A Novel Cell-Based Approach for Pulp-Dentin Complex Regeneration Using iPSC-Derived Endothelial and Epithelial Cells with DPSCs

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Directed by Professor Euiseong Kim

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감사의 글

학위 과정 동안 저를 항상 이끌어 주시고 아낌없는 지원을 해 주신 김의성 지도교수님께 깊은 감사의 말씀을 드립니다. 오랜 시간 동안 실험을 진행하며 여러 면에서 부족함과 어려움을 느낀 순간이 많았습니다. 그럴 때마다 교수님께서는 아낌없이 조언해 주시고 따뜻한 격려로 지도해 주셨기에, 제가 학위 논문을 무사히 완성할 수 있었습니다.

또한, 논문이 더 나은 방향으로 진행될 수 있도록 다양한 방법으로 도와주신 김선일 교수님과 이석준 교수님께도 감사드립니다. 교수님들의 가르침과 애정 덕분에 무사히 학위 과정을 마칠 수 있었습니다. 부족한 제 논문을 세심하게 지도해 주신 정한성 교수님과 신수정 교수님께도 깊은 감사의 말씀을 드립니다.

5년 전 보존과 전공의 과정을 시작하여 펠로우 생활을 마치기까지, 보존과 의사로 성장할 수 있도록 지도해 주신 모든 보존과 교수님들께 감사드립니다. 보존과 의국 동기와 선후배들에게도 감사의 말씀을 전하고 싶습니다.

마지막으로, 오랜 기간 학업을 이어가는 동안 아낌없는 사랑과 격려를 보내주신 아빠, 엄마, 언니, 형부, 그리고 귀여운 조카 이한이에게도 감사의 말씀을 드립니다. 특히, 저를 치과의사의 길로 인도해 주신 이모부 최성호 교수님께도 깊이 감사드립니다.

2024년 12월

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ABSTRACT

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This study investigates the odontogenic potential of DPSCs, endothelial cells, and epithelial cells. In order to treat immature permanent teeth with periapical lesion, regenerative endodontic therapy (RET) has evolved, yet challenges remain, particularly with immature permanent teeth. While traditional RET methods, such as revascularization, have shown promise, they often result in repair rather than true regeneration. This research highlights the necessity of incorporating diverse cell types to achieve effective pulp-dentin complex regeneration. Through single-cell analysis of human dental pulp, we elucidated the cellular composition and gene expression profile, revealing a unique endothelial cell population that expresses both endothelial and epithelial marker genes. Subsequently, we successfully differentiated iPSCs into endothelial and

epithelial cells, which were co-cultured with dental pulp stem cells (DPSCs). This co-culture method demonstrated significant odontogenic potential *in vivo* and *in vitro*, underscoring the importance of utilizing a multi-cellular approach for enhancing regenerative outcomes in endodontics. Our findings provide a foundation for developing targeted therapies aimed at restoring the functional integrity of the pulp-dentin complex.

Keywords : regenerative endodontic therapy, dental pulp stem cell, endothelial cell, epithelial cell, gene expression analysis, mouse transplantation, odontogenic potential

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I. Introduction

The groundbreaking study by Kakehashi et al. established microorganisms and their byproducts as the main causes of pulpal and periapical pathosis, and from that point on, the primary goals of root canal treatment have been well-defined (Kakehashi et al., 1965). These fundamental objectives include removing irritants from the root canal system mechanically and chemically, obturating the cleaned and shaped root canals, and preventing recontamination of filled root canals. However, treating necrotic immature permanent teeth with apical periodontitis becomes increasingly

difficult because of the difficulties they pose because of their thin, fracture-prone dentinal walls and open apices (Trope, 2010).

As a result, regenerative endodontic therapy (RET) has emerged as a promising advancement in this field, aiming to restore the structural and functional integrity of pulp-dentin complex. Banches and Trope published a case report of a procedure known as “revascularization” for treating necrotic immature permanent teeth that resulted in healing of periapical tissue and continued root development (Banchs & Trope, 2004). Although RET procedures such as revascularization have yielded exceptional clinical outcomes, recent studies have found drawbacks such as lack of continued root growth, recurrence of apical periodontitis, and intracanal obliteration (Lee & Song, 2022; Song et al., 2017). Histological analyses of extracted teeth that underwent revascularization revealed that the root canals were filled with connective tissue resembling periodontal ligament and hard tissue akin to bone and cementum, rather than true pulp or dentin (Becerra et al., 2014; Martin et al., 2013). This indicates that healing occurred through repair, not true regeneration, underscoring the challenge of achieving the ultimate goal of RET.

Due to the limitations mentioned above, more researchers are focusing on developing cell-based RET methods. At the forefront of this cell-based approach are dental pulp stem cells (DPSCs), which were initially acquired from third molars and validated as mesenchymal stem cells (MSCs) for their ability to differentiate into numerous types of cells (Gronthos et al., 2002). DPSCs, originating from the neural crest (Mattei et al., 2021), are of ectodermal origin (Nuti et al., 2016) and can also differentiate into functionally active neurons, showing potential use in treating neuronal disorders (Arthur et al., 2008). Notably, a study showed that DPSCs can regenerate dentin-like mineralized tissue with tubules lined with odontoblasts and pulp-like fibrous tissue with blood vessels (Gronthos et al., 2000).

However, although previous studies have attempted RET using DPSCs and have shown

promising outcomes, the complexity of the pulp-dentin tissue architecture suggests that relying solely on DPSCs may not suffice for true regeneration. Therefore, research is ongoing to identify additional cell types that could be beneficial when used alongside DPSCs. To better understand the composition of human pulp tissue and guide strategies for regeneration, dental pulp tissue was analyzed at a single-cell level in this study. This analysis provided detailed insights into its cellular composition and gene expression profiles. These findings are expected to help identify the most suitable cell combinations to recreate a microenvironment that closely mimics native pulp tissue, thereby enhancing the potential for true pulp-dentin complex regeneration.

Advancements in stem cell research have also introduced induced pluripotent stem cells (iPSCs) which have a huge potential in regenerative medicine, including endodontics. iPSCs are reprogrammed somatic cells that exhibit pluripotency, similar to embryonic stem cells (ESCs), but without the ethical concerns of ESCs (Takahashi & Yamanaka, 2006). Their ability to differentiate into a wide range of cell types has been demonstrated in various studies, such as the generation of kidney organoids from human iPSCs, which successfully modeled human nephrogenesis and contained multiple cell lineages (Takasato et al., 2015). This research highlights iPSCs' potential to replicate complex tissue environments, a key feature for developing regenerative therapies for tissues like the pulp-dentin complex. iPSCs offer several advantages in regenerative medicine. They provide a patient-specific source of cells, which minimizes the risk of immune rejection after transplantation, especially when differentiated iPSCs are used (Guha et al., 2013). Additionally, the self-renewal capacity of iPSCs allows for an unlimited, renewable source of cells that can be differentiated into a variety of lineages as needed. This versatility makes iPSCs an ideal candidate for addressing the complex cellular interactions required in pulp-dentin regeneration, offering a promising avenue for creating customized, effective therapies.

The objective was to conduct single-cell analysis of pulp tissue to examine the gene

expression profile and identify suitable cell types for assessing odontogenic potential when combined with DPSCs. Based on the results, these cell types were generated using iPSCs and then co-cultured with DPSCs to evaluate their odontogenic potential *in vitro* and *in vivo*.

II. Materials & Methods

1. Single cell preparation

Dental pulp tissue was isolated from the caries-free third molar of a 23-year-old female patient. After carefully removing the entire pulp tissue from the tooth, it was digested for one hour at 37°C using a solution of 3 mg/ml collagenase type I (Gibco, Brooklyn, NY, USA) and 4 mg/ml dispase (Gibco). To create single-cell suspensions, the cells were run through a 70-μm strainer (Falcon, NY, USA). Cells with 90% or more viability were used for single cell RNA-Seq analysis.

2. Single-cell encapsulation, library preparation and sequencing

Using the Chromium Single-Cell 3′ Reagent v2 Kit (10× Genomics, Pleasanton, CA, USA), droplet-based single-cell partitioning and RNA sequencing libraries were created according to the instructions of manufacturer that utilize the proprietary 10× GemCode technology. In summary, the RT-PCR master mix was combined with a single-cell suspension (less than 4 μl) containing about 2000 cells/μl. The mixture was then swiftly loaded into a Single-Cell 3′ Chip containing Single-Cell 3′ Gel Beads and Partitioning Oil. Unique molecular identifiers (UMIs), poly(dT) sequences, and 10× cell barcodes were all present in the Gel Beads. After that, the chip was inserted into a Chromium Controller (10× Genomics) to generate single-cell GEMs and perform barcoding. Full-length, barcoded cDNA was created using Clontech's SMART technology by reverse-transcribing RNA transcripts from individual cells inside the droplets. Once the emulsion was broken, one sample's cDNA was combined and pre-amplified. Following the fragmentation of amplified cDNAs, adaptor and sample indices were added to completed libraries that were suitable for Illumine next-generation short-read sequencing. Real-time quantitative PCR was used to quantify the final libraries, and a custom control sequencing library was used for calibration. The Agilent Bioanalyzer 2100

with a High Sensitivity DNA chip (Agilent, Santa Clara, CA, USA) was used to measure the size distributions of the pre-amplified cDNA and sequencing libraries.

3. Single-Cell RNA Data Analysis

Visualization and differentially expressed gene (DEG) analysis were conducted using the Seurat package in R. Cell-type annotation was carried out with the singleR package in R (R Foundation for Statistical Computing, Vienna, Austria).

4. Cell Isolation and Culture

The experimental protocol received approval from the Institutional Review Board of Yonsei University Dental Hospital (IRB number: 2-2017-0002). Dental pulp stem cells (DPSCs) were sourced from caries-free premolars of donors aged 16 to 24. The pulp tissues were immediately detached from the teeth and rinsed twice using Dulbecco's Modified Eagle Medium (Gibco) with 3% penicillin-streptomycin (wash medium). The separated pulp was then cut into small pieces, approximately 0.5 mm in size, using sterilized micro-scissors and placed in 6-well cell culture plates (SPL, Gyeonggi-do, Korea) containing Dulbecco's Modified Eagle Medium enriched with 15% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (complete culture medium). The DPSCs were kept with 5% CO_2 at 37 °C in a humidified environment until they reached subconfluence. In this study, passage 4 DPSCs were utilized.

5. iPSC reprogramming using DPSCs

Dental pulp stem cells (DPSCs) were reprogrammed to iPSCs with the Epi5™ Episomal iPSCs Reprogramming Kit (ThermoFisher Scientific, Waltham, MA, USA) in conjunction with the 4D-Nucleofector® X Unit (Lonza, Basel, Switzerland), according to manufacturer's guidelines. The

transfected cells were then placed into a 6-well Geltrex-coated (Gibco) plate and cultured in DMEM supplemented with GlutaMAX™-I (Gibco), 10% ESC Qualified FBS (Gibco), and 1% MEM Non-Essential Amino Acids Solution (Gibco) for 24 hours. On the first day, the medium was changed to an N2B27/basic fibroblast growth factor (bFGF) medium, which included DMEM/F12 with HEPES, B27 supplement, N2 supplement, GlutaMAX™-1, β-mercaptoethanol (55 μM), and MEM-non-essential amino acids (MEM-NEAA) (all Gibco), along with bFGF (100 ng/ml) (Peprotech, Cranbury, NJ, USA). The medium was replaced with serum-free defined Essential 8 (E8) medium (STEMCELL Technologies, Vancouver, BC, Canada) on day 15. By day 42, iPSC colonies were selected and moved to 24-well plates (SPL) coated with Geltrex, where they were maintained in StemFlex medium (ThermoFisher Scientific).

6. *In vitro* embryoid body formation and tri-lineage differentiation

Embryoid body (EB) formation protocol was used to carry out tri-lineage differentiation, and during the process, the hanging drop technique was employed. In summary, TrypLE™ express enzyme 1X (ThermoFisher Scientific) was used to trypsinize iPSC colonies cultivated in a feeder-free environment before transforming them into a single-cell suspension. Then, ES medium without the pluripotent cytokine bFGF was used to resuspend the iPSCs. On the lid of the culture dish, drops of 20 μl, each containing 10^4 cells, were created. The lid was gently turned upside down and placed over the dish for it to be incubated at 37 °C in a 5% CO_2 atmosphere for 48 hours.

Two days following EB formation, the induction of endodermal differentiation was carried out by culturing the EBs in a differentiation medium (composed of 80% DMEM Knockout without L-glutamine, 1% Glutamax 100X, 20% FBS, 1% non-essential amino acids, 0.1 mM β-mercaptoethano, and 1% penicillin-streptomycin), and after transferring the EBs to 8-well chamber slides (Millipore, Darmstadt, Germany) coated with 0.1% gelatin. In addition, a differentiation medium

supplemented with 100 μM ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA) was used to culture EBs for mesodermal differentiation. In order to achieve ectodermal differentiation, EBs were finally moved to 8-well chamber slides (Merk) coated with Matrigel. They were then cultivated in a specially prepared differentiation medium that contained DMEM F-12, 1% penicillin-streptomycin, 1% Glutamax 100X, 1% B27 supplement (Gibco), and 0.5% Gibco N2 supplement (Gibco). For all three differentiation procedures, the medium was changed every other day over the course of three weeks.

7. Differentiation of iPSC into Endothelial cell

iPSCs were plated in dishes coated with Geltrex. Pro-survival factor (Millipore) was added to E8 Medium for culturing the iPSCs because it is a cell-permeable pyrrolidine that supports the survival of individual human iPSCs or stem cells. From the dish, the iPSC colonies were carefully scraped off and transferred to dishes coated with Matrigel. After allowing the cells to attach for 24 hours, the medium was switched to DMEM/F12 with 4 μM Chir99021 (Millipore). Endothelial Cell Basal Medium (PromoCell, Heidelberg, Germany), supplemented with growth factors bFGF (PeproTech) and VEGFA (R&D Systems, Minneapolis, MN, USA) or Activin A (PeproTech) and BMP4 (PeproTech) was used as the medium two days later. Four more days later, EMV2 (PromoCell) media enriched with VEGFA was used in place, which was changed every other day.

8. Differentiation of iPSC into Epithelial cell

Epithelial cells were differentiated from iPSCs. From day (D0) to day 4 (D4), a single layer of iPSCs was moved onto 96-well plates (Akita Sumitomo Bakelite Co., Ltd, Japan) for producing EBs with 10^4 cells per well. On fibronectin-coated dishes (Corning Incorporated, Corning, NY, USA), 192 EBs were incubated for ectodermal induction from D4 to D8 in a mixture that contained

DMEM/F12, 1×N2 (Gibco), 25 ng/mL BMP4 (R&D Systems), 1 mM RA (Sigma-Aldrich), and 1% penicillin/streptomycin (Gibco). Then, using keratinocyte serum-free media (K-SFM; Gibco) supplemented with 20 ng/mL EGF (PeproTech) and 100 ng/mL NOGGIN (PeproTech), DEC1 was generated from D8 to D12. Subsequently, K-SFM with an addition of 25 ng/mL BMP4 and 20 ng/mL EGF was used to culture DEC2 from D12 to D16.

9. Co-culture of cells for *in vitro* and *in vivo* application

The co-culture experiment was conducted in a 12-well transwell plate. DPSCs were placed in the bottom wells at a density of 1×10^5 cells, while endothelial and epithelial cells (also 1×10^5 cells) were added to the upper inserts (8-μm pore size; SPL). The medium used was a 1:1:1 mixture of DMEM (Gibco), endothelial cell growth medium (PromoCell), and epithelial cell growth medium (PromoCell). The cells were co-cultured for 3 and 7 days, with the medium being replaced every 3 days.

10. Transplantation of Differentiated iPSC and DPSC

The transplantation pellet was created using a combination of DPSCs, endothelial cells, and epithelial cells. All animal experiment was approved by the committee and adhered to the Guidelines for the Care and Use of Laboratory Animals of Yonsei University's Institute of Laboratory Animal Resources (protocol number 2023-0028). After being anesthetized under 100% oxygen using 3% isoflurane (Hana Pharm, Seoul, Korea), the mice were further sedated using Rompun (Bayer Korea, Seoul, Korea). Careful manipulation was applied to implant the pellet mixture into the kidney capsule. Four weeks post-surgery, the mice were euthanized, and a high-resolution micro-computed tomography (micro-CT) scanner (SkyScan 1173, Bruker, MA, USA) was used to measure the volume of the hard tissue.

11. Total RNA Extraction and Reverse Transcription Polymerase Chain Reaction

Total RNA was extracted using Trizol Reagent (Life Technologies Corporation, Carlsbad, CA) on day 7 of the induction process. From 1,000 ng of the isolated RNA, complementary DNA (cDNA) was synthesized using the SuperScript™ IV first-strand cDNA synthesis kit (Invitrogen, Waltham, MA) following the manufacturer's protocol. TaqMan gene expression assays (Applied Biosystems) and Quantstudio 3 System (Applied Biosystems, Waltham, MA, USA) were utilized to carry out reverse transcription polymerase chain reaction (RT-PCR). GAPDH (Hs02786624_g1) served as the housekeeping gene. As markers of osteogenesis, bone sialoprotein (BSP, Hs00913377_m1), osteocalcin (OCN, Hs01587814_g1), dentin sialophosphoprotein (DSPP, Hs00171962_m1), and alkaline phosphatase (ALPL, Hs01029144_m1) were measured. For endothelial cell markers, platelet and endothelial cell adhesion molecule 1 (PECAM1, Hs01065279_m1), TEK Receptor Tyrosine Kinase (TEK, Hs00945150_m1), and lymphatic vessel endothelial hyaluronan receptor 1 (LYVE1, Hs00272659_m1) were used. Keratin 8 (KRT8, Hs01595539_g1), keratin 14 (KRT14, Hs00265033_m1), and paired like homeodomain 2 (PITX2, Hs04234069_mH) were utilized as epithelial cell markers. The $2^{-\Delta\Delta Ct}$ technique based on GAPDH expression was used to calculate the relative expression changes for each reaction, which was carried out in triplicate.

12. Immunostaining

For immunofluorescence staining, slides were treated with primary antibodies including OCT3/4 (Santa Cruz Biotechnology, sc-5279), SOX2 (Santa Cruz Biotechnology, sc-365823), NANOG (abcam, ab109250), TUB3 (BioLegend, 801201), AFP (abcam, ab133617), SMA (abcam, ab7817), PECAM1 (R&D Systems, BBA7), CD146 (abcam, ab75769), KRT8 (Millipore,

MABT329), KRT14 (abcam, ab7800), PITX2 (ThermoFisher Scientific, PA5-41089), NESTIN (Santa Cruz Biotechnology, sc-23927), SOX17 (Santa Cruz Biotechnology, sc-130295), and brachyury (ThermoFisher Scientific, PA5-46984) overnight at 4°C. After that, DAPI (ThermoFisher Scientific) was used to counterstain the samples to highlight the nuclei after being treated for an hour at room temperature with secondary antibodies labeled with Alexa Fluor 488 and 555 (ThermoFisher Scientific). A confocal laser microscope (LSM 700, Carl Zeiss Microscopy, Oberkochen, Germany) was used to analyze the samples. Hematoxylin and eosin (Cancer Diagnostics, Durham, NC, USA) staining was on the slides for histological examination.

13. Western bolt

Throughout the study, a conventional Western blot procedure was used. Following cell harvesting using 1 × sample buffer, SDS-PAGE was carried out using the Bio-Rad mini gel system. On a PVDF membrane, the proteins from the gels were transferred. As directed by the manufacturer, the membrane was blocked with TBST containing 5% non-fat dry milk and then incubated for overnight with primary antibodies diluted in TBST with 5% BSA. The membrane was thoroughly washed with TBST, then incubated with secondary antibodies conjugated with horseradish peroxidase for an hour at room temperature. This was followed by further washing with TBST buffer. The signals were detected using an ECL system (Pierce Biotechnology, Waltham, MA, USA).

14. Statistical analysis

To analyze the differences in the amount of hard tissue formation between groups, the nonparametric Mann-Whitney U test was employed. This analysis was conducted using IBM SPSS (version 28.0; IBM, Armonk, NY, USA), with a significance threshold set at $p < 0.05$.

III. Results

1. Single-Cell Analysis of Human Dental Pulp

A single-cell level RNA library of human dental pulp was generated and analyzed. The analysis revealed that human dental pulp is composed of nine clusters of cells including tissue stem cells, neurons, endothelial cells, smooth muscle cells, and immune cell population – macrophages, neutrophils, T cells, natural killer cells, and monocytes (Figure 1). Validation of the endothelial cell population was confirmed as it expressed PECAM (Figure 2a), an endothelial cell marker. Notably, dentin sialoprotein (DSP) and epithelial cell marker KRT18 were expressed in that same population (Figure 2b, c).

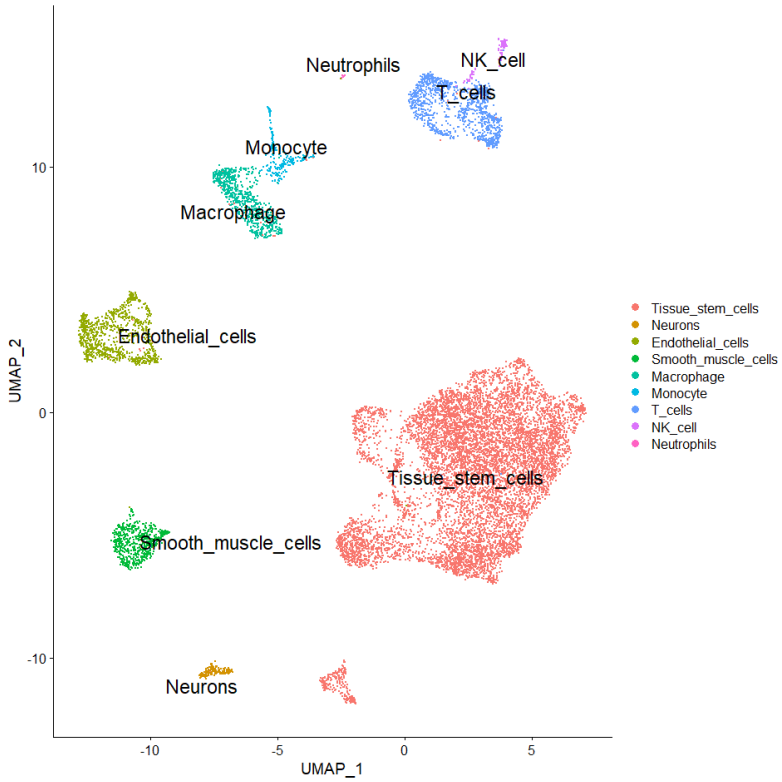


Figure 1. UMAP of human dental pulp tissue. Based on the RNA-seq data, cells isolated from dental pulp tissues showed nine clusters: tissue stem cells, neurons, smooth muscle cells, endothelial cells, monocytes, macrophages, neutrophils, T cells and natural killer cells.

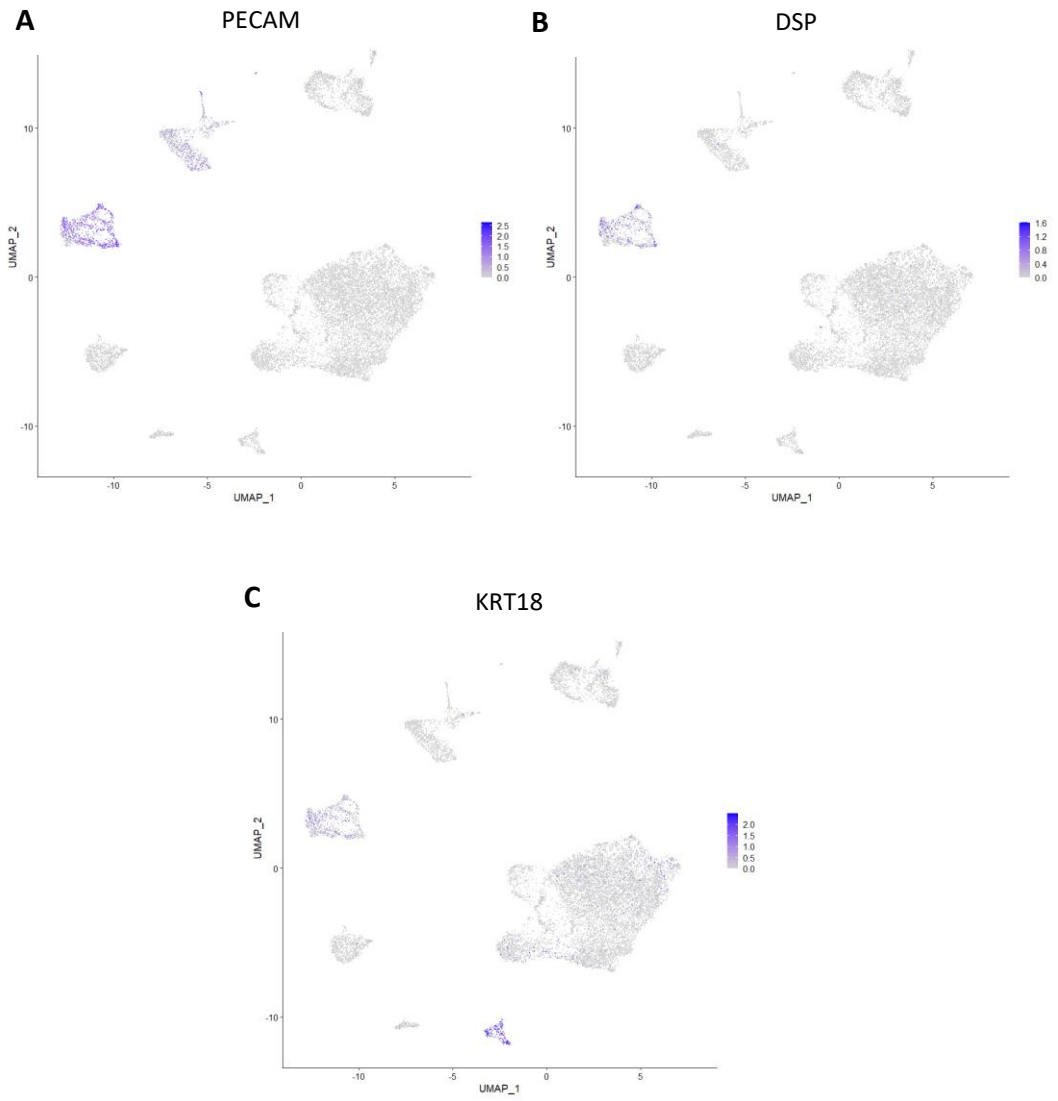


Figure 2. UMAP of human dental pulp tissue with marker genes (A) Endothelial cell marker gene, PECAM is highly expressed from endothelial cell population. (B) DSP is expressed mostly from endothelial cell population. (C) Epithelial cell marker gene, KRT18, is expressed in endothelial cell population and tissue stem cell population.

2. Reprogramming DPSCs to iPSCs and characterization of iPSCs

The successful reprogramming of DPSCs into iPSCs was confirmed through morphological evaluation and the expression of pluripotency markers. Approximately 35 days post-extrapolation, colonies of iPSCs began to emerge, and by day 41, iPSC colonies were ready for transfer (Figure 3). Both Western blot analysis and immunofluorescence (IF) staining expressed pluripotency markers Oct3/4, Sox2, and Nanog in the reprogrammed iPSCs (Figure 4, 5). To verify the ability of iPSCs to differentiate into three embryonic germ layers, iPSCs were differentiated into endoderm, mesoderm, and ectoderm. IF staining of the differentiated cells verified the expression of specific markers – AFP and SOX17 for endoderm, SMA and Brachyury for mesoderm, TUB3 and NES for ectoderm (Figure 6).

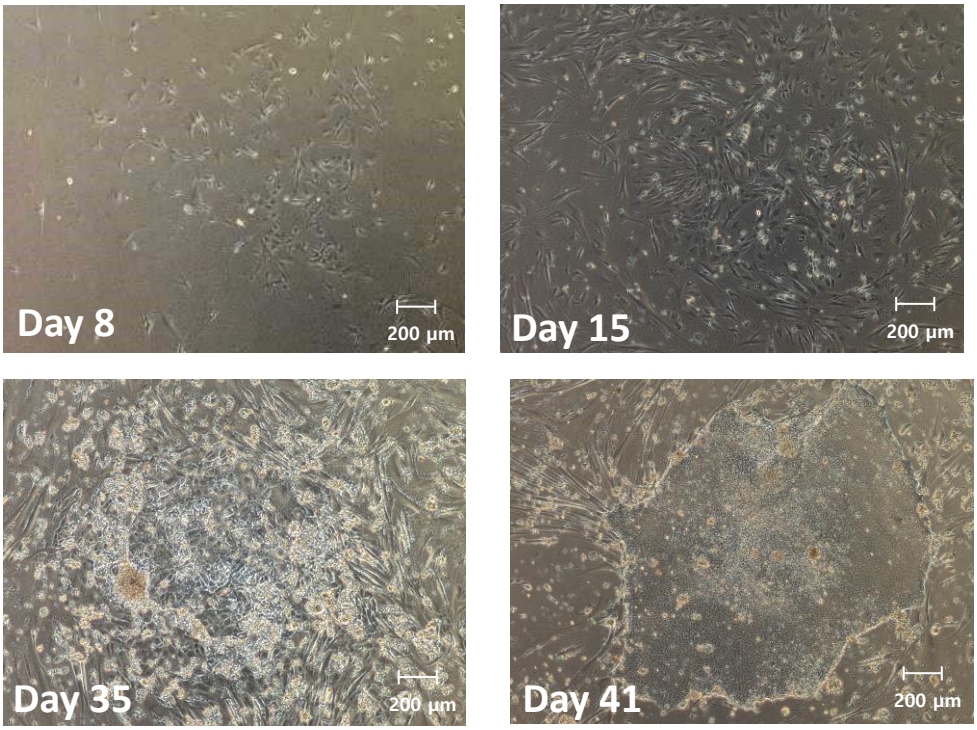


Figure 3. Reprogramming of DPSCs to iPSCs. DPSCs were reprogrammed to iPSCs. iPSC colony began to form 35 days post-extrapolation. Colonies with well-defined margin ready for transfer at day 41.

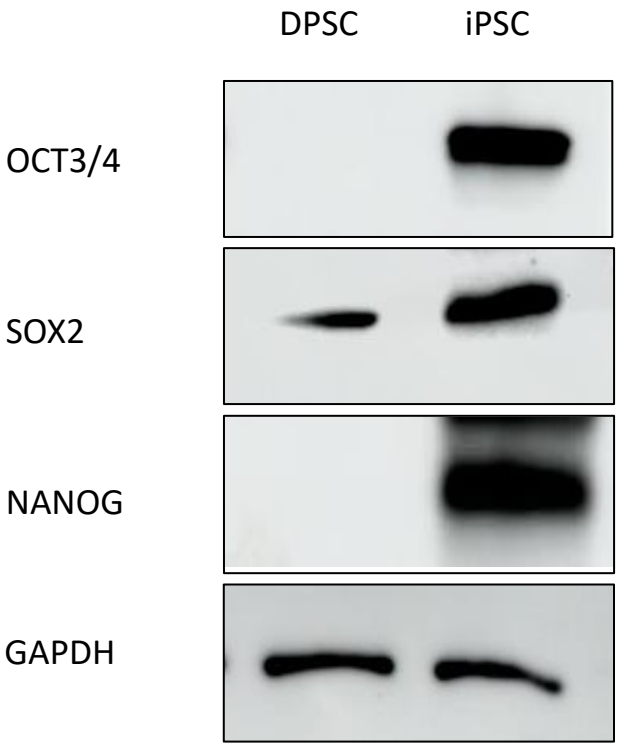


Figure 4. Western blot analysis of pluripotency markers genes on DPSC and iPSC. Oct3/4, SOX2, Nanog are expressed for iPSC. GAPDH served as internal control. Oct3/4, Octamer-binding transcription factor 3/4; SOX2, SRY-box 2; NANOG, Nanog Homeobox

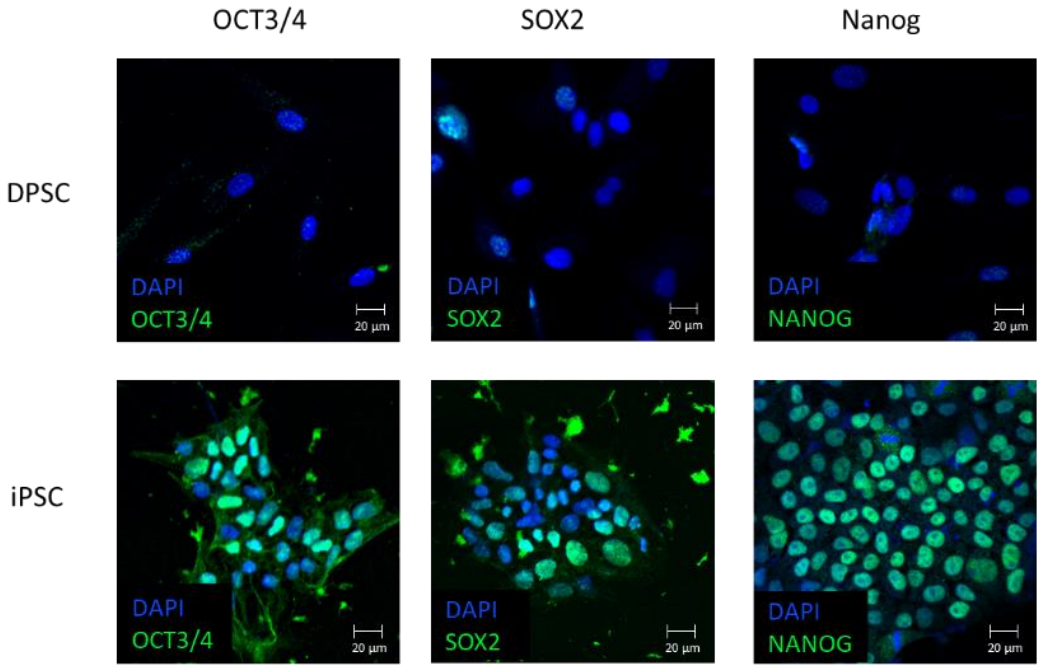


Figure 5. Immunofluorescence staining of DPSC and reprogrammed iPSC with pluripotency marker genes. DAPI staining (in blue) ensures clear visualization of nuclei for both DPSC and iPSC. IF staining shows the expression of pluripotency markers Oct3/4, Sox2, Nanog (green) in iPSCs.

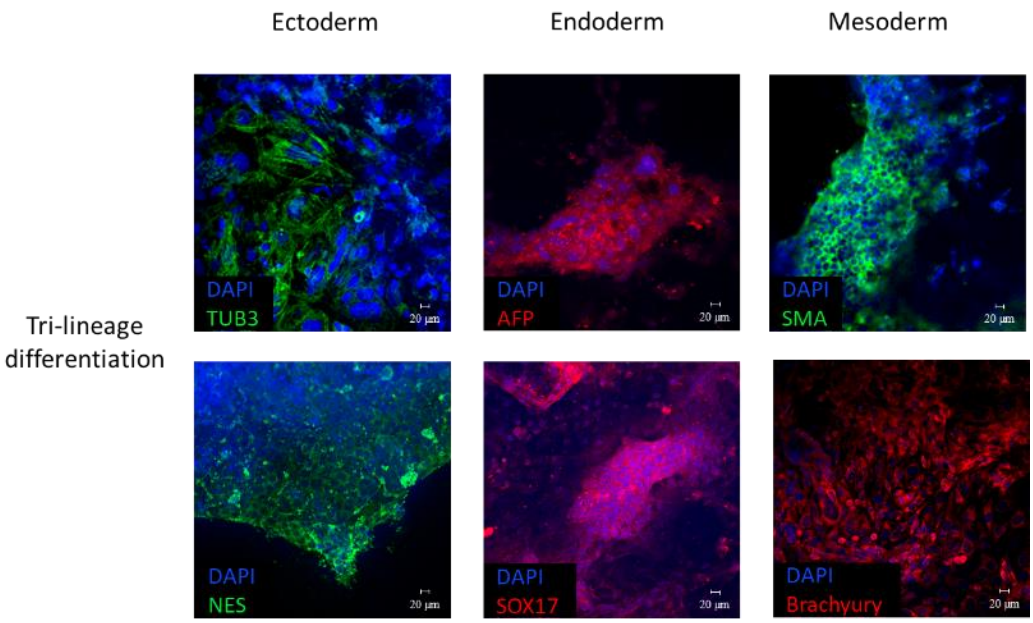


Figure 6. Immunofluorescence staining of following tri-lineage differentiation of iPSCs. For all three germ layers, nuclei are stained with DAPI (blue). iPSC differentiated to ectoderm expresses TUB3, ectoderm marker (green) and NES (green). iPSC differentiated to endoderm is stained by AFP (red) and SOX17 (red). iPSC differentiated to mesoderm expresses SMA (green) and brachyury (red). TUB3, β-tubulin III; AFP, alpha-fetoprotein; SMA, smooth muscle actin; SOX17, SRY-Box Transcription Factor 17; NES, Nestin; brachyury, T (Brachyury)

3. Differentiation of iPSCs into Endothelial Cells and Characterization

The iPSCs were differentiated into endothelial cells (iPSC_EDs) using a stepwise protocol involving the application of endothelial-specific growth factors. The functional outcome was assessed by tube formation assay, and within 24 hours, iPSC_EDs formed a vascular-like structure successfully, unlike DPSCs and iPSCs (Figure 7). This indicates that the iPSCs were effectively differentiated into endothelial cells, capable of contributing to angiogenesis, a critical aspect of tissue regeneration. iPSC_EDs were analyzed by quantitative polymerase chain reaction (qPCR), and qPCR showed higher expression of endothelial markers PECAM1, TEK, and LYVE1 in iPSC_EDs compared to DPSCs and iPSCs (Figure 8). IF staining of iPSCs and iPSC_EDs with endothelial markers PECAM, KDR, and CD146 showed higher uptake of the markers for iPSC_EDs (Figure 9).

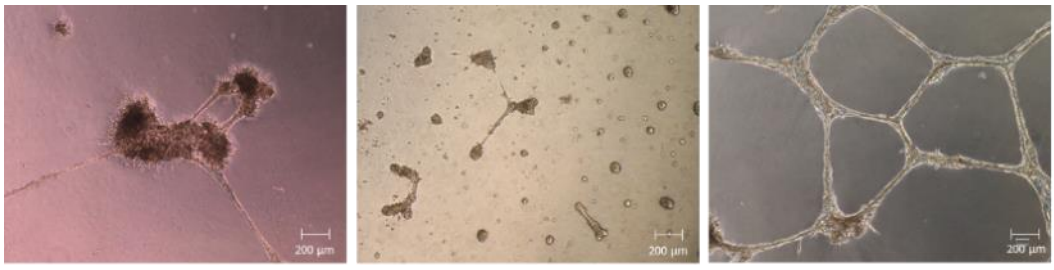


Figure 7. Tube formation assay at 24 hours. In contrast to DPSC and iPSC, iPSC_ED displays the tubular structures that endothelial cells develop throughout a 24-hour period.

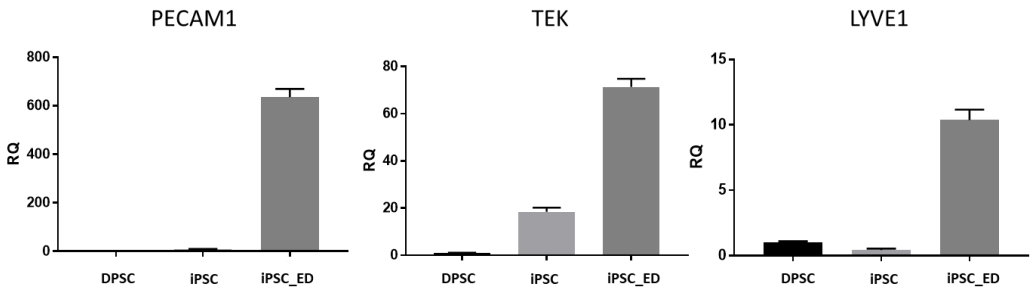


Figure 8. Relative expression of endothelial markers in DPSC, iPSC, and iPSC_ED. The expression of PECAM1, TEK, LYVE1 genes was observed in iPSC_ED. DPSC did not express PECAM1. DPSC and iPSC showed expression of TEK and LYVE1. PECAM1, platelet endothelial cell adhesion molecule 1; TEK, TEK receptor tyrosine kinase; LYVE1, lymphatic vessel endothelial hyaluronan receptor 1

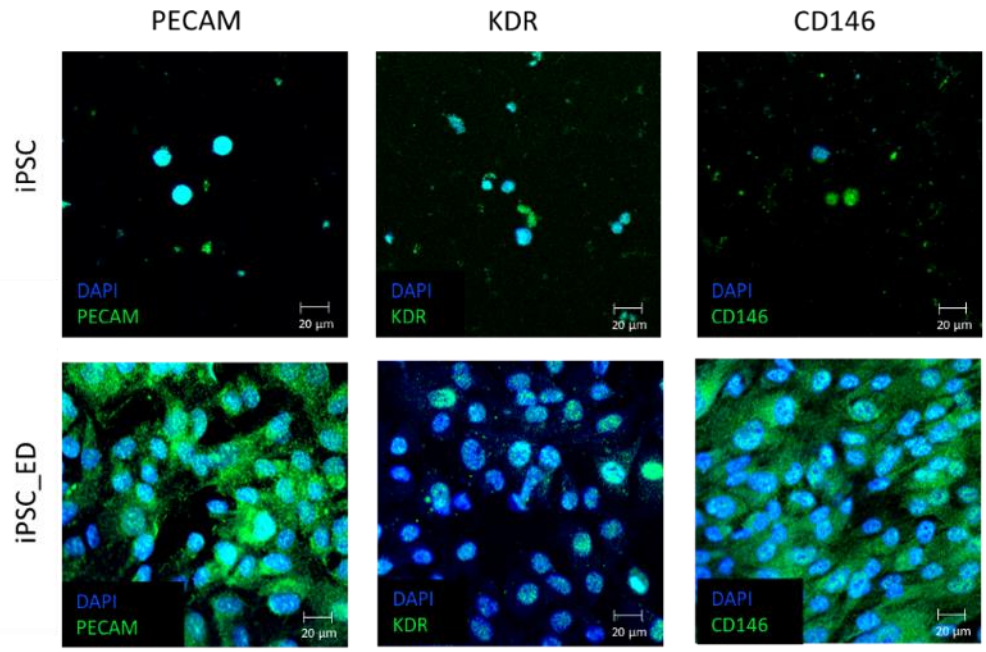


Figure 9. Immunofluorescence staining of iPSC and iPSC_ED. Undifferentiated iPSCs showing minimal expression of vascular markers, PECAM, KDR, and CD146 (green). iPSC_EDs demonstrate enhanced expression of endothelial markers, PECAM, KDR, CD146. PECAM, platelet endothelial cell adhesion molecule; KDR, kinase insert domain receptor; CD146, melanoma cell adhesion molecule

4. Differentiation of iPSCs into Epithelial Cells and Characterization

iPSCs were differentiated into epithelial cells (iPSC_EPs) and then characterized with qPCR and IF staining with epithelial markers KRT8, KRT14, and PITX2. KRT 8 was expressed in both iPSC and iPSC_EP. KRT 14 and PITX2 were expressed in DPSC, iPSC, and iPSC_EP. Epithelial cell marker genes, KRT8, KRT14, PITX2, were expressed in iPSC_EP ((Figure 10). IF staining showed higher expression of the markers in iPSC_EPs compared to undifferentiated iPSCs (Figure 11).

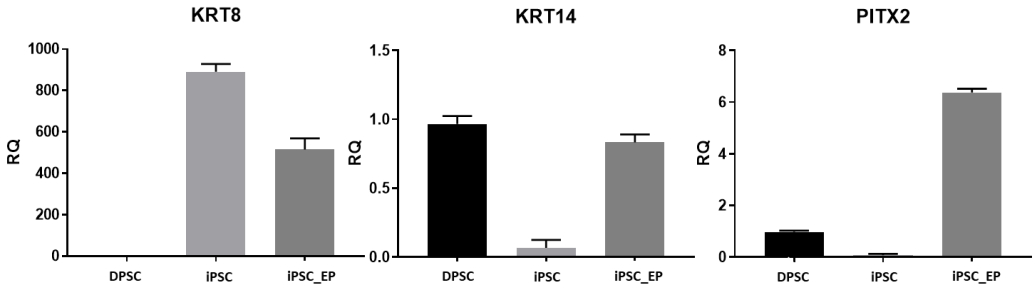


Figure 10. Expression of epithelial differentiation-specific genes and transcription factors in DPSC, iPSC, and iPSC_EP. KRT8 is expressed in iPSC and iPSC_EP. KRT14 and PITX2 are expressed in DPSC, iPSC, and iPSC_EP. KRT8, keratin 8; KRT14, keratin 14; PITX2, paired-like homeodomain transcription factor 2

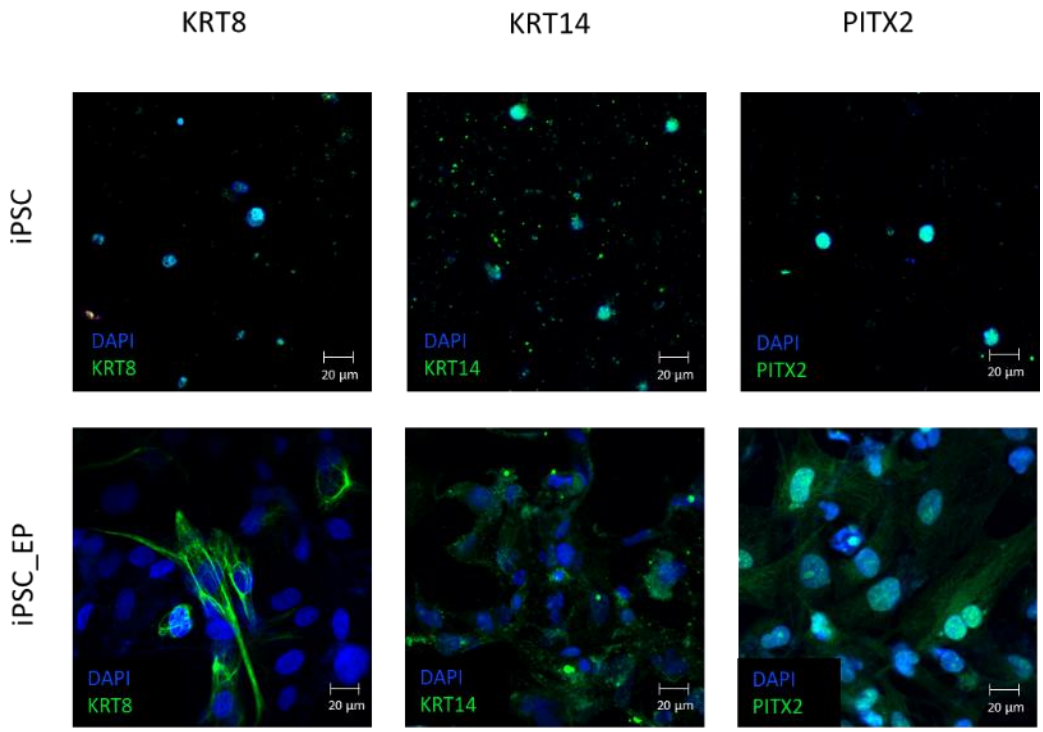


Figure 11. Immunofluorescence staining of epithelial markers in iPSC and iPSC_EP. Undifferentiated iPSCs show minimal expression of KRT8, KRT14, and PITX2. Cells are counterstained with DAPI (blue) to highlight nuclei. In iPSC_EPs, there is enhanced expression of epithelial call markers, KRT8, KRT14, PITX2 (green). KRT8, keratin 8; KRT14, keratin 14; PITX2, paired-like homeodomain transcription factor 2

5. *In Vitro* Analysis of Odontogenic Potential of Co-cultured Cells

iPSC_EDs, iPSC_EPs, and DPSCs were co-cultured to assess their odontogenic potential. The co-cultured cells were analyzed by qPCR for markers DSPP (Dentin sialophosphoprotein), ALP (Alkaline phosphatase), BSP (Bone sialoprotein), and OCN (Osteocalcin). By day 3, all three of the groups expressed markers ALP and OCN while the expression of DSPP was observed in DPSC and DPSC, iPSC_ED, iPSC_EP groups, with the highest expression of DSPP seen in the experimental group. By day 7, all three groups showed expression of BSP in addition to ALP, OCN, and DSPP.

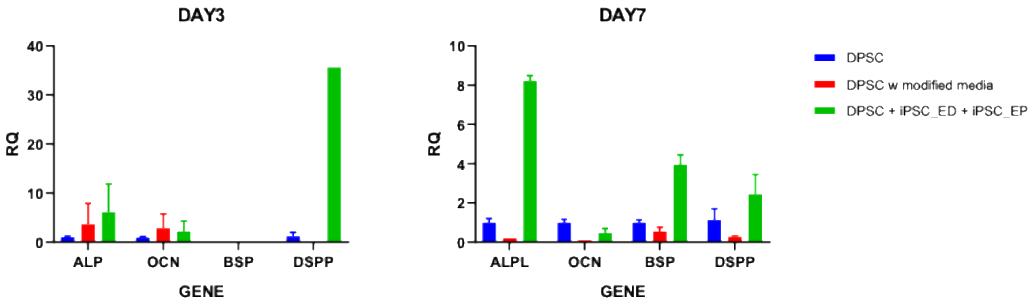


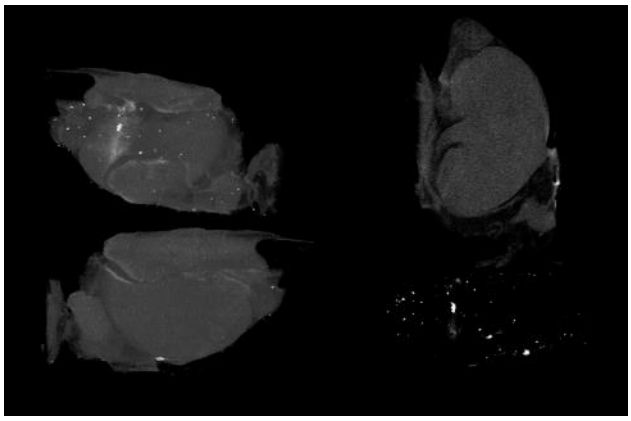
Figure 12. RT-PCR result of DPSC monoculture and DPSC, iPSC_ED, iPSC_EP coculture group. Expression of ALPL and OCN are observed in all three groups at Day 3 while expression of DSPP is observed in DPSC and DPSC, iPSC_ED, iPSC_EP groups. In addition to ALPL, OCN, DSPP, BSP are expressed in all three groups at Day 7.

6. *In Vivo* Analysis of Osteogenic Potential

Micro-CT imaging demonstrated hard tissue formation in both DPSC group (Figure 13B) and DPSC, iPSC_ED, iPSC_EP group (Figure 13A). However, when quantified, DPSC group showed higher volume of hard tissue formation with no significant difference (Figure 14). Table 1 shows the detailed data on the volume hard tissue formation in each sample.

Histologic analysis shows hard tissue formation in both DPSC group and DPSC, iPSC_ED, iPSC_EP group. Figure 15A and B shows hard tissue formation and contains parts that appears to be soft tissue within the newly formed hard tissue in DPSC, iPSC_ED, iPSC_EP group. Figure 15C and D shows hard tissue formation in DPSC group with no sign of newly formed soft tissue.

A



B

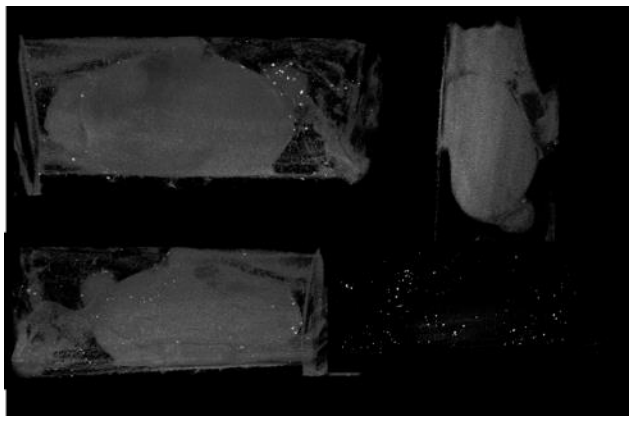


Figure 13. Hard tissue formation. (A) shows micro-CT image of kidney with hard tissue formation for the DPSC, iPSC_ED, iPSC_EP group. (B) shows micro-CT image of kidney with hard tissue formation for the DPSC monoculture group.

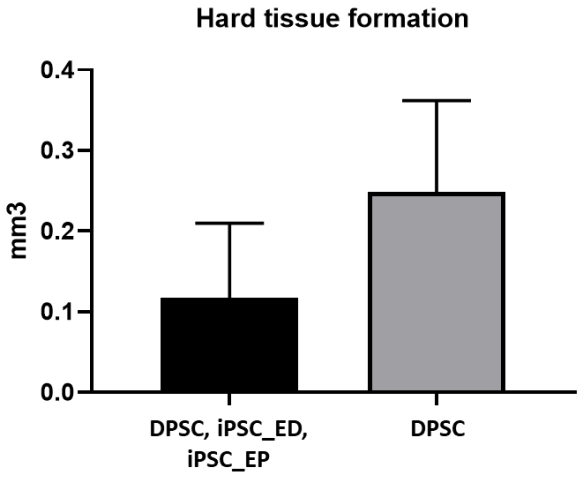


Figure 14. The volume of total hard tissue measured from micro-CT scan. A lower volume of hard tissues was observed in DPSC, iPSC_ED, iPSC_EP coculture group than DPSC monoculture group. There is no significant difference in the volume of hard tissue.

Table 1. The volume of hard tissue formed from each sample (mm^3)

DPSC, IPSC_ED, IPSC_EP	DPSC
0.18	0.17
0.02	0.30
0.06	0.27
0.24	0.09
0.07	0.42
	0.22

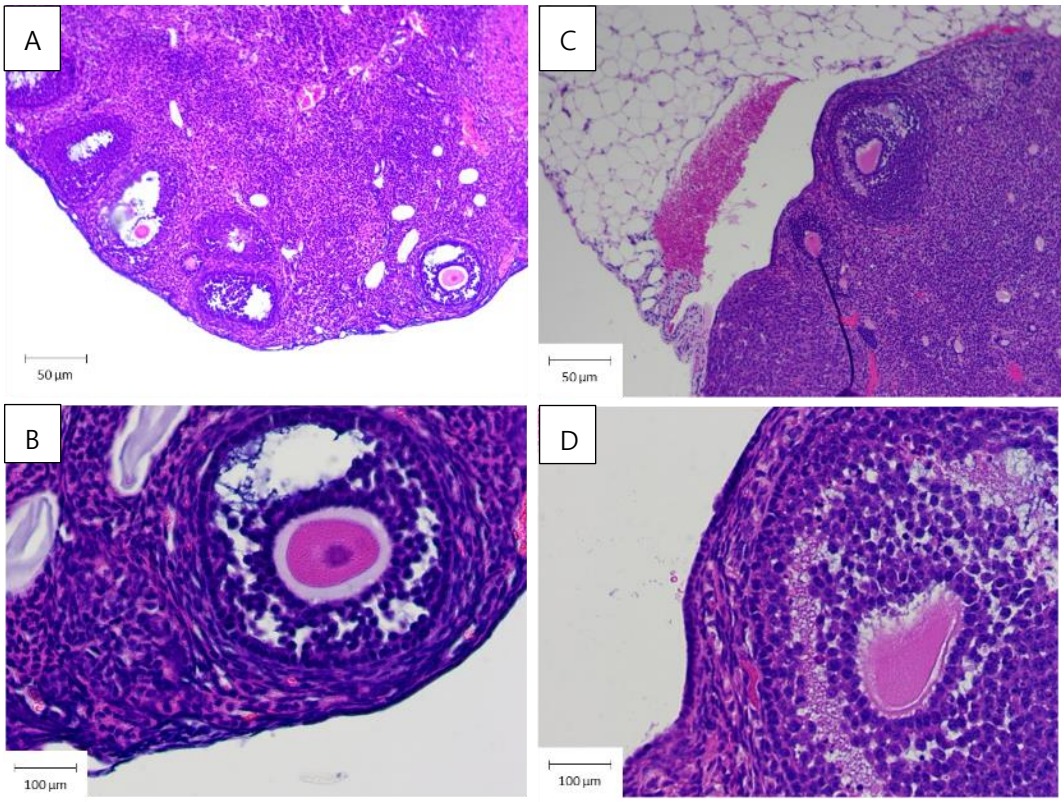


Figure 15. Hematoxlin-eosin staining of hard tissue. A and B are images of DPSC, iPSC_ED, iPSC_EP group at 50 μm and 100 μm, respectively. C and D are images of DPSC group at 50 μm and 100 μm, respectively.

IV. Discussion

In this study, human dental pulp tissue was analyzed at the single-cell level, and the analysis identified nine distinct clusters of cells. Based on the results, when excluding the immune cell population and smooth muscle cells, the population of cells that make up dental pulp are tissue stem cells, endothelial cells, and neurons. Although the composition of human dental pulp is already known, a key finding in this analysis is the gene expression within the endothelial cell population. The endothelial cell population expressed dentin sialoprotein (DSP), which stimulates odontoblastic differentiation, migration, and growth of dental pulp cells *in vitro* and also stimulates the migration and proinflammatory activation of immune system cells (Lee et al., 2012; Silva et al., 2004; Tani-Ishii et al., 1995). DSP expression from the endothelial cell population suggests that endothelial cells may contribute to the formation of dentin in addition to their role in angiogenesis. Interestingly, the expression of epithelial cell marker KRT18 was observed from both the endothelial and tissue stem cell populations. This finding aligns with an earlier study that found epithelial-like cells in human deciduous teeth (Nam & Lee, 2009), suggesting a possible role of epithelial cells in the function of dental pulp. Single-cell analysis of pulp tissue confirmed the expression of endothelial and epithelial markers, and co-culturing DPSCs with endothelial and epithelial cells may effectively mimic the natural architecture of human pulp tissue, potentially enhancing pulp-dentin regeneration.

Direct co-culture of DPSCs and endothelial cells enhanced the differentiation toward odontogenic and angiogenic phenotypes *in vitro* (Dissanayaka et al., 2012). In an *in vivo* study comparing DPSC-monoculture to DPSC and human umbilical vein endothelial cell (HUVEC) co-culture, while both groups exhibited vascularized pulp-like tissue, the cocultured group exhibited more extracellular matrix, vascularization, and mineralization than the monoculture group when transplanted into mice (Dissanayaka et al., 2015). Endothelial cells, one of the cells that make up

the dental pulp, seem to play a critical role not only in providing structural support through vascularization but also in promoting the migration of reparative cells necessary for pulp regeneration. For these reasons, the use of endothelial cells along with DPSCs most likely has yielded positive results. Moreover, when the tooth being treated with RET has somewhat constricted apical opening, securing an adequate amount of blood supply during RET is a challenging task. Therefore, using endothelial cells in pulp-dentin regeneration is likely to enhance the overall effectiveness.

While the presence of endothelial cells within dental pulp has been known, there has been limited information regarding the presence and role of epithelial cells in pulp tissue. In 2009, one study reported about finding epithelial cell-like cells from human deciduous dental pulp (Nam & Lee, 2009), and epithelial cell-like stem cells found in human deciduous pulp can help odontoblastic induction and lead to dentin formation (Lee et al., 2016). Additionally, when dental tissue-derived mesenchymal stem cells were cocultured with epithelial cells, the cocultured group showed significantly higher odontogenic differentiation capacity (Lee et al., 2024). These findings further underscore the need for future research into the possible role of epithelial cells in pulp regeneration. It is well-known that epithelial cells are crucial for odontogenesis (Rombouts et al., 2017). Consequently, the inclusion of both epithelial and endothelial cells along with DPSCs seemed desirable for a more comprehensive approach to pulp-dentin complex regeneration.

Building on the successes and challenges of using DPSCs, researchers have also turned their attention toward iPSCs which offer a more versatile cell source for regenerative endodontics. iPSCs, derived from adult somatic cells reprogrammed to a pluripotent state, can differentiate into a wide variety of cell types essential for pulp-dentin complex regeneration. iPSCs have been

successfully differentiated into endothelial cells expressing markers like PECAM1, KDR, and CD146, as well as epithelial cells expressing markers such as KRT8, KRT14, and PITX2. This pluripotency of iPSCs offers the possibility of creating patient-specific regenerative therapies, minimizing the risk of immune rejection (Guha et al., 2013). This study aimed to analyze the effect of DPSCs, endothelial cells, and epithelial cells on pulp-dentin regeneration so using cells from multiple donors seemed less desirable, which is the reason why iPSC was chosen to differentiate them into endothelial and epithelial cells in hopes of minimizing the risk of immune rejection.

In this study, scaffolds were not included because there are several issues concerning the use of scaffolds. They do not replicate the actual environment; thus, remnants of scaffolds or degraded material often hinder cell proliferation and angiogenesis (Sung et al., 2004). A 2018 study demonstrated that using only DPSCs in a 3D culture system without any scaffolds or growth factors resulted in successful pulp regeneration, highlighting the potential of scaffold-free regenerative strategies (Itoh et al., 2018). This is an essential consideration for the advancement of RET, as simplifying the process by eliminating the need for additional materials may lead to more efficient clinical applications. The current study's findings align with this scaffold-free approach, showing that co-culturing DPSCs with endothelial and epithelial cells shows odontogenic potential *in vitro* and *in vivo* without requiring external scaffolds or growth factors.

In *in vitro* analysis of odontogenic potential of the coculture group, the expression of the DSPP gene along with ALPL, OCN, BSP was observed. The expression of ALPL, OCN, BSP, DSPP observed in DSPCs alone serves as a baseline that demonstrates the intrinsic odontogenic properties of these cells. Previous studies have shown that DPSCs have characteristics of the osteoblastic phenotype, ALP, OCN, BSP (Mori et al., 2010; Tsukamoto et al., 1992). Unlike those markers, the DSPP gene is recognized as a specific marker gene for odontoblast formation (Svandova et al., 2020). Interestingly, the modification of the culture media negatively affects the expression level of the

genes in DPSCs, showing that the gene expression level of the coculture group is not altered by the media. The increased expression of odontogenic markers, including DSPP, in the coculture group indicates that the combination of endothelial and epithelial cells with DPSC could have a potential for pulp-dentin regeneration.

From micro-CT analysis of hard tissue formation *in vivo*, although no statistically significant difference was observed, the numeric data reveals a trend where the co-culture group tended to form slightly less hard tissue compared to the DPSC monoculture group. Interestingly, a previous study on *in vivo* observations on the impact garcinol has on the odontogenic potential of DPSCs has mentioned a reduction in hard tissue formation when garcinol was used (Jang et al., 2023). The result of this study suggests that the combination of iPSC_ED and iPSC_EP with DPSC might influence the balance between hard tissue and soft tissue regeneration. While dentin formation is necessary for the successful regeneration of the pulp-dentin complex, the regeneration of functional pulp tissue is crucial rather than promoting excessive formation of hard tissue. True regeneration of dentin and pulp would require both revascularization and the restoration of functional odontoblast and/or nerve fibers (Mao et al., 2012). In the histological analysis, the DPSC, iPSC_ED, and iPSC_EP groups contained tissues that appeared as soft tissue within hard tissue. In contrast, the DPSC group was composed solely of hard tissue. These results are consistent with the findings from micro-CT scan, which showed a slightly higher amount of hard tissue in the DPSC group.

V. Conclusion

1. Single-cell analysis of dental pulp tissue revealed that the endothelial cell population expresses not only endothelial marker genes but also epithelial marker genes and dentin sialoprotein.
2. Reprogrammed iPSCs were successfully differentiated into endothelial and epithelial cells for co-culture, and co-culturing iPSC-derived endothelial and epithelial cells with DPSCs showed odontogenic potential in vitro and in vivo.

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Abstract in Korean

유도 만능 줄기 세포 유래 내피 및 상피세포와 사람 치수 줄기 세포를 이용한 치수-상아질 복합체 재생을 위한 새로운 세포 기반 접근법

최 명 락

연세대학교 대학원 치의학과
(지도교수: 김 의 성)

이 연구는 단일 세포 분석과 유도 다능성 줄기 세포(iPSCs)의 적용을 통해 치수 조직의 재생 가능성을 조사했다. 미성숙 영구치에 발생한 치근단 병변을 치료하기 위해 재생적 근관 치료 (RET)가 발전했지만, 미성숙 영구치에 여전히 도전 과제가 남아 있었다. 전통적인 RET 방법인 재관혈화는 유망한 결과를 보였지만, 진정한 재생보다는 수리로 이어지는 경우가 많았다. 이 연구는 효과적인 치수-법랑질 복합체 재생을 위해 다양한 세포 유형을 포함할 필요성을 강조했다. 단일 세포 분석을 통해 인간 치수 조직의 세포 구성과 유전자 발현 프로파일을 밝혔고, 내피 세포 집단이 내피와 상피 마커 유전자 모두를 발현하는 것을 발견했다. 그 후, iPSCs 를 내피 세포와 상피 세포로 성공적으로 분화시켰고, 이를 치수 줄기

세포(DPSCs)와 공동 배양했다. 이 공동 배양은 실험실 내외에서 상당한 치아 형성 잠재력을 보여주었고, 재생적 결과를 향상시키기 위해 다세포 접근 방식을 사용하는 것이 중요하다는 점을 강조했다. 우리의 발견은 치수-상아질 복합체의 기능적 무결성을 회복하는 맞춤형 치료법 개발을 위한 기초를 제공했다.

핵심되는 말 : 재생 근관치료, 치수 줄기세포, 내피세포, 상피세포, 유전자 발현 분석, 쥐 이식, 치아 생성 잠재력