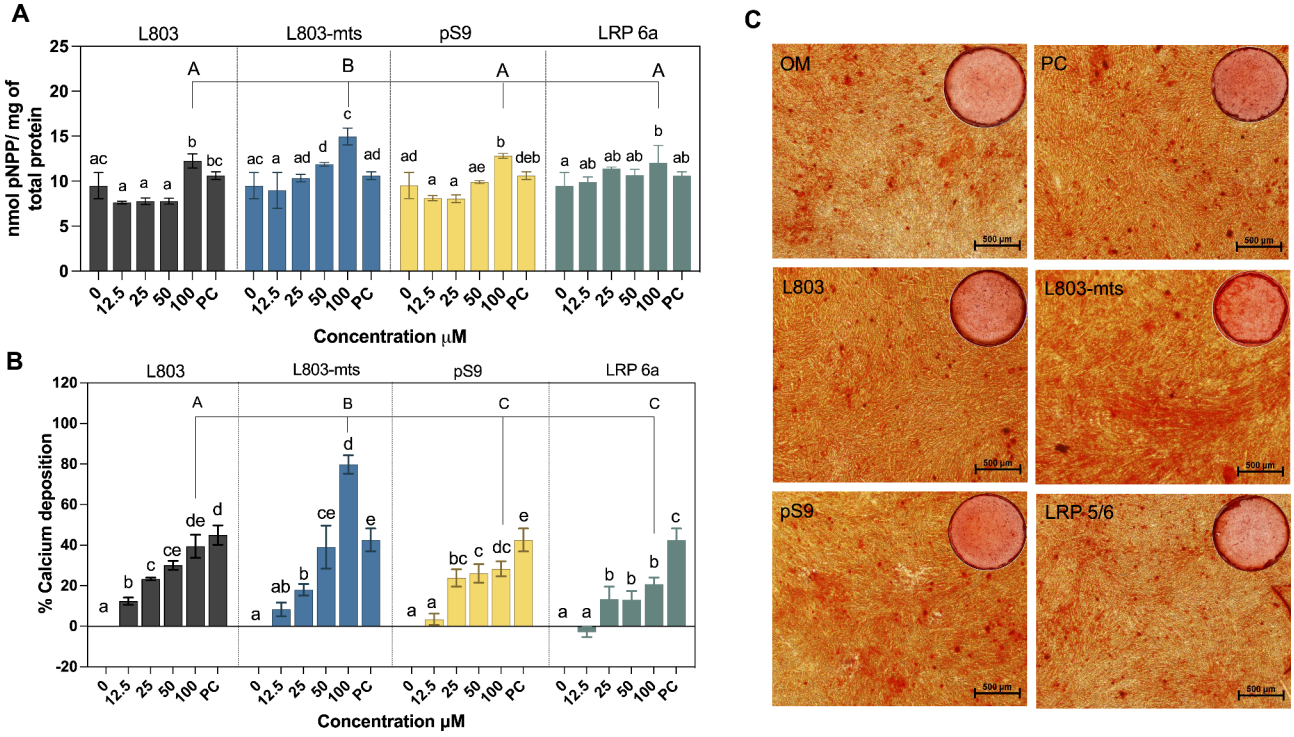

Fig. 4 Effect of the peptides on the odontogenic differentiation of hDPSCs. (**A**) The peptides pS9, LRP 6a, L803 and L803-mts showed a significant increase in ALP expression at 100 μM ($P < 0.05$) compared to the negative control (OM: Odontogenic media). (**B**) Quantitative data of Calcium deposition to detect the mineralisation of DPSC treated with peptides. The graph shows the significant increase in the calcium deposition in the peptide-treated groups ($P < 0.05$) compared to the control (**C**) Mineralisation of DPSC following treatment with peptides, with L803-mts showing increased mineralisation compared to the other peptides and the positive control (PC: Positive control, 100 nM Tideglusib). Multiple comparisons were made between the groups. Different lowercase letters indicate a significant difference ($P < 0.05$) between the control and the treated groups, while different uppercase alphabets indicate a significant difference ($P < 0.05$) between the peptides at 100 μM concentration

Discussion

Activation of the Wnt signaling is known to be an early and immediate response to tissue injury [28]. Stimulating this pathway has been shown to enhance reparative dentine formation by activating the resident mesenchymal stem cells [7, 29]. However, the investigation of Wnt agonists in dentistry has predominantly focused on small molecules [30, 31]. Small molecule-based Wnt agonists have demonstrated excellent dentine reparative properties in both in vitro and in vivo studies [25–28]. Yet, the potential influence of peptide-based substrate competitive inhibitors of GSK-3β in the dental field remains largely unexplored. Peptide-based inhibitors possess superior physiochemical characteristics compared to small molecules, which can enhance their biological activity and effectiveness in dental applications [17].

The results of this study showed that L803-mts increased the mineralization, odontogenic differentiation, and migration of HDPSCs through the activation of the Wnt signaling pathway. However, the peptide did not have an effect on the proliferation of hDPSCs. Therefore, the null hypothesis has to be partially rejected. To the best of the author's knowledge, this is the first study to comprehensively evaluate the effects of peptide-based GSK-3β inhibitors on hDPSCs. The peptides tested in this study included pS9, an auto-inhibitory sequence that mimics the N-terminal end of GSK-3β [GRPRTT{(p)S}FAESCK] with phosphorylated serine [16, 17], LRP 6a, a peptide sequence [NPPP{(p)S}PA{(p)T}ERSH] that mimics the binding site of GSK-3β to the LRP 6a receptors on the cell surface [18], L803 [KEAPPAPPQ{S(p)}P], derived from the target substrate heat shock factor-1, and an advanced version of the same peptide (L803 Myristate/mts) with the addition of the fatty acid myristate group to the N-terminal to improve cell permeability [14, 15].

In contrast to small molecule, Wnt agonists that have shown cytotoxicity towards hDPSCs at nanomolar concentrations, all the peptides used in this study were found to be highly biocompatible, even at high concentrations of up to 200 μM [7]. The use of peptide-based inhibitors enables more specific and moderate inhibition of the GSK3β enzyme, which is preferred over complete inhibition due to the potential adverse effects of aberrant Wnt signaling [15].

The self-renewal and proliferation of stem cells are important for enhancing the dentine reparative process without depleting the hDPSC source. Hence, the efficacy of the peptides in enhancing the proliferation of

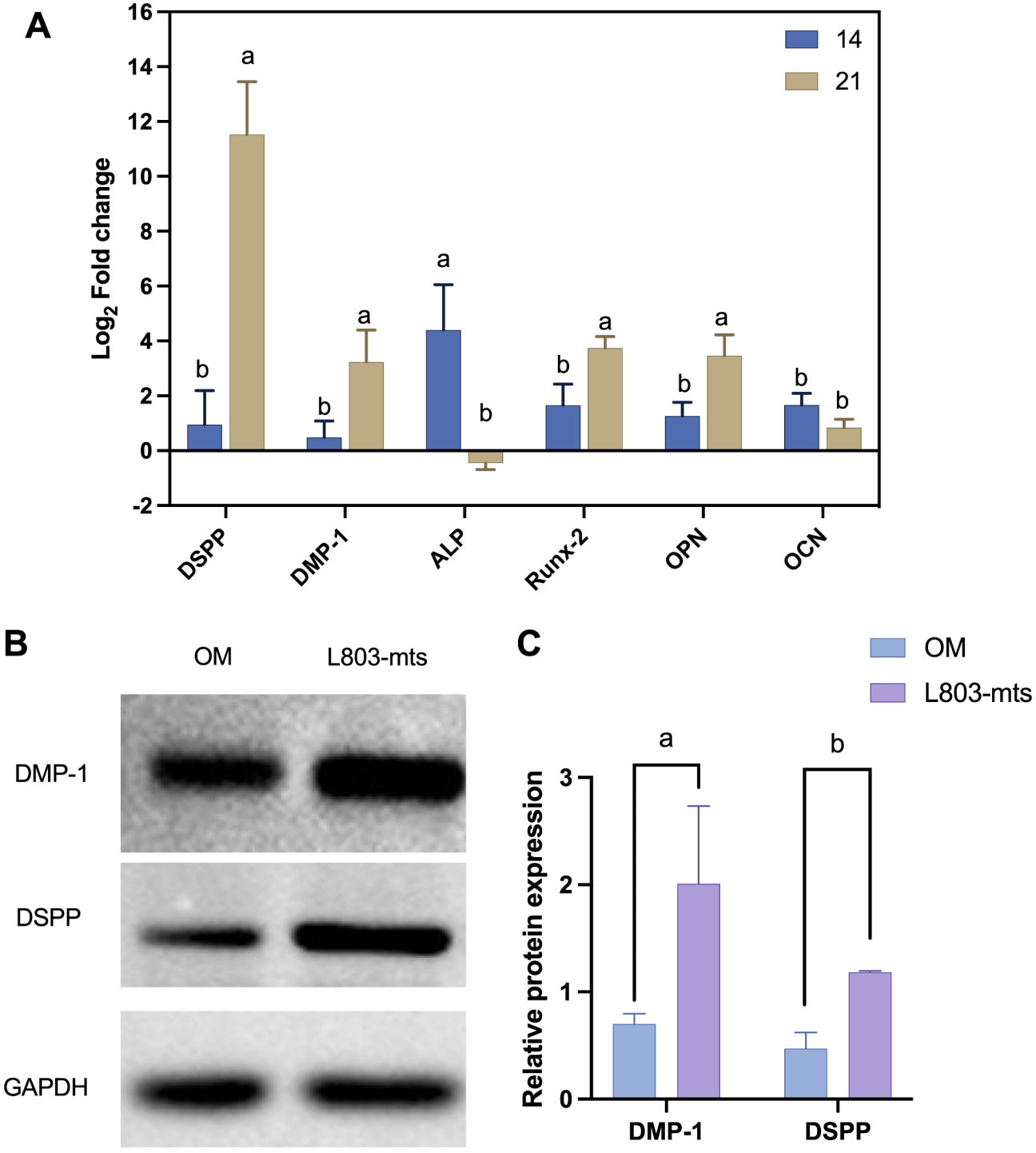

Fig. 5 Molecular assays to determine the odontogenic differentiation of hDPSCs following the treatment with the peptides. (**A**) Bar graph illustrating the logarithmic fold change in the expression of genes in the L803-mts treated hDPSC. (**B**) Western blotting analysis shows the increase in the expression of two odontogenic markers DSPP and DMP-1 (Full length blots are presented in additional file 2). OM indicates the hDPSC control culture in odontogenic media without any treatment. The gene expression was normalised to the control using the $2^{-\Delta\Delta Ct}$ method. Bars marked with "a" indicate that the values are statistically significant ($P < 0.05$) compared to the control while "b" indicates no significant difference ($P > 0.05$)

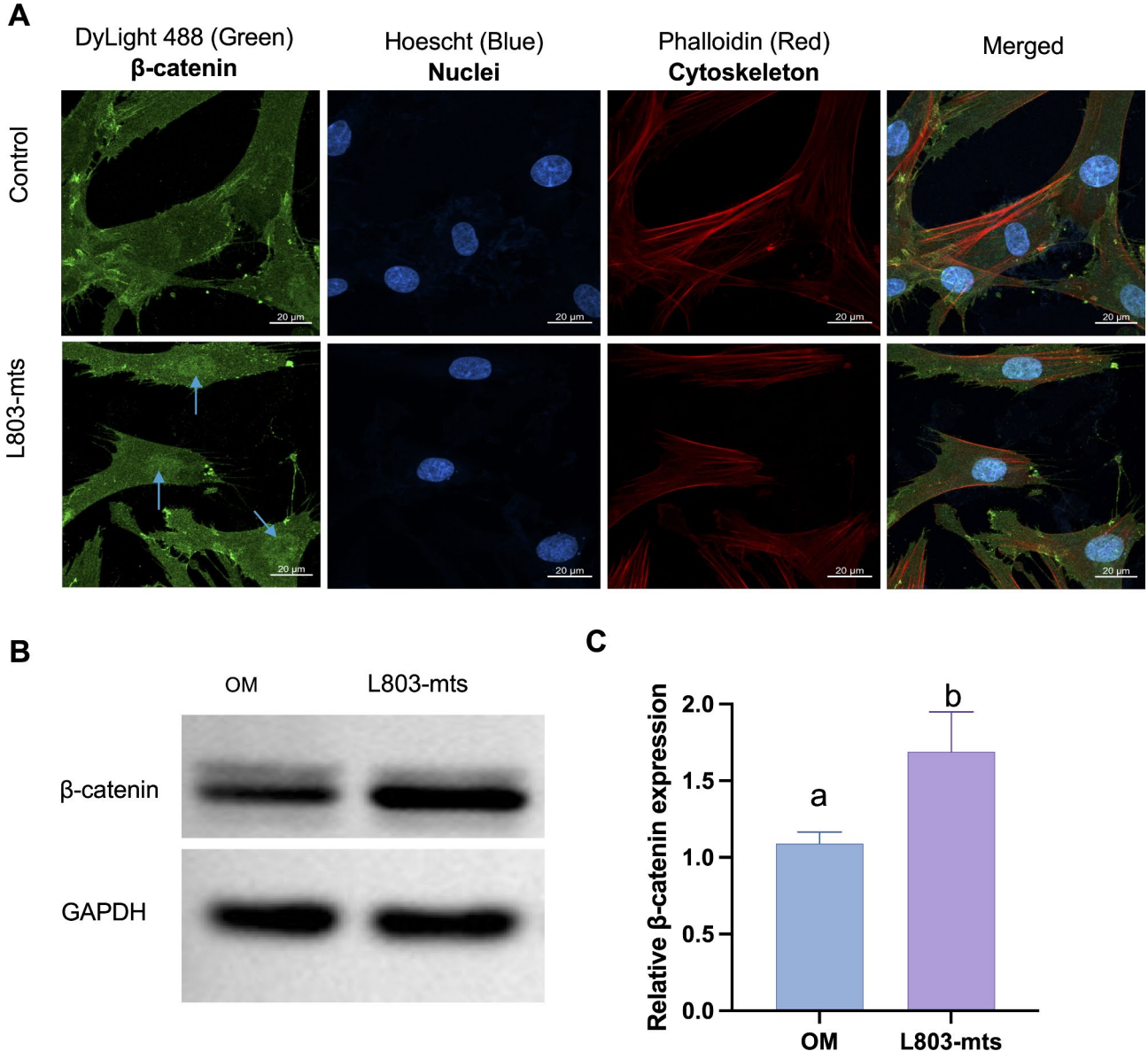

Fig. 6 Representative images showing an increase in the expression of β-catenin in hDPSCs following treatment with the peptides. (**A**) Immunofluorescence staining confirmed the nuclear localization of β-catenin. β-catenin was visualized in green, nuclei were counterstained with Hoescht (blue), and the cytoskeleton was stained with phalloidin (red). L803-mts increased the accumulation of β-catenin inside the nucleus compared to the control. (**B**) Western blotting analysis was conducted to assess the expression of β-catenin in hDPSCs treated with the peptides (Full length blots are presented in additional file 2). (**C**) Graphical representation of protein expression analysed using Image J software. Assays were repeated three times. Data represented in mean ± SD, Different letters indicate a significant difference ($P < 0.05$), blue arrow marks the increase in β-catenin inside the nucleus

hDPSCs was examined. Similar to Tideglusib [26], the GSK-3β inhibitory peptides did not significantly increase cell proliferation compared to the control, providing further evidence for the theory that Wnt signaling plays a more significant role in the differentiation of the human embryonic stem cells (hESC) than the proliferation/self-renewal process [31].

Wnt signaling has been known to enhance the odonto/osteogenic differentiation of MSCs [7]. In this study, all the peptides showed a dose-dependent increase in the ALP activity and calcium deposition, indicating their potential for promoting odontogenic differentiation. Out of all the peptides tested, 100 µM of L803-mts demonstrated the highest level of activity. As a result, further characterization of the differentiation properties was conducted specifically using this concentration. The odontogenic differentiation property of L803-mts was further confirmed by evaluating the mRNA and protein expression of differentiation-related markers. The superior activity of L803-mts in promoting odontogenic

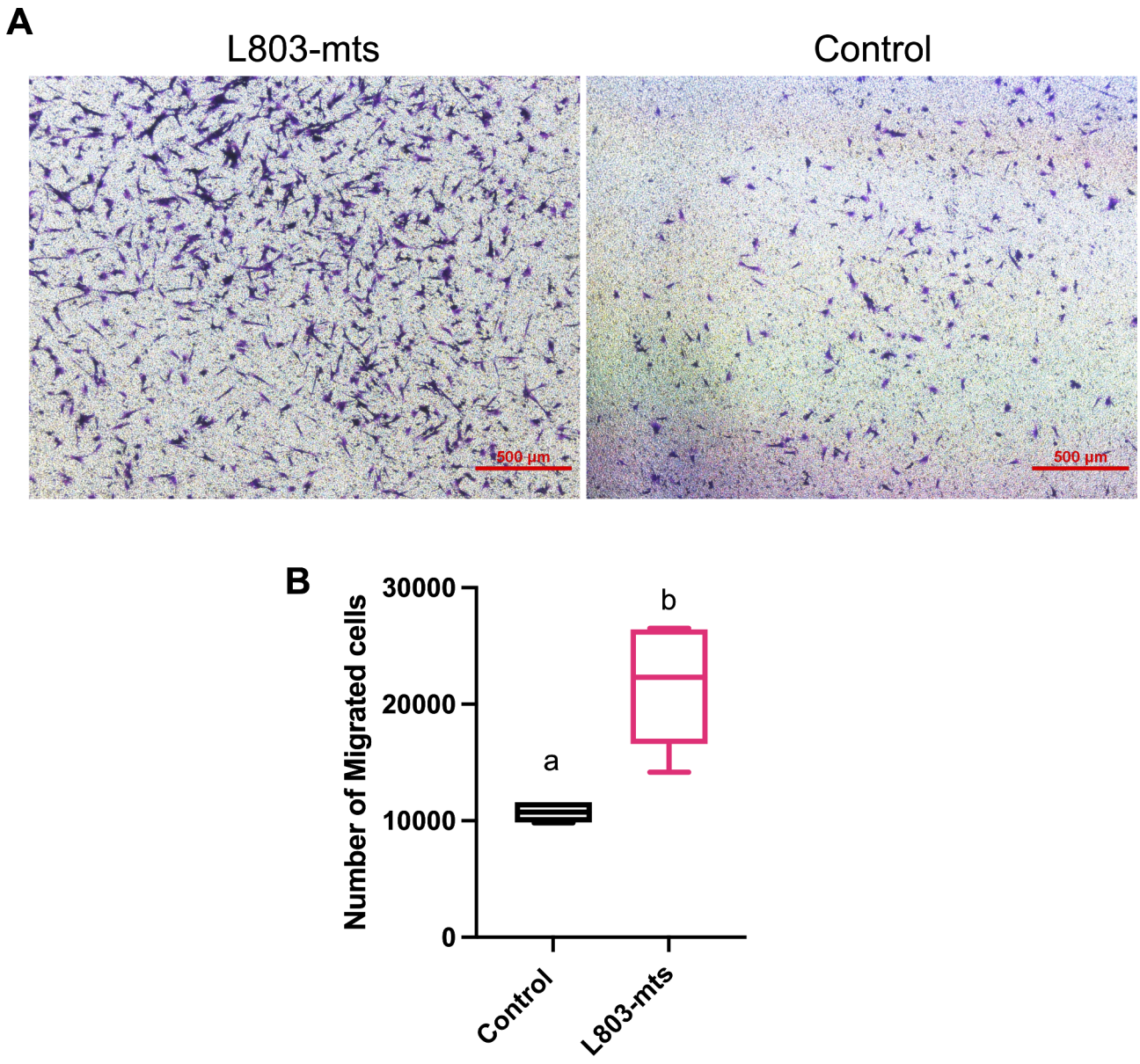

Fig. 7 Migration of hDPSCs in a transwell chamber following treatment with L803-mts. (**A**) Microscopic images (4×) of migrated hDPSCs, (**B**) The number of migrated cells was quantified using ImageJ software. Different letters indicate a significant difference ($P < 0.05$) in the migration of L803-mts treated hDPSCs compared to the control. Assays were repeated three times

differentiation of hDPSCs compared to the other tested peptides may be attributed to the addition of the myristate (mts) fatty acid group at the N-terminal. Previous studies have shown that the addition of this fatty acid increases the serum stability of the peptide, facilitates its subcellular localisation, and improves its binding ability to the cytoplasmic GSK-3β enzyme [14, 15].

The expression of genes related to odontogenic differentiation, such as DSPP, DMP-1, Runx-2, OPN, and OCN showed a significant increase of multiple-fold, along with an increase in protein expression of the primary odontogenic markers DSPP and DMP-1. Additionally, alkaline phosphatase (ALP) gene expression was significantly increased on day 14 but decreased on day 21, which is consistent with the time course of ALP activity. During the early stages of differentiation and odontoblast maturation, the expression of this enzyme is expected to be high during, but it diminishes during the later stages of mineralization [24].

L803-mts increased the localisation of β-catenin inside the nucleus, indicating the activation of the Wnt signaling pathway in hDPSCs [25, 26]. This suggests that the significant increase hDPSC differentiation observed with L803-mts treatment is attributed to the activation of the

Wnt signaling pathway. Migration of MSCs towards the site of injury is crucial for tissue repair, and it is facilitated by chemoattractant molecules, such as chemokines, cytokines, and growth factors. Previous studies have shown that the SDF-1/CXCR4 axis interacts with the FAK/PI3K/Akt and GSK3β/β-catenin pathways to regulate the migration of hDPSCs [30]. Treatment of hDPSCs with the Wnt agonist AR-A014418, which is an ATP-competitive inhibitor, enhanced the migratory potential of hDPSCs via the β-catenin/PI3K/Akt signaling pathway and upregulation of the CXCR4 axis [27]. Therefore, it is likely that the chemotactic migration of hDPSCs upon activation of Wnt signaling by L803-mts is also mediated by the activation of β-catenin/PI3K/Akt signaling pathway [27, 32].

This study provides novel insights into the effects of peptide-based GSK-3β inhibitors on hDPSCs, highlighting their potential for enhancing the regenerative properties of dental pulp. The findings suggest that L803-mts has the potential to be developed as a direct pulp capping agent to promote dentine bridge formation in clinical settings. Incorporating the bioactive molecules into current gold standard pulp capping materials is a readily available approach, as it can increase the biological properties of the current treatment approaches, and it can be easily translated into clinical application. For instance, BMP 2 and 7 were incorporated into MTA, which showed enhanced dentin deposition, and it was also able to surpass the initial cytotoxicity exerted by MTA because of its alkaline nature [33, 34]. In addition to this, many peptides based therapeutic have opted for commercially available collagen sponge for delivery, providing a biodegradable scaffold, which will eventually be replaced by a native tissue structure. Previous research has shown the ability of collagen sponge to be degraded within 6 weeks into application in the pulp cavity and replaced by native dentin structure [6, 35]. Hence, the above-mentioned delivery approaches can all be potentially designed for the application of L803-mts in the clinical setting.

However, it is important to note that the use of primary hDPSCs from human third molars in these experiments may not fully represent in vivo responses. The dose-dependent nature of the Wnt signaling response could pose limitations on the application of L803-mts, as achieving a precise concentration to activate the pathway optimally may be challenging. Moreover, the susceptibility of the peptide to various proteases released at the injury site adds complexity to optimizing its efficacy under in vivo and clinical conditions. Additionally, the interaction between macrophages and hDPSCs is known to play a significant role in reparative dentinogenesis. Therefore, future studies should focus on characterizing the in vivo reparative effects of dentine and examining the effects of the L803-mts peptide on regulating the immune response by modulating the hDPSC-macrophage crosstalk. These investigations will provide a more comprehensive understanding of the therapeutic potential of L803-mts peptide in dentin-pulp regeneration.

Conclusions

The L803-mts peptide demonstrated promising effects on enhancing odontogenic differentiation, mineralization, and migration of hDPSCs through the activation of the Wnt signaling pathway. The biocompatibility and specific cellular targeting properties of these inhibitors make them promising candidates for future therapeutic applications in dental tissue regeneration.

Abbreviations

hDPSCs	Human dental pulp stem cells
GSK-3β	Glycogen synthase kinase-3β
MSC	Mesenchymal stem cells
ALP	Alkaline phosphatase
DMP-1	Dentin matrix protein-1
DSPP	Dentine sialophosphoprotein
OCN	Osteocalcin
OPN	Osteopontin
Mts	Myristate

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

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Author contributions

Vidhyashree Rajasekar: Conceptualization, methodology, data curation, statistical analysis, and writing—original draft preparation. Mohamed Mahmoud Abdalla: Methodology, data curation, writing—original draft preparation, and review and editing. Mohammed S. Basabrain: Data curation, methodology. Prasanna Neelakantan: Conceptualization, Supervision, Methodology, Project administration, Writing - review & editing. Cynthia Yiu: Conceptualization, Formal analysis, Methodology, Supervision, Funding acquisition, Project administration, Resources, Writing - original draft, Writing - review & editing.

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Data availability

All data generated and analyzed during this study are included in this published article and its supplementary files. The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
The protocol for isolation of dental pulp stem cells from human dental pulp tissue was approved by the University of Hong Kong, Institutional Review Board (20221026-003-000) under the title "Enhancing dentin regeneration with a multi-functional bioactive scaffold" on 26 January 2023. The ethics approval statement and patient consent document have been submitted with the manuscript.

Consent for publication
Not applicable.

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