Supplementary Information

Cell Membrane-Coated Nanomicrospheres Mimicking Stem Cell Functions Enhance Angiogenesis for Dental Pulp Regeneration.

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1. Experimental Section

1.1 SHEDs Culture and Characterization: The exfoliated deciduous teeth were obtained from healthy donors undergoing routine dental treatment at Tianjin Stemmatological Hospital, with approval from the Ethics Committee of Tianjin Stemmatological Hospital. This study selected extracted healthy deciduous teeth from donors aged approximately 6-8 years, with less than two-thirds root resorption. The pulp tissue was rinsed thrice with PBS buffer containing 2% double antibody. Under sterile conditions, the dental pulp tissue was minced, and 1 mL of 20% α-MEM complete medium was added dropwise to the culture flask. Small pieces of pulp tissue were adhered to the flask bottom. The following day, an additional 0.5 mL of medium was added, and the pieces of tissue were removed once surrounded by crawling cells. All cells were cultured in α-MEM complete medium containing 1% penicillin/streptomycin and 20% fetal bovine serum, and SHEDs were incubated at 5 °C and 37 % CO_2. Following three passages, SHEDs were collected for identification purposes. Roughly 1 × 106 cells were suspended in PBS and treated with antibodies with fluorescent tags [CD105-PE, CD90-APC, CD45-PerCP, CD14-PerCP, CD44-FITC, CD19-APC] for a duration of 30 minutes at 4°C in darkness. After two washes with PBS, the cells were resuspended in 100 μL PBS and analyzed by flow cytometry. To induce osteogenic and adipogenic differentiation, cells underwent culture in osteogenic differentiation medium for two weeks and in adipogenic differentiation medium for four weeks, respectively. Following fixation using 4% paraformaldehyde, staining was performed using Oil Red O for 30 minutes at ambient temperature for adipogenic differentiation assessment, and Alizarin Red S for 30 minutes at 37 °C for osteogenic differentiation evaluation. Prior to visualization under light microscopy, the samples were washed thrice with PBS.

1.2 Cell Membrane Extraction: Following the centrifugation of SHEDs cells, the collected sediment was rinsed three times with chilled PBS and then reconstituted in a hypotonic buffer (comprising 1 mM $NaHCO_3$, 1 mM PMSF, 0.2 mM EDTA·2Na, and 1×PIC in an H_2O solution) at 4 °C throughout the night. Through sonication in an ice bath, cell membrane fragments were produced until the mixture became translucent. To isolate pure cell membranes, the mixture was first spun at 3200 g for 5 minutes, followed by a subsequent centrifugation of the supernatant at 16,000 g for 30 minutes. The resulting pellet, comprising cell membranes, was washed once more with PBS and then dissolved in PBS. Subsequently, the protein concentration within the cell membrane solution was quantified using a BCA assay kit. The prepared cell membrane solution was stored at -80 °C for future use.

1.3 Preparation and characterization of CF: After three passages, 3×10^6 SHEDs (stem cells from human exfoliated deciduous teeth) were incubated in T75 flasks containing 10 mL of α-MEM complete medium for 24 hours. Subsequent to apposition, the existing medium was exchanged for 10 mL of serum-free α-MEM and incubated under conditions of 21% O_2 and 1% O_2, in turn. At specified intervals (1, 3, and 5 days), CF-containing medium from both normoxic and hypoxic environments was harvested, filtered through a 0.45-micrometer membrane to

eliminate cellular debris and contaminants before being collected in a sterile centrifuge tube. The filtered CF was then preserved at -80 °C for subsequent analyses. APPLIED PROTEIN TECHNOLOGY (Shanghai, China) conducted a proteomic analysis of the CF obtained, comparing the protein composition between noCF-5d and hyCF-5d through LC-MS. Proteins exhibiting an average label-free quantification (LFQ) intensity that differed by at least two-fold across groups were deemed differentially expressed, achieving statistical relevance with a P value less than 0.05. For functional categorization, we conducted an unsupervised enrichment analysis on these proteins, utilizing the Kyoto Encyclopedia of Genes and Genomes (KEGG) for reference. mRNA levels of key proangiogenic factors were assessed through quantitative reverse transcription PCR (qRT-PCR) following 5 days under both hypoxic and normoxic conditions with SHEDs, as detailed in Table S1 of the Supporting Information. The relative expression levels of mRNA were calculated using the 2-Δ(Δt) method and normalized against GAPDH expression.

1.4 Evaluation of CF on functional vascularization of HUVECs in vitro: To evaluate the effect of hypoxic conditions on SHEDs' paracrine secretion, 1, 3, and 5 days of hy-CF and no-CF treatments were analyzed for their ability to migrate and form tubes for HUVECs. In the transwell migration assay for HUVECs, 1×10^4 cells were seeded in the upper chamber, featuring an 8-μm pore size membrane, above a 24-well plate. Culture medium enriched with CF was then introduced into the lower chamber. Migration was permitted for 12 hours at 37 °C in a 5% CO_2 atmosphere. Cells that did not migrate through the membrane were subsequently eliminated from the upper side. Those that migrated were fixed using 4% paraformaldehyde (PFA) for 15 minutes and stained with 0.1% crystal violet for 30 minutes. The stained cells were observed and counted using a microscope. During the tube formation assays, liquid matrix gel was allocated into 48-well plates, followed by a solidification process achieved through incubation at 37°C for 30 minutes. Following this, 1×10^4 HUVECs were inoculated on the solidified matrix gel and added to the collected CF-containing medium. After 12 hours of co-culture, the cells were washed twice with PBS and fixed with 4% paraformaldehyde (PFA). Observations were made using a microscope. Capillary-like structures formed in randomly selected fields of view were quantified using ImageJ software.

1.5 Evaluation of Odontogenic Differentiation of DPSCs by CF in vitro: To assess the ability of hy-CF and no-CF over 1, 3, and 5 days to induce odontogenic differentiate in DPSCs, the deposition of mineralization in vitro was evaluated using ALP staining and Alizarin Red S staining. When DPSCs reached 70% confluency, either CF-containing medium or serum-free α-MEM medium (control) was added. One week post-cultivation, cells underwent fixation with 4% paraformaldehyde (PFA), were subsequently rinsed, and stained using an Alkaline Phosphatase Chromogenic kit for 30 minutes, with subsequent microscopic examination. Quantification was achieved by employing an Alkaline Phosphatase Detection kit, with optical density readings taken at 405 nm. Following a three-week period, cells were again fixed with 4% PFA, cleansed, and stained for 30 minutes with Alizarin Red S. This allowed for the microscopic observation of mineralized nodule formation. Quantitative assessment was

conducted after dissolving the samples in a 10% cetylpyridinium chloride solution (CPC), with optical density measurements obtained at 490 nm. To analyze the mRNA levels of various odontogenic differentiation genes of DPSCs by hyCF-5d and noCF-5d, mRNA expression of treated DPSCs was determined by (qRT-PCR). Refer to Table S1 in the Supporting Information. The relative expression levels of mRNA were quantified using the 2-Δ(Δt) method and normalized against GAPDH expression.

1.6 Preparation and Characterization of Bionic Dental Pulp Stem Cells：Utilizing a water/oil/water (w/o/w) emulsion method, CF-encapsulated PLGA microparticles (PLGA-MPs) were synthesized. A 500 μL volume of concentrated hyCF-5d, serving as the inner aqueous phase (w1), was combined with 6 milliliters of dichloromethane containing 100 milligrams of PLGA and subjected to sonication at 500 W for 25 seconds in an ice bath. This mixture formed the primary emulsion, which was subsequently incorporated into 40 mL of 0.7% polyvinyl alcohol (PVA) water solution, crafting a w1/o/w2 emulsion. The secondary emulsion underwent continuous stirring at a rate of 1500 revolutions per minute for seven minutes at ambient temperature to facilitate the evaporation of the solvent. The PLGA-MPs were cleansed thrice with distilled water at 3000 rpm to remove any residual surfactant. For the membrane coating process, 4.2 μg (60 μL) of cell membranes were combined with 2.5 mg of PLGA-MPs and underwent sonication for 20 minutes. After centrifugation and three washes, Bionic Dental Pulp Stem Cells were produced. Scanning electron microscopy was employed to scrutinize the surface morphology of both PLGA-MPs and Bionic Dental Pulp Stem Cells. Additionally, BSA labeled with FITC was integrated into PLGA-MPs, while cell membranes stained with DiI were coated onto the surface of PLGA-MPs. The samples were then examined using a confocal laser scanning microscope. An analysis using flow cytometry was performed to assess the expression of surface markers on SHEDs and Bionic Dental Pulp Stem Cells. This involved incubating samples with fluorescently-labeled antibodies (CD105-PE, CD44-FITC, CD90-APC, CD45-PerCP, CD14-PerCP, CD19-APC) for 30 minutes at 4 °C, followed by triple washes with PBS. Each sample was then analyzed using flow cytometry after the washing steps.

1.7 In Vitro Release Kinetics: To evaluate the CF release pattern, pellets (4 mg/mL), encapsulated and non-encapsulated samples were immersed in 20 mL of PBS buffer (pH 7.4) and subjected to incubation at 37°C, with a mild agitation set at 150 rpm. Samples of 100 μL were taken at predetermined intervals, centrifuged at 4000 rpm for 5 minutes, and then replaced with an equivalent volume of fresh PBS to maintain the original volume. Protein release from these samples was quantified using the BCA protein assay. Additionally, the fractions released into the solution by the Bionic Dental Pulp Stem Cells were assessed using an ELISA kit. For this purpose, the solution released from the pellet, concentrated at 20 mg/mL in 5 mL of PBS buffer, underwent processing. A 100 μL aliquot of this solution was applied to a 96-well plate precoated with primary antibodies and left to incubate for 1 hour. Following this incubation, secondary antibodies, specifically anti-VEGF and anti-TGF-β1, were introduced and incubated for 15 minutes. A colorimetric reaction was then initiated, and absorbance was measured at 450 nm using an enzyme marker to determine the levels of released proteins.

1.8 In Vivo Evaluation in the Acute Hindlimb Ischemia Model: All animal experiment protocols were authorized by the Animal Ethics Committee of Nankai University, in strict adherence to the protocols recommended by the Tianjin Laboratory Animal Use and Care Committee. Under anesthesia, male BALB/c nude rats, weighing between 20-25 grams and aged 8-10 weeks, underwent surgical procedures on one hindlimb following a previously established protocol by our team. In summary, the left femoral artery was surgically exposed, and ligations were applied both proximally and distally with 6-0 silk. Incisions were then performed between these ligation points. The skin was finally sutured using 6-0 nylon stitches. To assess the viability and retention of Stem cells from human exfoliated deciduous teeth (SHEDs) and Bionic Dental Pulp Stem Cells in ischemic tissues, both cell types were modified for visualization: SHEDs were tagged with luciferase and Bionic Dental Pulp Stem Cells with Cy5. For the study, 100 μl containing 1 million SHEDs and 1 million Bionic Dental Pulp Stem Cells were injected into ischemic areas. The fluorescence from Bionic Dental Pulp Stem Cells was captured using the IVIS Lumina II system, and SHEDs were imaged 15 minutes post D-luciferin injection. Signal reduction over time was analyzed from the initial measurement point. Following injection, the mice were categorized into five groups, each receiving an intramuscular injection of PBS, CF (an amount equivalent to the Bionic group), PLGA-MP-BSA (1 million particles), SHEDs (1 million cells), or Bionic Dental Pulp Stem Cells (1 million particles). Hindlimb blood flow was monitored using Laser Doppler at predetermined intervals, and average blood perfusion was determined using the PeriCam PSI System. The outcomes regarding limb preservation, necrosis, and loss were evaluated visually using criteria that had been established beforehand. Limb condition was rated from salvage (highest) to loss (lowest). Rates of each condition were separately tallied.

Muscle tissue from mice in acute hypoxia-ischemia was collected at designated times for further analysis. Using anti-CD31-PE for staining, tissue sections underwent angiogenesis assessment. Images from various treatments (minimum of 3 mice, over 5 sections each) were taken for ImageJ software analysis, focusing on vessel presence in stained muscle, quantified by the positive area percentage.

1.9 In Vivo Assessment of Pulp Regeneration Model : Single-rooted premolar teeth, freshly extracted from healthy individuals aged 15 to 30, were obtained with the approval of the institutional review board from the Oral and Maxillofacial Surgery Clinic at Tianjin Stomatological Hospital. The preparation of the root segments mirrored previously reported methodologies. A root segment, measuring 5-6 mm in length from the tooth root's middle third and with an orifice width of 2-3 mm, was precisely cut using sterilized burs. After removing any remaining soft tissue, the roots were treated with 17% EDTA for 10 minutes at room temperature to eradicate the smear layer, subsequently sterilized using hydrogen peroxide for 5 minutes and 5.25% NaClO for 10 minutes. The roots were then cleansed with sterile PBS and kept at 37 °C for a period of 3-7 days to thoroughly remove any residual disinfectants.

The animal studies conducted received approval from the Animal Ethics Committee at Nankai University and adhered to the Tianjin Laboratory Animal Use and Care Committee's guidelines,

in line with protocols we have previously described. In brief, subcutaneous pouches were fashioned through blunt dissection on anesthetized male BALB/c nude mice, aged 6-8 weeks and weighing 20-25 g. Initially, root segments were inserted into the dorsal pockets of the mice. This was succeeded by administering an injection of 100 μL of hydrogel, which included PBS, PLGA-MP-BSA (1×10^6 particles), CF (in an amount equivalent to the Bionic Dental Pulp Stem Cells group), SHEDs (1×10^6 cells), or Bionic Dental Pulp Stem Cells (1×10^6 particles), into these segments. Each mouse had two root segments transplanted subcutaneously on its back, and the surgical site was then sutured closed. Four weeks post-operation, all animals were euthanized, and the segments were harvested, fixed in 4% PFA for 24 hours, decalcified in 10% EDTA for three months, embedded in paraffin, and sectioned into 10-μm slices. These sections were stained with H&E and Masson's trichrome for further analysis. Quantitative assessments were carried out using ImageJ software, focusing on the regenerated pulp tissue area percentage within the root canal and the vascular area percentage within the pulp.

1.10 Statistical Analysis: The data is presented as means with deviation. To determine the difference between two groups we used Student's t test. For comparing groups we employed one way analysis of variance (ANOVA) followed by multiple comparisons testing. All statistical analyses were performed using a two approach and *P* values, below 0.05 were deemed statistically significant. Statistical calculations were conducted using GraphPad Prism version 8.0 software by GraphPad Software.

2. Supplementary Table

Table S1. DNA sequences used in this work

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
GAPDH	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGGA
HIF-1α	CACCACAGGACAGTACAGGAT	CGTGCTGAATAATACCACTCACA
VEGF	AGGGCAGAATCATCACGAAGT	AGGGTCTCGATTGGATGGCA
ANG-1	AACTTTCGGAAGAGCATGGAC	CGAGTCATCGTATTCGAGCGG
PGF	GAACGGCTCGTCAGAGGTG	ACAGTGCAGATTCTCATCGCC
FGF	AGAAGAGCGACCCTCACATCA	CGGTTAGCACACACTCCTTTG
TGF-b1	GTGGGTTTCCACCATTAGCAC	GTGGGTTTCCACCATTAGCAC
OCN	CTCACACTCCTCGCCCTATTG	GCTTGGACACAAAGGCTGCAC
ALP	GACAAGAAGCCCTTCACTGC	AGACTGCGCCTGGTAGTTGT
DMP-1	CTCCGAGTTGGACGATGAGG	TCATGCCTGCACTGTTCATTC

3. Supplementary Figures

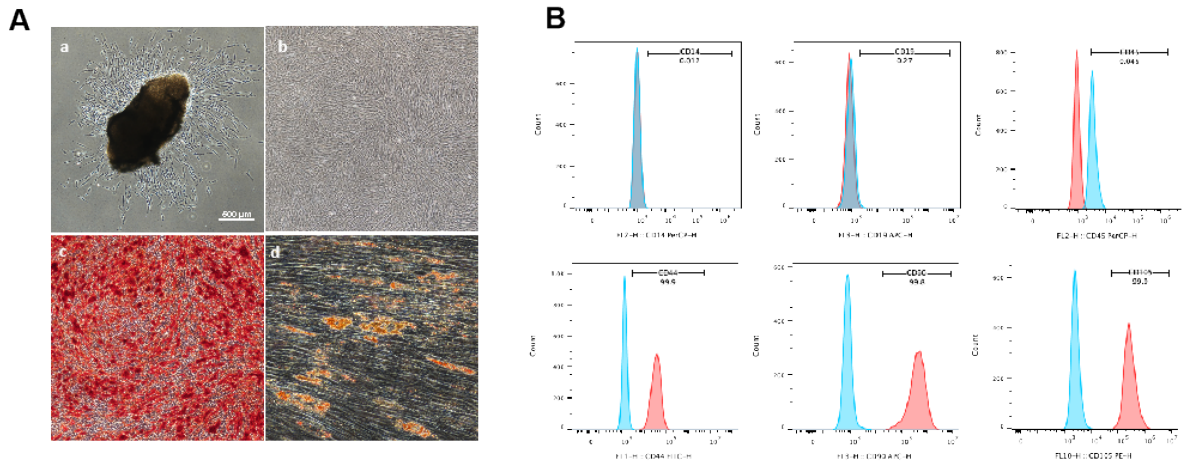

Figure 1S . Stem cells of human deciduous exfoliated teeth separation and extraction
A) Multidirectional differentiation of SHEDs; B) Flow cytometric characterization of SHEDs

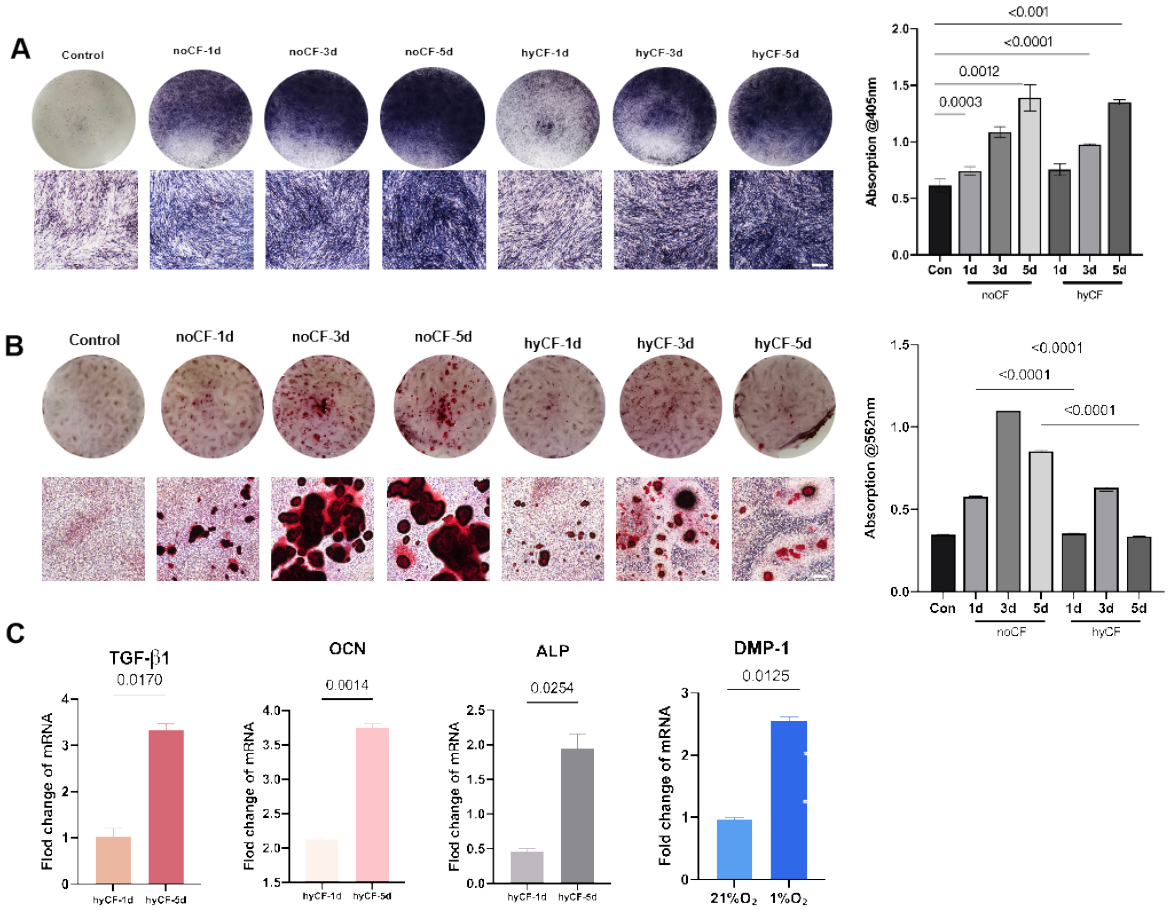

Figure 2S. In vitro mineralization behavior of DPSCs by secreted factors from SHEDs under hypoxic conditions. A) Odontogenic differentiation of DPSCs after treatment with noCF and hyCF for 7 days :ALP-stained images and inverted microscope images with quantitative analysis (scale bar 500 μm); B) Odontogenic differentiation of DPSCs after 14 days of noCF and hyCF treatment: alizarin red stained images and inverted microscope images and

quantitative analysis (scale bar 500 μm); C) The expression of relevant genes (TGF-β1, ALP, OCN, DMP-1) in DPSCs treated with hy-CF for 7 days was detected through qPCR.
(n = 3 independent samples, assessed by one-way ANOVA.)

4.A glossary of abbreviations

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Abbreviations	Explanation
RCT	Root canal therapy/treatment
SHED	Stem cells ofhuman deciduous exfoliated teeth
CM/CF	Conditioned media/factor
LC-MS	Liquid Chromatography Mass Spectrometry
noCF	noroxia-Conditioned media/factor
hyCF	hypoxia-Conditioned media/factor
PLGA	Poly lactic-co-glyeolie acid microspheres
ALP	Alkaline Phosohatase
HE	Hematoxylin-eosin staining
ELISA	Enzyme linked immunosorbent assay
HUVEC	Human Umbilical Vein Endothelial Cells
VEGF	Vaseular Endothelial Growth Factor
TGF-β1	Iransforming growth factor betal
ANG-1	Angiopoietin 1
EGF	Epidermal Growth Factor
FGF	Fibroblast Growth Factor
PGF	Placental Growth Factor
IGF-1	Insulin-like growth factor 1
BMP	Bone morphogenetie protein
HIF-1a	Hypoxia-inducible factor 1 alpha
MSC	Mesenchymal Stem Cells
DDS	drug delivery systems
KEGG	Kyoto Encyclopedia ofGenes and Genomes