Scientific Research Report

Secreted Peptidome Profiling of Human Dental Pulp Stem Cells–Conditioned Medium During Odontogenic Differentiation

Yanhua Huang [a,b], *Shu Liu* [c], *Huaying Wu* [a], *Zheng Zhang* [a], *Haiyan Chen* [d], *Kaipeng Xie* [d*], *Shoushan Bu* [b*]

[a] *Department of Stomatology, Women's Hospital of Nanjing Medical University, Nanjing Women and Children's Healthcare Hospital, Nanjing, China*
[b] *Department of Stomatology, The First Affiliated Hospital of Nanjing Medical University, Nanjing, China*
[c] *Department of Radiology, Nanjing Stomatological Hospital, Affiliated Hospital of Medical School, Institute of Stomatology, Nanjing University, Nanjing, China*
[d] *Department of Public Health, Women's Hospital of Nanjing Medical University, Nanjing Women and Children's Healthcare Hospital, Nanjing, China*

ARTICLE INFO

Article history:
Received 9 February 2026
Received in revised form
31 March 2026
Accepted 12 April 2026
Available online xxx

Key words:
hDPSCs
P1DC
Secreted peptide
LC-MS/MS
Odontogenic differentiation

ABSTRACT

Introduction and Aims: Odontogenic differentiation of human dental pulp stem cells (hDPSCs) is critical for pulp repair. This study aims to characterise the secretory peptidome of human dental pulp stem cells (hDPSCs) during odontogenic differentiation, thereby providing insights into potential dental pulp repair strategies.

Methods: We employed tandem mass tag labelling coupled with liquid chromatography–tandem mass spectrometry to compare the peptide profiles of undifferentiated and differentiated hDPSCs–conditioned medium (CM), followed by in-depth bioinformatics analysis. A Cell Counting Kit-8 assay was conducted to screen for functional peptides present in the hDPSCs-CM. 5-Ethynyl-2′-deoxyuridine staining, flow cytometry, transwell migration, alkaline phosphatase activity, and alizarin red S staining, quantitative real-time polymerase chain reaction, and Western blot analyses were performed to preliminarily investigate the functions of the selected peptide.

Results: A total of 90 peptides were differentially expressed in differentiated hDPSCs-CM compared with undifferentiated hDPSCs-CM. Gene Ontology analysis suggested that the differentially expressed peptides were closely associated with biological processes, including cell proliferation, growth, and reproduction. Kyoto Encyclopedia of Genes and Genomes enrichment analysis suggested that the precursor proteins of these peptides were significantly enriched in the PI3K-Akt signalling pathway, carbon metabolism, and extracellular matrix–receptor interactions. Furthermore, we identified a secreted peptide that promotes the proliferation, migration, and odontogenic differentiation of hDPSCs and named it P1DC (peptide 1 derived from CFL1 protein).

Conclusions: Our study is the first to identify differentially expressed peptides between undifferentiated and differentiated hDPSCs-CM. Furthermore, we discovered a novel secreted peptide, P1DC, which significantly enhances hDPSC function.

Introduction

Dental caries is recognised as one of the most prevalent chronic diseases.[1] As the carious lesion advances into dentin, bacteria and their toxic by-products invade the pulp tissue via dentinal tubules, leading to damage of varying severity.

* Corresponding authors.
E-mail addresses: kaipengxie@njmu.edu.cn (K. Xie), bushsh@sina.com (S. Bu).

https://doi.org/10.1016/j.identj.2026.109616

Dental pulp tissue is central to maintaining the defensive, formative, sensory, and nutritional functions of the tooth.[2] Following the removal of compromised pulp tissue, the tooth loses its vitality, potentially leading to complications such as fracture.[3] Human dental pulp stem cells (hDPSCs) are a population of mesenchymal stem cells capable of odontogenic differentiation, enabling them to produce reparative dentin and promote pulp repair.[4] Numerous physical and chemical factors, including light exposure, hypoxia, mechanical stress, growth factors, bioactive materials, and hormones, have been reported to influence the odontogenic differentiation of hDPSCs. However, the factors influencing hDPSC differentiation and the underlying mechanisms still require more in-depth investigation.[5]

An increasing number of reports have indicated that hDPSCs can secrete many bioactive factors and extracellular vesicles into the conditioned medium (CM), which is of great significance in the processes of tissue repair, angiogenesis, anti-inflammatory response, and immune regulation.[6] Recently, hDPSCs-CM have been used to direct odontogenic and osteogenic differentiation. Swanson et al[7] demonstrated that exosomes derived from hDPSCs upregulate odontogenic gene expression and enhance mineralisation in vitro. Furthermore, they promote reparative dentin bridge formation in vivo, exhibiting superior efficacy compared to glass-ionomer cement alone. Barone et al[8] reported that hypoxic dental pulp stem cells–CM exhibit a greater capacity to induce human umbilical vein endothelial cell adhesion to fibronectin, migration, and the formation of capillary-like structures compared to normoxic dental pulp stem cells–CM. These findings indicate that the secretory profile of hDPSCs is complex and varies under different physiological conditions. Therefore, further investigation is required to identify additional hDPSC-secreted factors and elucidate their regulatory mechanisms.

Advances in mass spectrometry technology have significantly expanded the research scope of peptidomics.[9] Short peptides, typically comprising 3 to 50 amino acids, are a class of bioactive substances capable of interacting with DNA and histone proteins, thereby influencing key cellular processes such as proliferation, differentiation, and apoptosis.[10] Peptidomics analysis can provide important information about proteolytic activity under relevant environmental conditions, as well as details about potential bioactive peptides. Furthermore, certain mesenchymal stem cells have been shown to secrete functional antimicrobial peptides, including LL-37, hepcidin, and β-defensin-2, which contribute to combating bacterial infections and promoting wound healing.[11] However, there are no studies to elucidate the peptides of hDPSCs-CM in directing hDPSC odontogenic differentiation.

Here, we combined the tandem mass tag (TMT) labelling method with liquid chromatography (LC)–tandem mass spectrometry (MS/MS) analysis for comprehensive analysis and quantification of the secreted peptides from undifferentiated and differentiated hDPSCs-CM. This approach allowed us to explore the profiling of secreted peptidomes related to odontogenic differentiation, providing a basis for their use in reparative dentinogenesis and pulp repair.

Method

Ethics approval

Human teeth were derived from patients who underwent treatment at the stomatology department of Women's Hospital of Nanjing Medical University (Nanjing Maternity and Child Health Care Hospital). Each participant provided written informed consent. The Ethics Committee of the Women's Hospital of Nanjing Medical University (Nanjing Maternity and Child Health Care Hospital) approved this study (No. 2022KY-001-01).

Preparation of hDPSCs

Inclusion criteria were (1) derivation of teeth for orthodontic treatment or extraction of the third molars; (2) healthy, nondecayed teeth; and (3) age between 10 and 25 years, no history of arthritis or systemic diseases, and no immunosuppressants or antibiotics taken within 2 months. Eighteen healthy permanent teeth from 10 patients were collected. Dental pulp tissues were stripped under sterile conditions, mixed together, and then subjected to primary cell isolation and culture.

The hDPSCs were obtained by enzymatic digestion and the tissue explant adherent method, as previously reported.[12] The freshly extracted teeth were ground with a turbine in the neck of the tooth to create a guiding groove, and then the teeth were cut off at the guiding groove using a cutting forceps in a laminar flow hood. The acquired pulp tissue samples were cut into pieces approximately 0.5 to 1.0 mm^3 in size, and 3 mg/mL collagenase I (Sigma) and 4 mg/mL dispase (Sigma) were added. The mixture was digested at 37 °C for 30 minutes, and the cells and tissue fragments were transferred to a culture flask. Cells were cultured in Mesenchymal Stem Cell Medium (ScienCell) consisting of basal medium, foetal bovine serum, and mesenchymal stem cell growth supplement in a humidified CO_2 (5%) incubator maintained at 37 °C. When the cells reached the third passage (P3), cells in good condition were selected for all subsequent experiments to ensure consistent cell sources and experimental reproducibility.

Cell differentiation

Odontogenic differentiation of hDPSCs was induced with Mesenchymal Stem Cell Osteogenic Differentiation Medium (MODM; ScienCell) consisting of 500 mL basal medium, 25 mL foetal bovine serum, 5 mL Mesenchymal Stem Cell Osteogenic Differentiation Supplement, and 5 mL penicillin/streptomycin solution. We seeded hDPSCs at passage 3 in 6-well plates and allowed them to reach 80% confluency. MODM was used for 21 days and subsequently exchanged every 2 to 3 days. Alizarin red S (ARS A5533; Sigma-Aldrich) was used after the cells had been fixed for 30 minutes with 4% paraformaldehyde. Mineral nodules were observed and recorded under a light microscope (Observer D1; Zeiss).

Adipogenic differentiation was performed according to the OriCell Kit (Cyagen). We seeded hDPSCs at passage 3 in 6-well plates and allowed them to reach 100% confluency. Then, adipogenic differentiation was guided using medium A and medium B containing glutamine, dexamethasone, 3-isobutyl-

1-methylxanthine, rosiglitazone, and insulin for 21 days. Oil red O staining was performed after the cells had been fixed for 30 minutes with 4% paraformaldehyde. Lipid droplets were observed and recorded under a light microscope (Observer D1; Zeiss).

Flow cytometry analysis

Cells were washed with phosphate-buffered saline (PBS) 3 times, resuspended, and counted (1×10^5 cells/sample). For flow cytometry identification of hDPSC surface markers, cells were then aliquoted at 1×10^5 cells/100 μL into centrifuge tubes; 2 μL of CD29-PE, CD146-PE, CD90-PE, CD105-PE, CD34-PE, and CD45-PE (Abcam, Cambridge, UK) were added, respectively, and incubated in the dark at room temperature for 1 hour. For flow cytometry analysis of the cell cycle, 1 mL ice-cold 70% ethanol was added and fixed at 4 °C for 12 hours; then, 4 μL of Red Nucleus I staining solution was added and incubated in the dark at 37 °C for 10 minutes. After centrifugation and filtration of all cell samples, a flow cytometer (Gallios Flow Cytometer; Beckman Coulter) was used to analyse the cells.

Preparation of the undifferentiated and differentiated hDPSCs-CM

For undifferentiated hDPSCs-CM collection, 15 Petri dishes (10 cm) of P3 hDPSCs were cultured to 90% confluence. After discarding the complete medium and washing 3 times with PBS, serum-free Mesenchymal Stem Cell Medium—phenol red free (ScienCell) basal medium was added. Supernatants were collected after 24 hours, divided into 3 groups (5 dishes per group), with 50 mL per group.

For differentiated hDPSCs-CM collection, 15 Petri dishes (10 cm) of P3 hDPSCs were cultured to 80% confluence, then induced with MODM for 14 days. After discarding MODM and washing 3 times with PBS, serum-free Mesenchymal Stem Cell Medium—phenol red free was added. Supernatants were collected after 24 hours, divided into 3 groups (5 dishes per group), with 50 mL per group.

Collected serum-free medium was centrifuged (4 °C, 1000g, 10 minutes and 12,000g, 10 minutes) to remove cell debris, and a protease inhibitor (Roche) was added. Lastly, undifferentiated and differentiated hDPSCs-CM were snap-frozen in liquid nitrogen and stored (−80 °C) for further analysis.

Peptide extraction and purification

A total of 6 CM samples (3 undifferentiated hDPSCs-CM and 3 differentiated hDPSCs-CM) prepared as above were analysed by LC-MS/MS. Each sample was subjected to only 1 independent instrumental run, with no technical replicates. All collected samples were treated with 10 mM DL-dithiothreitol (Promega) and incubated at 56 °C for 1 hour. After the reaction was complete, the mixture was cooled to room temperature, and 55 mM iodoacetamide was added and incubated in the dark for 45 minutes. Thereafter, the peptide solution was desalted using a Strata-X C18 column (Phenomenex), frozen, and dried. The dried peptide samples were resolubilised with 300 μL 0.1% formic acid), vortexed for 1 minute, and centrifuged at 20,000g for 5 minutes, and the supernatant was collected. Then, the samples were filtered twice using a 10-kDa molecular-weight cutoff filter tube (Merck Millipore, UFC501096) to collect the filtrate. All filtrates were lyophilised to obtain the final peptide samples. The key information of the specific experiment is shown in the Table.

Table – Experimental setup.

Item	Parameter
Donor number	A total of 18 healthy permanent teeth from 10 patients
Passages used	P3
Number of CM batches	6 (3 undifferentiated hDPSCs-CM and 3 differentiated hDPSCs-CM)
Number of MS injections	1

Abbreviations: CM, conditioned medium; hDPSC, human dental pulp stem cell; MS, mass spectrometry.

TMT labelling and LC-MS/MS analysis

Each sample was labelled according to the manufacturer's instructions for the TMT labelling kit (Thermo Fisher Scientific). Analysis of labelled peptides was performed on a Q Exactive Orbitrap LC-MS/MS system (Thermo Fisher Scientific). The mass spectrometry data were retrieved using the MaxQuant (version 1.5.3.30) software, and the database search algorithm employed was Andromeda. Qualitative and relative quantitative analyses of the detected peptides were performed using the SWISSPROT human database from Uniprot.

Bioinformatics analysis

Based on the differentially expressed peptides, an in-depth bioinformatics analysis of their precursor proteins was carried out. The online tool pI/MW (http://web.expasy.org/compute.pi/) was applied to calculate the molecular weight (MW) and isoelectric point (pI) of the identified peptides. The Gene Ontology (GO) (http://www.geneontology.org/) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/) enrichment analyses were carried out to predict the functions of precursor proteins for differentially expressed peptides. IPA (Ingenuity Pathway Analysis) software was used to further analyse the functions and molecular networks. The UniProt database (http://www.uniprot.org/) and Peptide Ranker (http://distilldeep.ucd.ie/PeptideRanker/) were used to analyse the functional domains and bioactivity of peptides, respectively.

Synthetic peptides

All the peptides used in this study were synthesised by Scipeptide. The purity of each peptide was 95%, as determined by high-performance LC-MS. The peptides were dissolved in distilled water with a final concentration of 50 mM/μL and stored at −40 °C.

Cell Counting Kit-8

The Cell Counting Kit-8 (CCK-8) assay (DOJINDO) was employed to determine the effect of peptide concentration on the viability of hDPSCs. Briefly, 5000 cells/100 μL were plated into 96-well plates, and various concentrations of peptide were added and cultivated for 0, 24, 48, and 72 hours in a humidified CO_2 (5%) incubator maintained at 37 °C. Then, CCK-8 solution (10 μL) was added to each well and cultured for another 2 hours. Finally, the absorbance of each well at 450 nm was measured by an enzyme-labelled instrument hybrid reader (H4; Synergy).

5-Ethynyl-2′-deoxyuridine staining assays

Cell light Edu Apollo kit (Ribobio) was employed to further determine the effect of the selected polypeptide on cell proliferation. Briefly, 1×10^5 cells were plated into 96-well plates, and peptides were added and cultivated. Then, 100 μL of 50 μM 5-ethynyl-2′-deoxyuridine (EdU) was added to the cells, and the cells were incubated at 37 °C for 2 hours. Then, cells were fixed, decolorised, and stained. All images in the same figure panel were acquired with the same exposure setting on a fluorescence microscope and processed identically in ImageJ (National Institutes of Health).

Transwell assay

Transwell chambers (Corning) were placed in a 24-well plate with 8-μm pores to assess migration. The hDPSCs were suspended using serum-free Dulbecco's modified Eagle's medium containing 2.5 μM peptide 1 derived from CFL1 protein (P1DC) or vehicle or 2.5 μM cell-penetrating peptides (CPP), adjusted to a concentration of 4×10^4 cells/mL and seeded separately into the upper chamber for cell migration. At the same time, 650 μL Mesenchymal Stem Cell Medium (ScienCell) was added to the lower chamber. The cells were incubated for 48 hours at 37 °C in an incubator with 5% CO_2. The chamber was removed, washed with PBS, fixed in methanol for 1 hour at room temperature, and stained with 0.1% crystal violet for 15 minutes. The upper cells of the microporous membrane were carefully removed using a cotton swab, and images were observed and collected using an inverted optical microscope. Four fields of view were randomly selected to calculate the number of cells passing through the microporous membrane.

Alkaline phosphatase activity test and staining

The hDPSCs were seeded into 6-well plates at a density of 1×10^5 cells/well. Then, they were divided into 3 groups for induction for 7 days: control group with MODM alone, CPP group with MODM containing 2.5 μM CPP, and P1DC group with MODM containing 2.5 μM P1DC. For the alkaline phosphatase (ALP) activity test, the cells were lysed overnight at 4 °C in 0.2% Triton X-100 and incubated with the ALP activity kit (Sigma-Aldrich; Merck KGaA) following the manufacturer's instructions. The absorbance value of each sample was measured at a wavelength of 520 nm using an automatic microplate reader. For ALP staining, cells were fixed in 4% paraformaldehyde for 15 minutes at room temperature and incubated following the protocol of the NBT/BCIP Staining Kit (Beyotime). The stained cells were observed and imaged using an inverted microscope.

Real-time fluorescence quantitative polymerase chain reaction

The hDPSCs were lysed with TRIzol reagent (Invitrogen, Thermo Fisher Scientific), and total RNA was extracted using the phenol-chloroform extraction method. The RNA concentration was detected by an ultraviolet spectrophotometer, and A260/A280 was measured. Then, the extracted total RNA was reverse transcribed into complementary DNA according to the instructions of the reverse transcription kit (Vazyme). Real-time fluorescence quantitative polymerase chain reaction was completed using the SYBR Green (Thermo Fisher Scientific) method. Primer sequences were the following:

DSPP: forward, 5′-ATATTGAGGGCTGGAATGGGGA-3′; reverse, 5′-TTTGTGGCTCCAGCATTGTCA-3′

GAPDH: forward, 5′-AGTCCACTGGCGTCTTCACC-3′; reverse, 5′-TGATCTTGAGGCTGTTGTCATACTTC-3′

Relative expression level was computed using the $2^{-\Delta\Delta Ct}$ method.

Protein extraction and Western blotting

The hDPSCs were solubilised in lysis buffer (Beyotime) for 20 minutes, followed by centrifugation at 4 °C at 14,000g for 20 minutes. The protein concentrations were detected using a Protein Assay kit (Beyotime). The prepared protein samples were separated on sodium dodecyl sulfate −polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Millipore). Membranes were then blocked in 5% skim milk powder (Biofroxx) at room temperature for 2 hours and then incubated with primary antibody at 4 °C overnight. Membranes were washed 3 times with TBST, incubated with secondary antibody for 2 hours at room temperature, washed 3 times with TBST again, and then developed with ECL luminescent solution (Millipore). The primary antibodies used were GAPDH (1:1000; cat. ab128915; Abcam) and DSPP (1:100; cat. sc 73632; Santa Cruz Biotechnology). The secondary antibody was horseradish peroxidase−linked goat anti-rabbit IgG (dilution in 1:5000; Cell Signaling Technology, #7074) or goat anti-mouse IgG (1:10,000; cat. 926 32210).

Statistical analysis

The differences between groups were assessed using an unpaired Student t test using the SPSS 20.0 software package. $P < .05$ was considered statistically significant. The threshold value | fold change | $\geq$1.5 or $\leq$0.667 and $P \leq .05$ were used to screen differentially expressed peptides. Since the peptides were identified only in the differentiated hDPSCs-CM group, they were defined as the upregulated group of differentially expressed peptides; conversely, they were defined as the downregulated group of differentially expressed peptides.

Results

Isolation and characterisation of hDPSCs

Pulp tissues obtained from teeth were used to extract the hDPSCs used in this study (Figure 1A). Systematic characterisation confirmed that hDPSCs retained typical morphologic features (spindle-shaped fibroblast-like morphology) (Figure 1B). After adding the respective inducers, the hDPSCs exhibited multidirectional differentiation potential, such as adipogenesis (detected using oil red O staining) and osteogenesis (detected using ARS staining) (Figure 1C). Additionally, hDPSCs were characterised using flow cytometry analysis, which revealed that isolated hDPSCs were positive for CD29, CD146, CD90, and CD105 staining and negative for CD45 and CD34 staining (Figure 1D). These results confirmed that the hDPSCs were successfully isolated.

Secreted Peptide profiling of undifferentiated and differentiated hDPSCs-CM

The peptides in hDPSCs-CM were extracted and identified by the TMT labelling method combined with LC-MS/MS (Figure 2A). A total of 1349 peptides were identified in the undifferentiated and differentiated hDPSCs-CM. The length of peptides ranged from 6 to 14 amino acids, but most peptide fragments fell into the 12 to 14 amino acid range (Supplementary Figure 1A). The MW of most identified peptides ranged from 500 to 1800 Da (Supplementary Figure 1B), and the pI values of peptides were uniformly distributed between 3 and 13 (Supplementary Figure 1C).

Characteristics of differentially expressed secreted peptides from undifferentiated and differentiated hDPSCs-CM

The fold change was defined as the ratio of the average concentrations between the 2 peptide groups. If the concentration of the differentiated hDPSCs-CM group was greater than that of the undifferentiated hDPSCs-CM group (| fold change | $\geq$1.5 or $\leq$0.667 and $P \leq .05$), and the peptides were only identified in the differentiated hDPSCs-CM group, they were defined as the upregulated group of differentially expressed peptides; conversely, they were defined as the downregulated group of differentially expressed peptides. As shown in the pie chart (Figure 2B and Supplementary Table 1), we identified 90 differentially expressed peptides, including 66 upregulated peptides and 24 downregulated peptides.

We also analysed the general characteristics of these differentially expressed peptides. As shown in Figure 2C, the MW distribution presented a wide range, mostly from 600 to 2000 Da. The pI values of the upregulated peptides were mainly in the ranges of 3 to 4, 5 to 6, and 9 to 10, whereas those of the downregulated peptides were mostly acidic and mainly in the ranges of 3 to 4, 5 to 6, and 6 to 7 (Figure 2D). Both the MW and pI of these peptides were broadly distributed (Figure 2E).

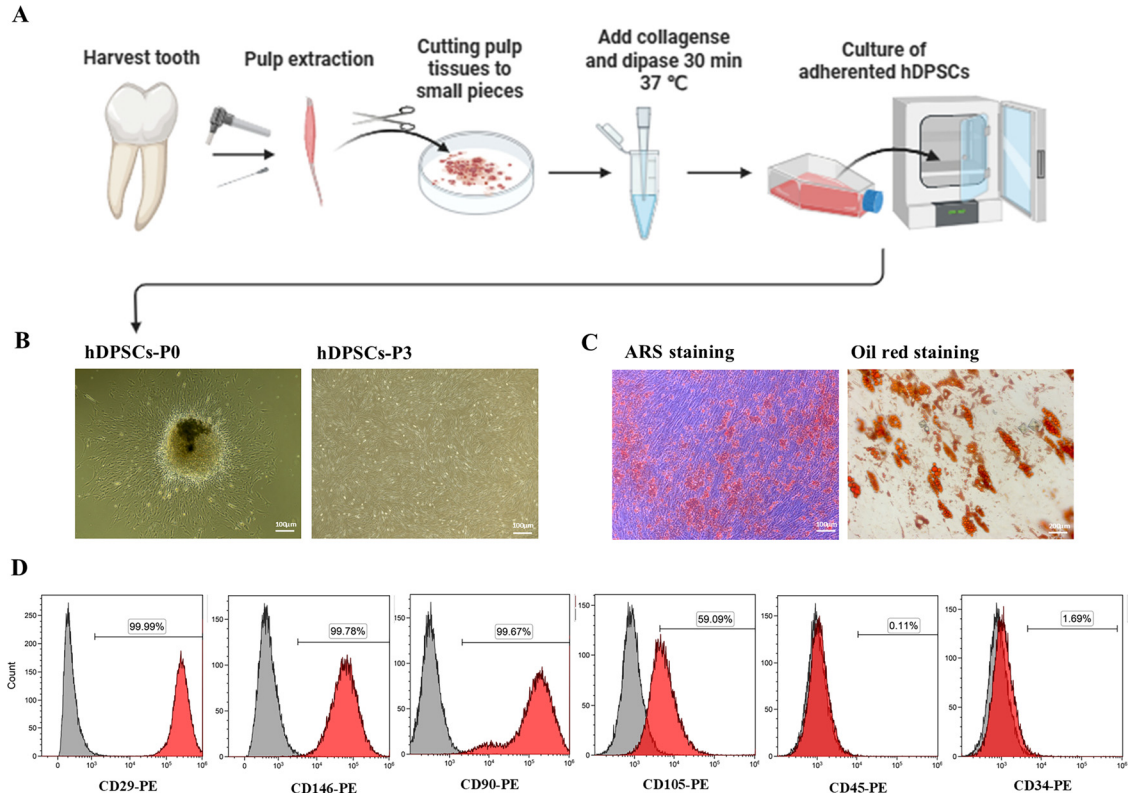


Fig. 1 – Isolation and characterisation of human dental pulp stem cells (hDPSCs). A, Schematic representation of the method for hDPSC isolation from pulp tissue. B, Light microscopy of hDPSCs at different culture passages (P0 and P3). C, HSPSCs exhibited the potential for osteogenesis (alizarin red S staining) and adipogenesis (oil red O staining). D, Flow cytometry for CD29, CD146, CD90, CD105, CD45, and CD34 surface markers.

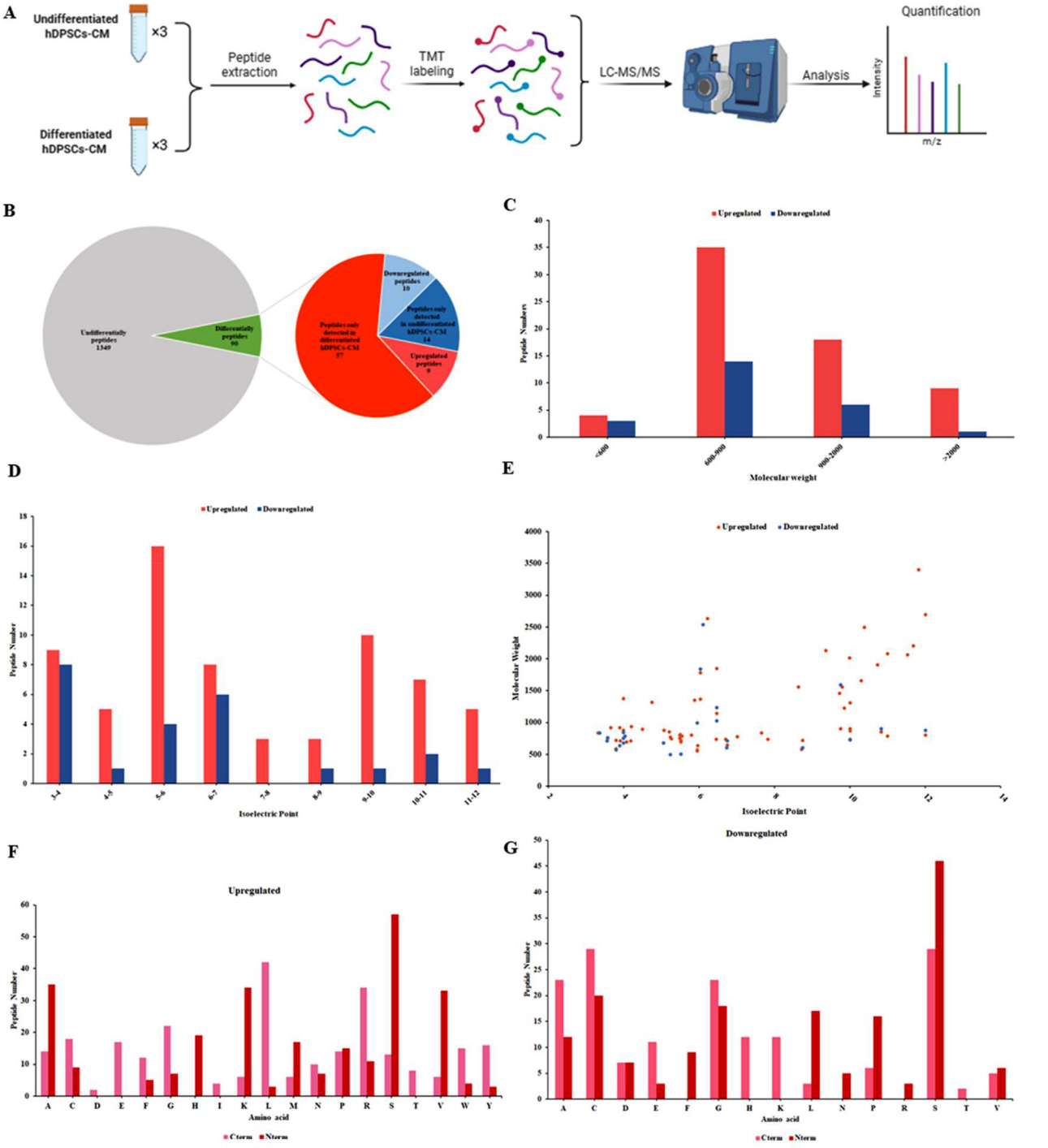


Fig. 2 – Secreted peptide characterisation of the undifferentiated and differentiated human dental pulp stem cells–conditioned medium. A, Schematic representation of the method of the secreted peptide isolated and identified by liquid chromatography (LC)–tandem mass spectrometry. B, Distribution of the differentially expressed peptides. C, D, Molecular weight (MW) and isoelectric point (pI) of the differentially expressed peptides. E, Scatterplot of MW versus PI of the differentially expressed peptides. F, G, Distributions of the N- and C-terminals of the differentially expressed peptides.

Pattern analysis of enzyme cleavage sites

The level of peptides is regulated by the activity of specific proteases, which release them from their respective precursor proteins. Therefore, the cleavage site characteristics of differentially expressed peptides at the C-terminus and N-terminus of the peptides were analysed. As shown in Figure 2F, G, the 2 dominant cleavage sites in the upregulated peptides were L (leucine) (17%) and S (serine) (22%), whereas those in downregulated peptides were C (cysteine)/S (serine) (18%) and S (serine) (28%).

Bioinformatic analysis of the precursor proteins of the differentially expressed peptides

Most peptides function as part of their precursors or as inactivators of their precursors.[13] To further investigate their potential function, GO functional annotation and KEGG pathway enrichment analysis of the precursor proteins of differentially expressed peptides were performed. The results of the GO function annotation enrichment analysis consist of 3 components: cellular component, molecular function ontologies, and biological process. As shown in Figure 3A, the top 3 cellular components involved cell junction, synapse, and synapse part; the top 3 molecular functions involved antioxidant activity, structural molecule activity, and transporter activity; the top 5 biological processes involved behaviour, cell proliferation, growth, reproduction, and reproductive process. KEGG pathway analysis (Figure 3B) showed that the precursor proteins of differentially expressed peptides were enriched in the longevity-regulating pathways of multiple species, PI3K-Akt signalling pathway, platelet activation, and extracellular matrix (ECM) −receptor interaction, among others. The protein-protein interaction was analysed by the UniProt database and STRING, the visualisation results of which are shown in Figure 3C. Within the protein-protein interaction network, the precursor proteins of differentially expressed peptides were divided into 3 clusters based on interaction strength and biological relevance. The red module mediates stress and DNA damage responses to govern cell fate, the blue epigenetic module modulates gene transcription via chromatin remodelling, and the green ECM module orchestrates the microenvironment to link intracellular signalling with tissue homeostasis. This modular organisation reveals the coordinated functions of precursor proteins during odontogenic differentiation, offering a mechanistic basis for their biological roles.

Screening of bioactive peptides

Based on the above bioinformatics results, we used UniProt to further predict the functions of the 4 most significantly upregulated precursor proteins in differentiated hDPSCs-CM. The heatmap of these 4 peptides in hDPSCs-CM is shown in Figure 3D. Combined with a literature review, we found that CFL1 (cofilin 1, actin filament depolymerising protein 1),[14] DNM3 (dynamin 3),[15] ANXA2P2 (annexin A2 pseudogene 2),[16] and KCNH7 (potassium voltage-gated channel subfamily H member 7)[17] are closely related to cell proliferation and differentiation. Therefore, we selected these 4 peptides to test their effects on hDPSCs to further investigate the function of the differentially expressed peptides. The characteristics of these 4 peptides were analysed using ProtParam (https://web.expasy.org/protp aram/) (Figure 3E).

Since these 4 peptides are all derived from the cell supernatant, we further added a CPP sequence (GRKKRRQRRRPPQQ) to the N-terminus of each of these 4 original peptides. Therefore, 8 peptides, including 4 original peptides and 4 peptides with the CPP sequence, were selected. These 8 peptides were then chemically synthesised and added to the culture medium of hDPSCs at different concentrations of 0, 1, and 10 μM. The CCK-8 results showed that peptide 1 (Ac-GRKKRRQRRRPPQQASGVAVSDGVIKVF-NH2), derived from the precursor protein CFL1 and named P1DC, could significantly increase the proliferation of hDPSCs. Therefore, we chose P1DC for our preliminary functional study (Figure 4A).

Using UniProt analysis, P1DC was determined to be located on the actin-binding domain of the precursor protein CFL1 at 2-15 aa with the aa sequence ASGVAVSDGVIKVF (Figure 4B). The secondary structure of P1DC was predicted using UCSF ChimeraX (version: 1.11rc202512130101) (Figure 4C). The original sequence of P1DC was highly conserved in humans, mice, *Bos taurus*, and rabbits (Figure 4D).

P1DC can significantly promote proliferation and migration of hDPSCs

To further explore the effects of P1DC on the proliferation of hDPSCs, CCK-8 was used to determine the optimal concentration of P1DC that promotes the proliferation of hDPSCs. The results indicated that the optimal concentration of P1DC for in vitro culture of hDPSCs was 2.5 μM (Figure 5A).

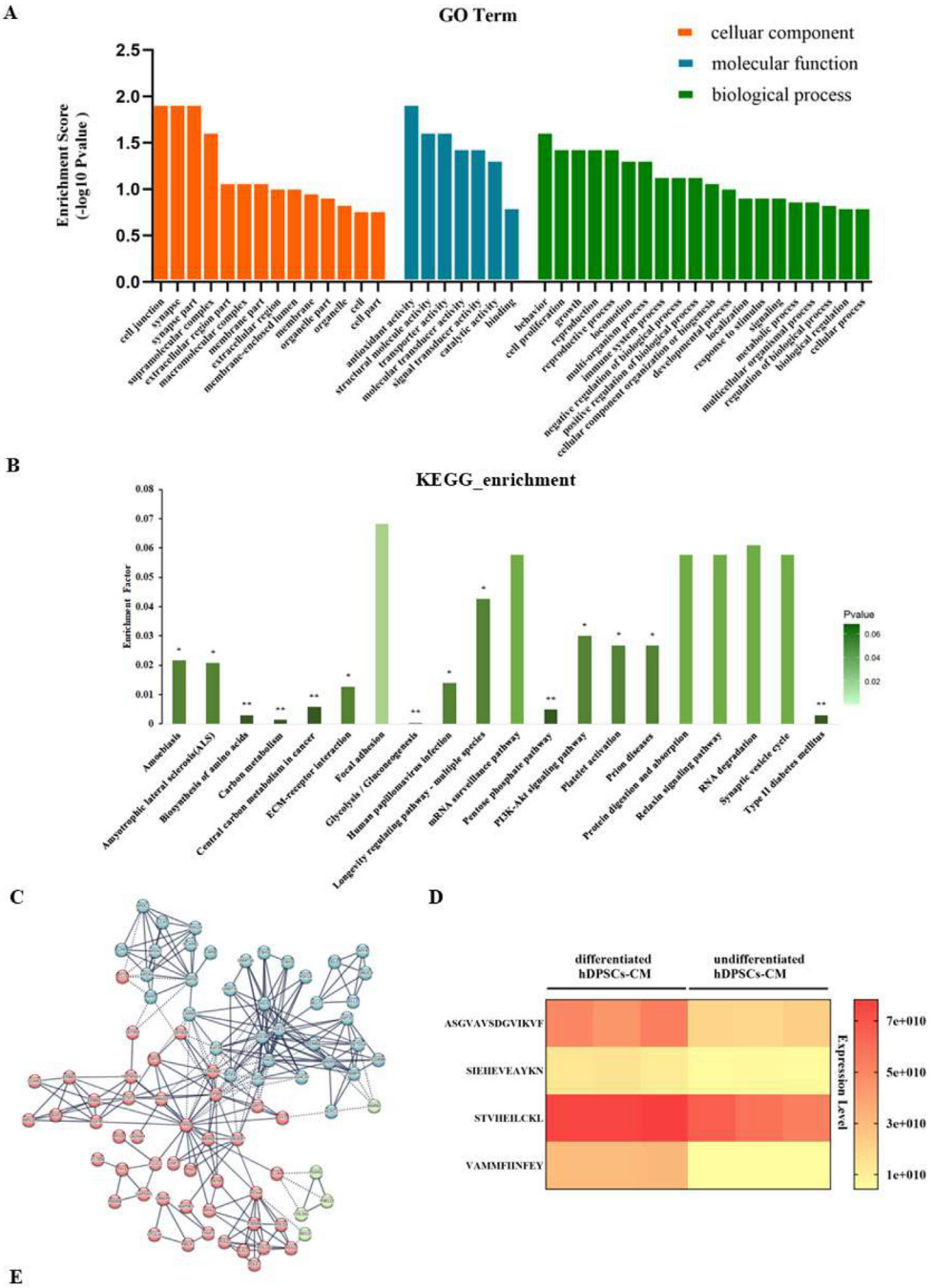
The results of the cell cycle analysis experiment showed that the proportion of cells in the G0/G1 and G2/M phases in the P1DC group was higher than in the control and CPP groups (Figure 5B). In the EdU assay, the nuclei of all cells were stained blue by Hoechst dye, and the nuclei of cells in the DNA replication phase could be stained red by EdU. By calculating the proportion of EdU-positive cells, it was found that the EdU-positive rate of the P1DC group was higher than in the control and CPP groups (Figure 5CD). The transwell assay showed that P1DC can promote cell migration of hDPSCs (Figure 5E). The above experimental results confirmed that the cells in the P1DC group had stronger proliferation activity than those in the control group; that is, P1DC promoted the proliferation and migration of hDPSCs.

P1DC can significantly promote odontogenic differentiation of hDPSCs

Cells in the P1DC, control, and CPP groups were simultaneously subjected to MODM. ALP staining and ALP activity were performed on day 7. On day 14, the messenger RNA and protein expression levels of the odontogenic differentiation-related gene DSPP were detected, and the degree of ARS staining and quantitative analysis were observed. The results showed that after the addition of P1DC, the ALP activity and mineral nodule formation ability of hDPSCs increased (Figure 6A-C), and the messenger RNA and protein expression levels of differentiation-related gene DSPP rose (Figure 6D-F).

Discussion

Odontogenic differentiation is of great significance for understanding the mechanism of pulp repair. hDPSCs play a key role in regulating odontogenic differentiation. In 2006, the International Society for Cell and Gene Therapy established the minimal criteria for identifying mesenchymal stem cells[16]: (1) static adherence, (2) specific surface antigen (Ag)



E

Peptide sequence	Precursor protein	Molecular weight	Isoelectric point	Stability	Fold change	P-value
ASGVAVSDGVIKVF	P23528/CFL1	1348.56	5.88	Stable	2.4230237	0.00110459
SIEHEVEAYKN	Q9UQ16/DNM3	1318.41	4.75	Unstable	1.8770812	0.00920109
STVHEILCKL	P07355/ANXA2P2	1142.38	6.46	Stable	3.8537007	9.88E-05
VAMMFIINFEY	Q9NS40/KCNH7	1377.68	4.00	Stable	4.9364705	0.00830531

Fig. 3 – Bioinformatic analysis of the precursor proteins of the differentially expressed peptides. A, Gene Ontology enrichment analysis results. B, Kyoto Encyclopedia of Genes and Genomes pathway enrichment. C, Protein-protein interaction network analysis based on STRING: red module mediates stress and DNA damage responses to govern cell fate, blue epigenetic module modulates gene transcription via chromatin remodelling, and green extracellular matrix module orchestrates the microenvironment to link intracellular signalling with tissue homeostasis. D, Heatmap of the 4 selected peptides in undifferentiated and differentiated human dental pulp stem cells–conditioned medium. E, The characteristics of 4 selected peptides. *P < .05 and **P < .01.

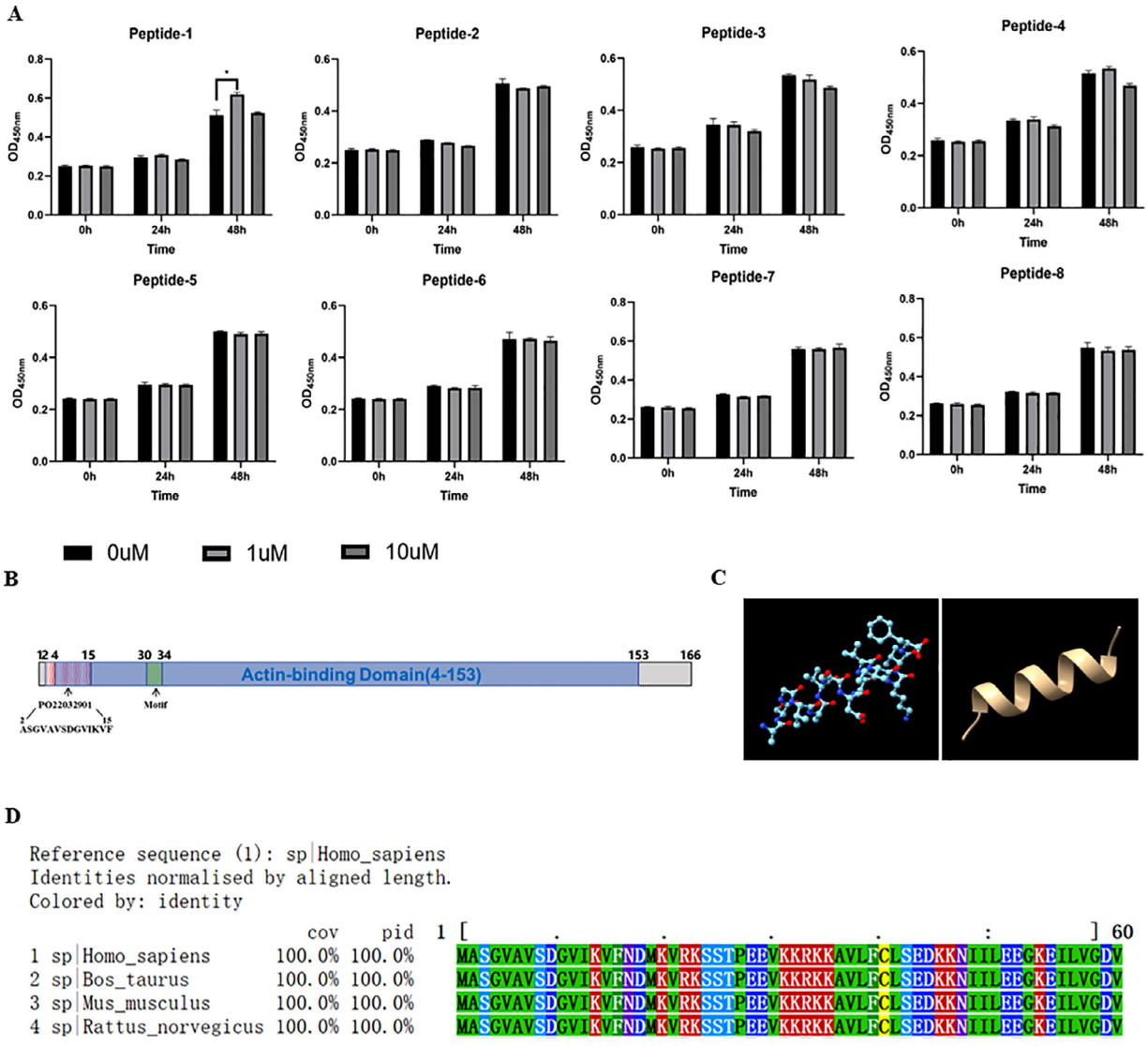


Fig. 4 – Identification of the novel functional peptide P1DC. A, CCK-8 assay of human dental pulp stem cells treated with 0, 1, and 10 μM peptides 1-8 for 0, 24, and 48 hours. B, The peptide P1DC is located on the actin-binding domain of the precursor protein CFL1 at 2-15. C, Secondary structure prediction of P1DC. D, Conservation analysis of the original sequence of P1DC. *P < .05.

expression, and (3) multipotent differentiation potential. Mesenchymal stem cells also constitute a heterogeneous population, and their surface antigen expression is nonspecific[18]; they may express markers associated with stromal, epithelial, and other cell types, such as STRO-1, CD44, CD29, and CD90, but do not express markers of hematopoietic cells such as CD34 and CD45. STRO-1 is expressed at low levels in hDPSCs, and Huang et al[19] found that STRO-1 can serve as an early surface marker for hDPSCs, with its expression decreasing as cell passage number increases. Therefore, this study assessed hDPSCs by examining the expression of CD29, CD44, CD90, CD45, CD146, and CD34, as well as their multipotent differentiation potential.

Advances in scientific research have established secreted peptides as functional biological entities. These peptides are synthesised within cells and subsequently released into the extracellular space, where they play pivotal roles in diverse physiological processes, including intercellular communication, differentiation regulation, and metabolic homeostasis. Their release is mediated through various mechanisms, encompassing classical secretory pathways, exocytosis, and passive diffusion.[20] Consequently, secreted peptides hold significant promise for therapeutic and biotechnological applications. He et al[21] also found that bone-derived mesenchymal stem cells secreting the antimicrobial peptide PK34 (Plent-PK34-BMSCs) can reduce infiltration and congestion of inflammatory cells in lung tissue, significantly reduce lung injury, and preserve lung structure better.

Furthermore, given the limited dynamic range of analytical techniques, a central challenge in peptidomics is the effective extraction of the complete peptide repertoire from complex clinical tissue samples. This process must preserve

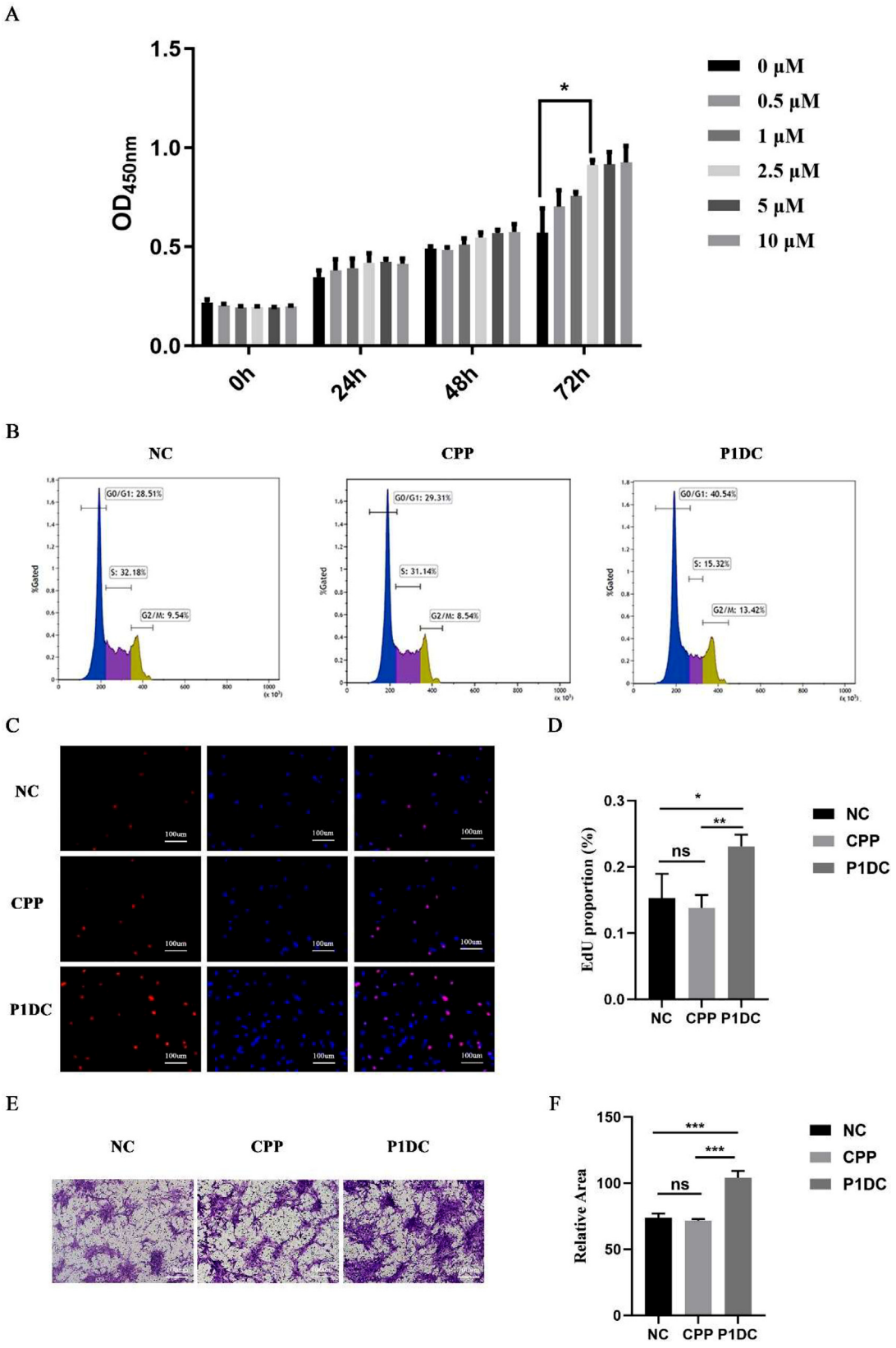


Fig. 5 – Promotion of human dental pulp stem cell (hDPSC) proliferation and migration by the P1DC peptide. A, The optimal concentration was determined through a CCK-8 assay. B, Flow Cytometric analysis of proliferation. C, EdU staining images of hDPSCs. D, Quantification of EdU-positive cell ratio. E, Transwell assay of hDPSCs. F, Quantification of Transwell assay. ns: $P > .05$, *$P < .05$, **$P < .01$ and ***$P < .001$. CPP, cell-penetrating peptide; P1DC, peptide 1 derived from CFL1 protein; NC, normal control.

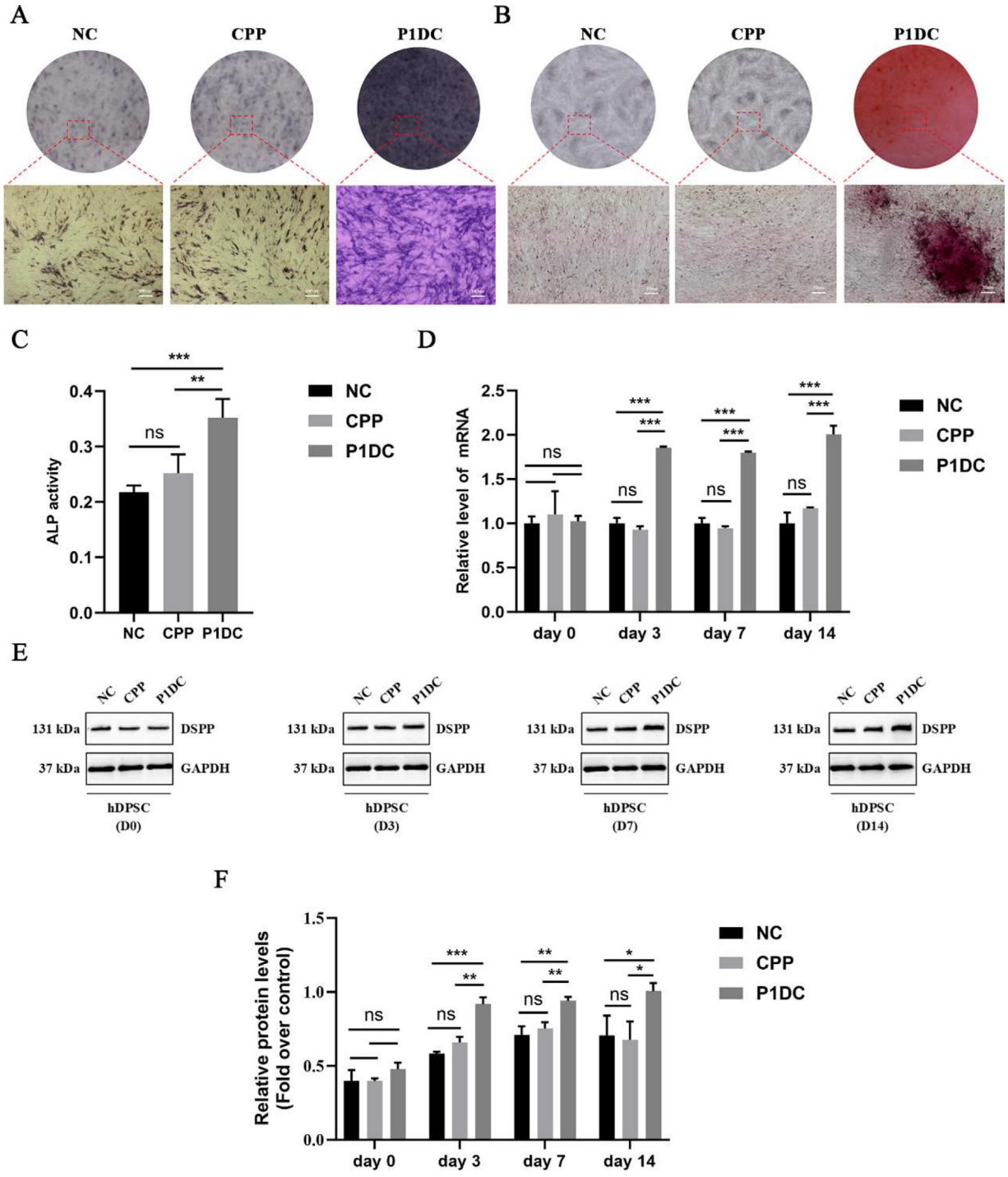


Fig. 6 – Promotion of human dental pulp stem cell (hDPSC) odontogenic differentiation by the P1DC peptide. A, Alkaline phosphatase (ALP) staining. B, Alizarin red S staining. C, Detection of ALP activity. D, The messenger RNA expression of the DSPP gene was detected by quantitative polymerase chain reaction. E, Western blot analysis of DSPP protein expression in hDPSCs at days 0, 3, 7, and 14 following NC or PIDC treatment. GAPDH was used as the control for normalisation. F, Quantitative analysis of DSPP protein levels based on Western blot band intensities. ns: $P > .05$, $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$. CPP, cell-penetrating peptide; P1DC, peptide 1 derived from CFL1 protein; NC, normal control.

their native state, avoid potential confounding factors, exclude interference from larger proteins, and enable the subsequent detection of all peptides present. This study used LC-MS/MS to analyse cell culture supernatants. Quantification was performed based on peptide peak intensity, peak area, and liquid chromatography retention time derived from primary mass spectrometry, facilitating the screening of differentially expressed peptides across

samples.[22] Thus, this approach contributes to a deeper understanding of the complex and specialised microenvironment of hDPSCs and aids in identifying novel biomarkers and therapeutic strategies.

To enhance peptide concentration and purity for LC-MS/MS analysis while minimising interference from larger proteins, molecular weight cutoff techniques were performed, which is a physical filtration-based method, offering advantages in specificity, simplicity, and speed.[23] Using a 10-kDa ultrafiltration device, 1349 peptides were identified across 6 samples in this study. Most detected peptides fell within a molecular weight range of 1500 to 2100 Da, with over 95% being under 3000 Da (Supplementary Figure 1). This result indicates efficient filtration by the 10-kDa ultrafiltration unit, supporting the suitability of the molecular weight cutoff method for peptide extraction from cell supernatant.

It has been recognised that the biological functions of peptides can be similar to those of their precursor proteins.[24] Bioinformatics analysis can effectively predict the involved signalling pathways; specifically, Wang and Gao[25] found that enrichment analysis of miR-641 target genes showed that they may participate in the mTOR and Ras signalling pathways to further regulate the progression of peri-implantitis. Our GO analysis indicates that the biological processes associated with the precursor proteins of differential peptides are closely linked to cell proliferation. These findings are consistent with the most relevant molecular functions and cellular components, namely "signal transduction" and the "membrane region." This suggests that these peptides, secreted by hDPSCs, participate in cell proliferation via signal transduction mechanisms. KEGG pathway enrichment analysis revealed that the precursor proteins of the differential peptides were closely associated with carbon metabolism and ECM-receptor interaction. It has been reported that the 1-carbon metabolite S-adenosylmethionine enhances m6A methylation and acts as a potential promoter of osteogenesis in bone marrow mesenchymal stem cells, significantly promoting osteogenic differentiation.[26] Research by Wu et al[27] also indicated that KLF6 facilitated glutamine influx into the tricarboxylic acid cycle, leading to increased citrate deposition in the ECM and thereby promoting reparative dentin formation. Bioinformatic analyses suggest these differentially expressed polypeptides may participate in signalling pathways regulating the odontogenic differentiation of hDPSCs, potentially via methylation or by modulating ECM formation. Previous studies have identified peptides secreted by adipose-derived stem cells that are also associated with cell cadherins and the extracellular matrix.[28] Our findings also suggest that the identified peptides primarily function in relation to the ECM. However, the secreted peptides exhibit considerable variation, likely attributable to differences between adipocytes and hDPSCs. Notably, the activation status and causal regulatory roles of these pathways have not been directly verified in the present study through protein-level detection or pathway-targeted intervention experiments. The mediating roles of these pathways represent only speculative results based on bioinformatics analysis, and their exact regulatory functions warrant further in-depth mechanistic studies for confirmation.

Peptide stability is a critical determinant of its in vivo half-life, biological activity retention, and translational potential, as unstable peptides are prone to rapid degradation by proteases in the physiological microenvironment, which significantly shortens their residence time and weakens their functional efficacy.[29] For dental tissue regeneration applications, the instability of the DNM3-derived peptide may limit its direct clinical utility, as sustained peptide activity is required to regulate odontogenic differentiation of hDPSCs and promote dentin repair.[30] To address this limitation, future studies could adopt structural modification strategies (eg, amino acid substitution, cyclization, or conjugation with biocompatible carriers) to enhance peptide stability, prolong its half-life, and improve its bioavailability—approaches that have been successfully applied to optimise the translational potential of bioactive peptides in tissue regeneration.[31,32] This modification would not only preserve the biological function of the secreted peptides involved in this article, including P1DC and DNM3-derived peptide, but also lay a foundation for its future clinical translation in dentin regeneration therapies, which is worthy of further research.

Polypeptide drugs have garnered significant research interest in recent years, owing to their low molecular weight, high specificity, low propensity for inducing drug resistance, and ease of modification.[33] Given the abundance of polypeptides in the human body, we analysed differentially expressed polypeptides during the differentiation of hDPSCs, identifying 66 upregulated and 24 downregulated species. Based on bioinformatics analysis, 4 peptides that were upregulated during differentiation and located within functional domains of interest were selected for synthesis. Since the polypeptides we identified were derived from the cell culture supernatant, the CPP sequence was conjugated to each of these 4 parent peptides to facilitate cellular uptake. We found that 2.5 μM P1DC significantly promoted hDPSC proliferation, migration, and differentiation, indicating that the peptide itself is not cytotoxic and can function effectively at relatively low concentrations. These results further suggest that bioactive peptides involved in odontogenic differentiation exist in vivo. Therefore, future studies should investigate the specific mechanisms by which P1DC promotes hDPSC odontogenic differentiation, and animal experiments are required to validate the function of P1DC.

The peptidomic analysis in this study has certain limitations. Some peptides were specifically present or absent in the conditioned medium of differentiated and undifferentiated hDPSCs, making it impossible to calculate valid *P* values for them. Therefore, multiple testing corrections such as the Benjamini-Hochberg false discovery rate were not performed, which may increase the risk of false-positive results in large-scale peptide screening. However, the main purpose of this study was not to identify biomarkers but to explore the biological functions of peptides. Subsequent experiments have confirmed that peptide P1DC exhibits clear biological functions.

Our study identified the differentially expressed peptides secreted by undifferentiated and differentiated hDPSCs-CM using TMT-based LC-MS/MS technology. In addition, through bioinformatics analysis, we explored a novel peptide named P1DC derived from differentiated hDPSCs-CM and demonstrated that it promoted proliferation, migration, and odontogenic differentiation of hDPSCs at a low concentration (2.5 μM) in vitro. This study expands our knowledge of the secretory components of hDPSCs and may provide insights into new treatments for reparative dentin and pulp repair.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author contributions

Yanhua Huang contributed to funding acquisition, methodology, project administration, resources, and writing—original draft. Shu Liu contributed to data curation, investigation, and visualisation. Huaying Wu contributed to funding acquisition. Zheng Zhang and Haiyan Chen contributed to investigation. Kaipeng Xie contributed to formal analysis, software, and writing—reviewing and editing. Shoushan Bu contributed to conceptualisation, supervision, validation, and writing—reviewing and editing.

Funding

This work was supported by grants from the Nanjing Health Science and Technology Development Fund project (YKK20138) and the Nanjing International/Hong Kong, Macao, and Taiwan Science and Technology Cooperation Project (202401038).

Data availability

All data used in this work can be acquired from the corresponding author upon reasonable request.

Ethics approval and consent to participate

This study was carried out following the Declaration of Helsinki and was approved by the Ethics Committee of the Women's Hospital of Nanjing Medical University (No. 2022KY-001-01). Informed consent was obtained from all participants involved in the study.

Supplementary materials

Supplementary material associated with this article can be found, in the online version at doi:10.1016/j.identj.2026.109616.

REFERENCES

1. Pitts NB, Zero DT, Marsh PD, et al. Dental caries. Nat Rev Dis Primers 2017;3:17030.
2. Li X, Fan W, Fan B. Dental pulp regeneration strategies: a review of status quo and recent advances. Bioact Mater 2024;38:258–75.
3. Quigley RM, Kearney M, Kennedy OD, et al. Tissue engineering approaches for dental pulp regeneration: the development of novel bioactive materials using pharmacological epigenetic inhibitors. Bioact Mater 2024;40:182–211.
4. Lu H, Mu Q, Ku W, et al. Functional extracellular vesicles from SHEDs combined with gelatin methacryloyl promote the odontogenic differentiation of DPSCs for pulp regeneration. J Nanobiotechnol 2024;22(1):265.
5. Ahmad P, Estrin N, Farshidfar N, et al. Isolation methods of exosomes derived from dental stem cells. Int J Oral Sci 2025;17(1):50.
6. Marcolli G, Barone L, Baranzini N, et al. Tissue regenerative potential of human dental pulp mesenchymal stem cell conditioned medium in wound healing of the medicinal leech. Stem Cell Res Ther 2025;16(1):673.
7. Swanson WB, Gong T, Zhang Z, et al. Controlled release of odontogenic exosomes from a biodegradable vehicle mediates dentinogenesis as a novel biomimetic pulp capping therapy. J Controlled Release 2020;324:679–94.
8. Barone L, Cucchiara M, Palano MT, et al. Dental pulp mesenchymal stem cell (DPSCs)–derived soluble factors, produced under hypoxic conditions, support angiogenesis via endothelial cell activation and generation of m2-like macrophages. J Biomed Sci 2024;31(1):99.
9. Madsen CT, Refsgaard JC, Teufel FG, et al. Combining mass spectrometry and machine learning to discover bioactive peptides. Nat Commun 2022;13(1):6235.
10. Abbas M, Lipiński WP, Wang J, et al. Peptide-based coacervates as biomimetic protocells. Chem Soc Rev 2021;50(6):3690–705.
11. AÉ Silva-Carvalho, MH Cardoso, Alencar-Silva T, et al. Dissecting the relationship between antimicrobial peptides and mesenchymal stem cells. Pharmacol Ther 2022;233:108021.
12. Gronthos S, Mankani M, Brahim J, et al. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proc Natl Acad Sci U S A 2000;97(25):13625–30.
13. Cao S, Zhou P, Shen G, et al. Binary peptide coacervates as an active model for biomolecular condensates. Nat Commun 2025;16(1):2407.
14. Chen L, Shi K, Frary CE, et al. Inhibiting actin depolymerization enhances osteoblast differentiation and bone formation in human stromal stem cells. Stem Cell Res 2015;15(2):281–9.
15. Ding W, Yousefi K, Goncalves S, et al. Osteopontin deficiency ameliorates Alport pathology by preventing tubular metabolic deficits. JCI insight 2018;3(6):e94818.
16. Huang R, Yang Q, Wang T. Norcantharidin-blocked anxa2p2 inhibits fibroblast proliferation by increasing ubap2l mRNA stability through lin28b. Life Sci 2021;279:119645.
17. Wang L, Li X, Xu C, et al. Unveiling novel cell clusters and biomarkers in glioblastoma and its peritumoral microenvironment at the single-cell perspective. J Transl Med 2024;22(1):551.
18. Zhang Y, Jia F, Li K, et al. Fibulin1 as a novel target for promoting the biological function of bone marrow mesenchymal stem cells and implant osseointegration in diabetic patients. Int Dent J 2025;75(6):103928.
19. Huang AH, Chen Y, Chan AW, et al. Isolation and characterization of human dental pulp stem/stromal cells from nonextracted crown-fractured teeth requiring root canal therapy. J Endod 2009;35(5):673–81.
20. Zhang T, Ai D, Wei P, et al. The subcommissural organ regulates brain development via secreted peptides. Nat Neurosci 2024;27(6):1103–15.
21. He X, Wang J, Chen Y, et al. Antimicrobial peptide pk34 modification enhances the antibacterial and anti-inflammatory effects of bone-derived mesenchymal stem cells in mycobacterium tuberculosis infection. Stem Cell Res Ther 2025;16(1):469.
22. Mary Celin S, Sharma B, Bhanot P, et al. Trends in environmental monitoring of high explosives present in soil/sediment/groundwater using LC-MS/MS. Mass Spectrom Rev 2023;42(5):1727–71.

23. Cassano A, Conidi C, Ruby-Figueroa R, et al. Nanofiltration and tight ultrafiltration membranes for the recovery of polyphenols from agro-food by-products. Int J Mol Sci 2018;19(2):351.
24. Chen G, Xu T, Yan Y, et al. Amyloid beta: structure, biology and structure-based therapeutic development. Acta Pharmacol Sin 2017;38(9):1205–35.
25. Wang M, Gao J. Mir31hg promotes the onset and progression of peri-implantitis by regulating the mir-641 levels. Int Dent J 2026;76(2):109375.
26. Zhang W, Bai Y, Hao L, et al. One-carbon metabolism supports S-adenosylmethionine and m6A methylation to control the osteogenesis of bone marrow stem cells and bone formation. J Bone Miner Res 2024;39(9):1356–70.
27. Wu W, Xu Z, Zhang Y, et al. Klf6-mediated glutamine metabolism governs odontogenic differentiation and matrix mineralization of dental pulp stem cells. Stem Cell Res Ther 2025;16(1):179.
28. Li J, Yin Y, Zou J, et al. The adipose-derived stem cell peptide adscp2 alleviates hypertrophic scar fibrosis via binding with pyruvate carboxylase and remodeling the metabolic landscape. Acta Physiol 2023;238(4):e14010.
29. Zhang L, Yao L, Zhao F, et al. Protein and peptide-based nanotechnology for enhancing stability, bioactivity, and delivery of anthocyanins. Adv Healthc Mater 2023;12(25):e2300473.
30. Xie Z, Jiang W, Liu H, et al. Antimicrobial peptide- and dentin matrix-functionalized hydrogel for vital pulp therapy via synergistic bacteriostasis, immunomodulation, and dentinogenesis. Adv Healthc Mater 2024;13(18):e2303709.
31. Najafi H, Jafari M, Farahavar G, et al. Recent advances in design and applications of biomimetic self-assembled peptide hydrogels for hard tissue regeneration. Biodes Manuf 2021;4(4):735–56.
32. Lai Z, Yuan X, Chen H, et al. Strategies employed in the design of antimicrobial peptides with enhanced proteolytic stability. Biotechnol Adv 2022;59:107962.
33. Chen J, Liu T, Wang M, et al. Supramolecular oral delivery technologies for polypeptide-based drugs. J Controlled Release 2025;381:113549.