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Direct Dental Pulp Tissue Grafting – a Novel Approach to Regenerative Endodontics

A thesis submitted in partial satisfaction
of the requirements of the degree Master of Science
in Oral Biology

by

Zhangrui Liang

2015

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ABSTRACT OF THE THESIS

Direct Dental Pulp Tissue Grafting – a Novel Approach to Regenerative Endodontics

By

Zhangui Liang

Master of Science in Oral Biology

University of California, Los Angeles, 2015

Professor Mo K. Kang, Chair

The translation of dental pulp stem cells (DPSC) transplantation into clinic practice is hindered by the Good Manufacturing Practice (GMP) approval for *in vitro* autologous DPSC expansion. In order to circumvent the laboratory procedure, we proposed an unprecedented approach for regenerative endodontics – direct dental pulp tissue grafting. This study has demonstrated that cells directly migrating out from dental pulp tissue explantation (DPTE), functioning as the putative cell source in our approach for tissue regeneration, exhibits stem cell properties such as elongated lifespan as well as multi-potent differentiation *in vitro* as equivalent to DPSC. Additionally, our data from *ex vivo* tissue engineering with either porous biodegradable scaffold (poly-L-Lactic acid) or injectable self-assembly scaffold (PuraMatrix Hydrogel) suggests that dental pulp tissue fragments are survivable and proliferative in three-dimensional culture, generating stem cell population with odontogenic differentiation potential as well as mineralization

capacity. Consequently, this preliminary investigation has established the fundamental rationale for the utilization of direct pulp tissue grafting in pulp-dentin complex engineering, charting out a blueprint for subsequent studies of a brand-new technique for regenerative endodontics.

The thesis of Zhangrui Liang is approved.

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2015

TABLE OF CONTENTS

INTRODUCTION

Endodontic treatment on mature permanent tooth yielded a success rate up to 89% according to strict radiographic criteria and 97% according to retention and functioning up to 8 years post-treatment in an epidemiological analysis(Salehrabi and Rotstein 2004)(Sjogren et al. 1990). However, management of immature permanent root with necrotic pulp and apical periodontitis presents several challenges to a successful treatment – limitation of conventional aggressive mechanical debridement, open apex providing no apical barrier for root filling material and, above all, the high susceptibility of fracture due to the undeveloped root structure(Trope 2010). Currently, the application of mineral trioxide aggregate (MTA) to create a hard tissue seal has become predictably successful in promoting periapical healing(Giuliani et al. 2002). However, teeth with thin dentinal walls are more susceptible to fractures that render them nonrestorable. It has been reported that approximately 30% of these teeth will fracture during or after endodontic treatment(Trope 2010). Consequently, heated investigation of newer techniques that can internally strengthening the teeth to increase their long-term survivability has taken place.

Pulp revascularization has been introduced as a way to revitalize teeth with necrotic pulp(Moreno-Hidalgo et al. 2004). The rationale lies in the possibility of further root development and reinforcement of dentinal walls by deposition of hard tissue. After adequate disinfection of the canal, a blood clot was induced into the canal space as a scaffold for the ingrowth of new tissue, followed by a double seal of MTA and bonded resin restoration. It has been demonstrated that success revascularization took place after approximately 45 days in canine animal study(Murray, Garcia-Godoy, and Hargreaves

2007). Case reports have also showed that in teeth with necrotic pulp and apical periodontitis revascularization is possible to increased root formation after periapical healing (Chueh et al. 2009; Jeeruphan et al. 2012). However, it is important to distinguish between revascularization and pulp regeneration. At present, it is likely that the tissue in the pulp space is more similar to periodontal ligament than to pulp tissue as histological studies indicated that the revascularized tissues formed in the canals composed of fibrous connective tissue, bone, and cementum-like tissues, instead of bone fide pulp-dentin complex(Martin et al. 2013; Becerra et al. 2014). Future research is still in need to stimulate pulp regeneration from the pluripotential cells residing in pulp and/or periapical region.

Dental pulp stem cells (DPSC) are the population of mesenchymal stem cells isolated from permanent teeth while stem cells from human exfoliated deciduous teeth (SHED) from primary teeth, both constitute postnatal pulpal MSC populations(Gronthos et al. 2000)(Heissig et al. 2002). Transplantation of such cells demonstrated promising outcome *in vivo* with the regenerated tissue resembling the architecture and function of a pulp-dentin complex(Cordeiro et al. 2008)(George T-J Huang et al. 2010). Iohara *et al*. first demonstrated successful regeneration of pulp-dentin complex in a pulpotomy model in dogs by transplantation of fractionated side-population (SP) cells enriched with the CD31-/CD146- immunophenotype (Iohara et al. 2011). In subsequent studies, the same group demonstrated regeneration of the entire pulp and pulp-dentin complex when MSCs from dental pulp were transplanted into pulp space – unfractionated whole pulp cells generated pulp-dentin complex, and pulp-dentin regeneration was enhanced when the transplanted cells were enriched for CD105+ MSCs (Murakami et al. 2013)(Iohara et al.

2011). On the contrary, absence of pulp cell transplantation failed to yield bone fide pulp-dentin regeneration. Therefore, this is the first evidence to show the requirement of pulp cell transplantation in "emptied" root canal space in order to achieve pulp-dentin regeneration. Preclinical studies using xenograft experiments in animals also revealed the requirement of DPSCs transplantation for bone fide regeneration of pulp-dentin complex (George T-J Huang et al. 2010)(Sakai et al. 2010).

However, one prominent question must be addressed regarding the cell-based approach: "How is it possible to bring the technology to a dental office?" This question is seemingly simple but it considers a multitude of complex issues in providing the practical logistics of MSC transplantation into root canals in the typical endodontic practice setting. Primarily the source and potency of MSCs are of paramount importance and could be limited by our available technology. Normal human somatic cells like pulpal MSCs undergo a finite number of cell divisions during *in vitro* culture until the cells exhaustively arrive at the terminal state called replicative senescence (Mo K. Kang et al. 2004). Our prior study showed a marked accumulation of senescent pulpal MSCs using *in vitro* culture and loss of essential odonto/osteogenic differentiation capacities in cells due to induction of $p16^{INK4A}$ and loss of Bmi-1, a stem factor (Mehrazarin et al. 2011).As such, *in vitro* expansion of pulpal MSCs in preparation for cell transplantation procedures would require an extensive number of cell doublings with congruent loss of differentiation capacity.

Clinical translation of the cell-based approach must also overcome the challenge of MSC expansion *in vitro* and the requirement of good manufacturing practice (GMP) facilities to ensure reliable cell production. This becomes a difficult requirement for

autologous MSCs due to time constraints during a dental appointment and the cost associated with individualized MSC cultivation at a GMP laboratory, if such facility is available at all. Therefore, the cell-based approach seems to be efficacious for bone fide pulp-dentin regeneration but feasibility for day-to-day practice is the limiting factor at present.

To this end, we proposed a new protocol namely pulp tissue grafting, which harnesses the regenerative potential of direct pulp tissue transplantation and the convenience of bypassing *in vitro* culture and cell expansion. This new approach allows direct insertion of pulp tissues with regenerative capacities without having to expand the DPSCs *in vitro*. In this study, we demonstrated the feasibility of pulp tissue grafting in vitro and ex vivo, by proving that a) minced pulp tissues yields outgrowth of MSCs named dental pulp tissue explant cells (DPTEs) that exhibit stem-cell characteristics and multi-potency; b) direct dental pulp tissue grafting with *ex vivo* tissue engineering technique sustains the vitality of the minced pulp and promotes DPTE outgrowth and differentiation into mineral-depositing cells on 3D scaffold. Attaining these goals, this study has established a solid foundation for in vivo application of pulp tissue grafting for regenerative endodontics.

MATERIALS AND METHODS

Sample collection and primary culture

Samples of discarded freshly extracted healthy 3rd molars (16-22 years old) were collected in Oral Surgery Clinic, School of Dentistry University of California Los Angeles. All primary culture of DPSCs and DPTEs were maintained with primary culture medium, α-MEM medium (Invitrogen, Carlsbad, CA) supplemented with 20% fetal bovine serum (Invitrogen), 15 μg/mL gentamicin sulfate (Gemini Bio-Products, West Sacramento, CA), and 20 mmol/L L-glutamine (Invitrogen). All passage cultures were maintained in basal medium, α-MEM with 10% FBS and 5 μg/mL gentamicin sulfate.

All teeth were stored in primary culture media in sterile Falcon tubes (Corning) on ice before promptly transferring to biosafety cabinet after extraction. It has been demonstrated that the vitality of the dental pulp could be preserved up to 24 hours after extraction under optimal condition. Teeth were washed three times with ice-cold PBS supplemented 15 μg/mL gentamicin sulfate, all attached periodontal and gingival tissue removed with scalpel. The enamel-cementum junction was cracked open with a wire-cutter and pulp carefully retrieved from chamber. The pulp were washed three times in 1xPBS before transport onto a 60mm petri dish and minced with micro-scissors into fine pieces (about 0.5 mm in length).

The minced pulp tissue from the same sample was subsequently divided into two groups. One group proceeded to DPSCs isolation while the other group was distributed evenly onto a six-well plate for DPTEs culture. For conventional DPSC enzyme digestion culture, pulp tissue was treated with a solution of 3 mg/mL type I collagenase and 4

mg/mL dispase (GIBCO) for 60 minutes in 37 degree water bath with vibration. After enzymatic digestion, the cell suspension was filtered through a 100 um cell strainer and washed three times by centrifugation in basal culture medium, and cell suspensions were seeded into 6-well cell culture plates (Corning). For the cells from dental pulp tissue explants (DPTE) outgrowth procedure, the minced tissue fragments were transferred directly onto petri dish with primary culture medium and incubated in 80% humidity, 5% Carbon dioxide atmosphere at 37 degree. Medium was changed every three days.

Morphological Analysis

Primary DPSCs and DPTEs were serially passaged at every 80% confluency level for subculture until proliferation arrested. Cumulative population doublings (PDs) and replication kinetics were determined based on the total number of cells in the beginning and at the end of each passage as previously described. After different time periods of culture, DPTEs and DPSCs were stained *in situ* for β -gal activity. For senescence-associated β – galactosidase assay, DPTEs and DPSCs (50,000) were seeded into 6-well culture plates and fixed in 2% formaldehyde/0.2% glutaraldehyde for 5 min at room temperature. The cells were then stained for β -gal activity in freshly prepared staining solution containing 1 mg/ml X-Gal (Sigma), 40 mM citric acid/Na phosphate buffer (pH 6.0), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, and 2 mM $MgCl_2$ for 12 h at 37°C without humidity and carbon dioxide. The dark green/blue color in the cytoplasm adjacent to nuclei indicated the presence of β -gal activity. The result was quantified under light microscopy.

Reverse transcription and quantitative real-time PCR (qPCR)

Total RNA was isolated from cells using TRIzol reagents (Invitrogen). After

centrifugation, the aqueous layer containing RNA is preserved. Isopropanol was added to the aqueous layer to form RNA precipitate. Lastly, total RNA was washed with 75% ethanol and its quality was assessed using NanoDrop Spectrophotometer (Thermo Fisher Scientific). cDNA was generated from 5 ug of extracted RNA utilizing SuperScript first-strand synthesis system (Invitrogen).

cDNA was amplified with SYBR Green I Master Mix (Roche) with LightCycler 480 II real-time PCR system (Roche) following manufacturer's protocol. All experiments were performed in triplicates with initiation of heat denaturation at 95 degree for 10 minutes, then 45 cycles of 95 degree for 10 seconds followed by 58 degree for 45 seconds and 72 degree for 10 seconds. GAPDH was used as an internal control. The second derivative of the Cq value was determined by comparing the gene of interest to GAPDH for fold-differences of amplification following the manufacturer's instructions (Roche).

Telomere length measurement with real-time PCR

Cell genomic DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen). DNA was quantified in triplicate using NanoDrop Spectrophotometer (Thermo Fisher Scientific). Relative telomere length was determined by protocols previously described. Briefly, PCR reactions were performed in triplicates in 10 ul reaction volumes for all the samples studied. The PCR reactions were performed using telomere and single copy gene primers in 96 well plate (Roche). The PCR mixture contained 10 pmoles of each of the primers (Tel and 36B4). The thermal conditions consisted of a initial denaturation of 5 minutes at 95 degree, followed by a total of 40 cycles at 95 degree for 5 seconds, 56

degree for 30 seconds, and 72 degree for 30 seconds and fluorescence acquisition. Crossing points (Cp) were determined using the LightCycler 480 software (Roche). The average of telomere versus single copy gene (T/S) ratio was calculated, which is proportional to the telomere length of each individual.

Measurement of telomerase activity

Telomerase activity was measured using the quantitative telomeric repeat amplification protocol (Q-TRAP) assay. Briefly, cell pellets were resuspended in 1X CHAPS buffer from TRAP-eze Telomerase Detection Kit (Chemicon, Temecula, CA) and incubated for 30 minutes on ice. After centrifugation at 12,000 x g for 20 minutes at 4 degree, aliquots of the supernatant were rapidly frozen and stored at -80 degree. The SYBR Green Q-TRAP assay was conducted with 0.25 ug cell lysates, 0.1 ug of telomerase primer TS and 0.1 ug of anchored return primer ACX, in 12.5 ul with SYBR Green I PCR Master Mix (Roche). Using the lightCycler 480 Real-Time PCR cycler (Roche), samples were incubated for 20 minutes at 25 degree, and amplified in 35 cycles of 30 seconds at 95 degree and 90 seconds at 60 degree. Each sample was analyzed in triplicate.

Western blotting

Whole cell extracts were isolated with cell lysis buffer (1% Triton X-100, 20mM Tris-HCl pH 7.5, 150mM NaCl, 1mM EDTA, 2.5 mM sodium pyrophosphate, 1μM β-glycerophosphate, 1mM sodium orthovanadate, 1mg/ml PMSF). The isolated extracts were fractioned by SDS-PAGE and transferred to polyvinyl difluoride protein membrane (Millpore). The membrane was incubated with a primary antibody overnight at 4 degree.

Secondary antibody was incubated at room temperature for tow hours. After incubation, the membranes were exposed to the chemiluminescence reagent (Bio-Rad) for detection of proteins. The antibodies detected were OCT-4 (ABcam), ALP and GAPDH (Santa Cruz Biotech). Horseradish-peroxidase (HRP) conjugate was used for secondary antibody (Santa Cruz Biotech).

Immunophenotypic characterization

DPTEs and DPSCs (10,000 cells) were seeded on chamber slides (Sigma) overnight before rinsing with PBS and fixation by incubating with 4% paraformaldehyde in PBS for 15 min at room temperature. Chamber slides were blocked in 5% bovine serum albumin (BSA) for 30 minutes in room temperature following 0.5% Triton incubation for 15 minutes. Primary antibodies, CD 146 (ABcam) and STRO-1 (R&D) were diluted to 1:100 in 1% BSA/0.05% Triton X-100/PBS and incubated overnight at 4°C. Fluorochrome-conjugated secondary antibodies were prepared 1:500 dilution (BD Biosciences) in 1% BSA/0.05% Triton X -100/PBS according to the manufacturer specification data sheet. All samples were incubated for 1 hour at room temperature in dark before counterstaining nuclei with 5ug/ml DAPI and fluorescent microscopy.

After up to 3 culture passages, DPSCs and DPTEs (50,000 cells) were pellet (10 minutes at 750 x g) and resuspended in 1x PBS with 0.1% sodium azide and 1% BSA as a marking solution. Cells were then incubated for 15 min with 10 mL of antibody diluted to 1:200 according to the manufacturer's protocol. Stem cell markers employed were the mouse anti-human antibodies (BD Biosciences) fluorochrome-conjugated antibodies for CD44 (hyaluronan receptor), CD90/Thy-1 (thymocyte antigen), CD105 (endothelial lineage), CD73 (T lymphocyte differentiation antigen) CD34 and CD45 (hematopoietic

stem/progenitor lineage marker). Flow cytometry was performed at the Flow Core of Jonsson Comprehensive Cancer Research at UCLA.

Odontogenic differentiation assay

DPTEs and DPSCs were cultured in basal medium before 90% confluency. Odontogenic induction media, conditioned with 100 μmol/L L-ascorbic acid 2-phosphate (Sigma, St Louis, MO), 9mmol/L KH_2PO_4, 10 mmol/L β-glycerolphosphate, and 9.8 nmol/L dexamethasone (sigma), was then added to cell culture with cells maintained in basal media as a control. Media was changed every other day.

Both cells lines were stained for ALP activity with the ALP Staining Kit (Sigma) at different days of induction culture. Solution A was prepared by melting 1 ml Fast Violet B Salt pill into 1 ml of sodium nitrite solution 0.1 M. Solution B was prepared by dissolving 1 ml of Naphtol AS-MX phosphatase alkaline solution in 48 ml distilled water, then A and B solutions were mixed in a single mixture. Cells were fixed with acetone/citrate 1:2.5 for 30 seconds, washed with distilled water for 45 seconds, added to alkaline-dye mixture for 30 seconds. Samples were incubated in the dark in sterile water for 10 min before photographic documentation.

Also, the cells were exposed to the aforementioned calcifying condition for 21 days and stained with Alizarin S Red for mineralization capacity assay. Briefly, induced cells were rinsed with PBS and fixed in 70% ethanol for 1 hour at 4° C. Cells were stained with 40 mM Alizarin red, pH 4.2, at room temperature for 10 minutes with gentle shaking. Cells were rinsed multiple times with water to remove unbound Alizarin S Red. Relative Alizarin S Red staining density was quantified by destaining in 10% acetylpyridinium chloride (Sigma) and measured at 562 nm on a multiplate

spectrophotometer.

Adipogenic differentiation assay

DPTEs and DPSCs were cultured in basal medium before 80% confluency. adipogenic induction media (D-MEM supplemented with 10% FBS, Invitrogen), conditioned with 60 μM indomethacin (Sigma), 0.5 mM 3-isobutyl-1-methylxanthine (Sigma), 1 μM dexamethasone (Sigma) and 5 μg/mL insulin (Invitrogen), was added to cell culture with cells maintained in basal media as a control. Media was changed every other day. Bone marrow mesenchymal stem cells (BMSCs) were used as a positive control in this assay, following identical protocol.

After 21 days of induction, all cells were fixed with neutral 10% formalin at room temperature for 30 minutes after gentle rinsing with PBS. Stock solution of Oil Red O was previously prepared with 0.3g of powder and 100 mL of 99% isopropanol. Upon washing fixed cells, 2 ml of 60% Isopropanol was then added into fixed cells and incubated at room temperature for 3 minutes. 30 mL of Oil Red O stock solution with 20 mL deionized water was mixed and filtered before adding into the culture plates and incubated for 10 minutes. Culture plates were observed under high magnification phase-contrast microscopy after final rinsing with double distilled water.

Tooth Slice/Scaffold Preparation

The freshly collected molars were transversally cut at the cervical region with a diamond-edged blade at low speed under cooling to obtain slices of approximately 1-mm-thickness.

The pulp cavity of each tooth slice was filled with sodium chloride (Sigma) before poly-L-lactic acid (PLLA) (Goodfellows, US) dissolved with chloroform was

poured into the chamber. After complete polymerization of PLLA and evaporation of chloroform in a fume hood overnight, the salt was completely removed with distilled water. DPTEs, DPSCs (50,000 cells in 10 uL medium) and freshly minced dental pulp tissue were seeded onto the PLLA carefully before incubation of 30 minutes for cell attachment. All dentin scaffolds was then placed into a new petri dish with fresh medium. For *ex vivo* histological analysis, after 7 days of culture, PLLA inside of the dentin slices were retrieved and fixed in 10% formalin at 4°C for 24 hours. Samples were mounted into paraffin blocks and sectioned into 5um thick slides before hematoxylin-eosin staining. For mineralization analysis, cell/tissue was cultured on scaffolds for 21 days in odontogenic induction media and subsequently proceeded to Alizarin S Red staining protocol as aforementioned.

For PuraMatrix hydrolgel (BD Biosciences) culture, cells were resuspended in a 1:1 mixture of hydrogel and cell culture medium to allow for their seeding in the scaffolds. The tooth slice/scaffolds containing cells were incubated for 30 minutes at 37°C to allow for the setting of the hydrogel. After 21 days of culture of DPSCs and minced dental pulp tissue in calcifying condition, the hydrogel-dentin slices scaffold was stained for minerals with protocols previously described.

RESULTS

Sample collection and tissue culture

Fourteen sets of freshly extracted third molars (patient age 16 to 22 years old, 6 female 8 male) were collected from UCLA oral and maxillofacial surgery clinic in compliance with university protocols. Collection was carried out through out the period from February 2014 to June 2015.

The pulp tissue was minced finely using microscissors and viewed under phase contrast micrograph at different magnifications. To reveal the abundance of cellular contenct within the pulp tissue, we labeled the nuclei with DAPI (4',6-diamidino-2-phenylindole) for 30 minutes and viewed under fluorescence microscope (Figure 1). DAPI is a blue-fluorescent nucleic acid that preferentially stains dsDNA, by binding to the AT clusters in the minor groove. Such interaction Binding of DAPI and dsDNA yields a 20-fold fluorescence enhancement, potentially due to the displacement of water molecules from both DAPI and the minor groove. The minced dental pulp exhibited numerous living cells homed in the tissue, even though the unstained tissue appeared like a lifeless collection of connective matrix. Such abundant cellularity in the dental pulp presumably serves as the cell source for the outgrowth tissue explant culture.

Pulp tissue from the same patient was subsequently divided into two groups after fine cutting and mincing. One group of minced pulp tissue was distributed evenly onto cell culture dish. After early as 4 days of culture we observed outgrowth of rapidly proliferating cells from the tissue explant, named DPTEs. The other group was enzymatically prepared using the standard protocol for DPSC isolation. Fourteen sets of samples yielded three pairs of DPTE/DPSC after primary culture and early passaging,

with the other eleven cell lines producing either DPTEs or DPSCs due to procedural errors i.e., contamination. In one of the three pairs, the DPTE were contaminated after P10, all results consequently annulled. Two pairs of cell lines were included in this project with each individual experiments triplicated. All in vitro experiments were performed with early passages (up to P5) of DPTEs and DPSCs to minimize the effect of in vitro cell expansion on cell property, unless mentioned otherwise.

DPTEs exhibited similar morphological change but different proliferative dynamics compared to DPSCs.

DPTEs and DPSCs were indistinguishable under light microscopy at early stage, with the spindle shape typical of mesenchymal cells. Both cell lines also underwent similar morphological change from exponentially replicating stage to senescence, at which time the cells exhibited a flattened and enlarged morphology (Figure 2).

In the aim of further elucidating the proliferation dynamics of DPTEs and DPSCs, replication kinetics were determined and plotted against time in culture. Rapidly proliferating cultures of DPSCs and DPTEs were serially sub-cultured until the cells spontaneously arrested their replication, replication kinetics documented. DPSCs demonstrated exponential replication for approximately 85 days in culture, completing 50 population doublings (PDs). However, DPTEs completed 40 times of population doublings in approximately 60 days with faster replication rate and arrested at PD 47. Population doubling time of the first and last 20 days of culture of both cell lines also indicated that DPTEs at an initial stage replicated faster than its counterpart but ceased proliferating earlier (Figure 3A).

Both DPTEs and DPCSs entered a stage called replicative senescence *in vitro* after rapid duplication. Cells in this physiological stage had overexpression and accumulation of the endogenous lysosomal β-galactosidase, which has been demonstrated to be associated with cell senescence (senescence-associated β-galactosidase A-gal, SA β-gal)(M K Kang et al. 2000). Under certain pH, SA β-gal can be readily stained and visualized *in situ* under light microscope. At different passages of DPTEs and DPSCs, cells were stained for SA β-gal, and results quantified. DPTEs had a higher percentage of senescent cells than DPSCs from PD30, suggesting that DPTEs enter senescence earlier than DPSCs in addition to its faster proliferation at earlier stage (Figure 3B and C).

Telomere is a highly conservative region of repetitive DNA sequence located at each end of the chromosome, which protects the functional genes at the polar region from erosion during DNA replication(Blackburn 1991). However, due to the limitation of semiconservative replication, telomere would be gradually truncated in DNA duplication. Such deterioration of the telomere is associated with aging and senescence. Both cultures of DPTEs and DPSCs demonstrated progressive shortening of telomere DNA during in vitro replication. This was correlated to the final morphological senescent stage in both cell lines. However, started at similar telomere length at day 10 from primary culture, the rate of erosion in DPTEs seems to be faster than that of the DPSCs. At the terminal stage, DPTEs telomere was significantly shorter than DPSC, even though DPSCs had a duplicated more doublings (Figure 4A).

Additionally cells that are able to replicate unlimitedly, such as human malignancies and stem cells, have a protective mechanism of elongating their telomeric DNA sequence via telomerase activation. hTERT is the gene coding the functional

catalytic enzyme of human telomerase reverse transcriptase (hTERT) (Poole, Andrews, and Tollefsbol 2001). Its expression is closely correlated with telomerase activity *in vitro and in vivo*. (Figure 4B) Additionally, telomerase activity can be detected by telomere repeat amplification protocol (TRAP assay). When we checked such mRNA expression level of these cells, we found that the both DPTEs and DPSCs had higher hTERT level than that of normal human oral fibroblasts (NHOF). NHOF is a terminally differentiated oral fibroblast cell line exhibiting limited proliferation capacity and no multi-potency. Furthermore, it has been proven that highly active telomerase serves as one of the mechanisms for the immortalization of HOK-16B, a human oral keratinocytes transformed by transfection with recombinant human papillomavirus type 16 (HPV-16) DNA(Park et al. 1991). It has 27 folds higher hTERT expression than that of the DPTEs and DPSCs. In conclusion, DPSCs and DPTEs undergo subculture-induced senescence through telomere shortening as a result of lack of ability to maintain a high level of telomerase activity. However, they both had more potent telomerase than NHOF, suggesting the molecular mechanism of a longer lifespan in DPTEs and DPSCs than terminally differentiated somatic cells (Figure 4C).

DPTEs demonstrated stem cell characteristics in consistent with DPSCs

DPSCs transplantation *in vivo* rendered promising outcomes, as the regenerated tissue resembles the architecture and function of a healthy dentin-pulp complex. To determine whether DPTEs also represent a regenerative cell population, we started looking into the cell surface markers consistent with MSCs of dental origin, notably, STRO-1 and CD146.

STRO-1 is proven to be present on precursors of various stromal cell types. In preosteogenic precursors, the expression of STRO-1 is progressively lost after cell proliferation and differentiation into mature osteoblasts *in vitro*.(Shi and Gronthos 2003) Therefore, STRO-1 has been established as a hallmark stem cell marker of many different mesenchymal stem cell populations including DPSCs. While the precise function of CD146 is still under investigation, it has been associated with greater differentiation potential in MSCs. As is shown in the figure, both DPSCs and DPTEs were positively stained for the MSC markers (Figure 5A).

Additionally, cell surface immunophenotype profile of both DPTEs and DPSCs was quantified by flow cytometry. As indicated, DPTEs exhibited similar surface immunophenotype profile as DPSCs. In DPTEs, CD44 (83%) and CD90(99%) were highly expressed while CD105 (1%), CD34 (1.1%), CD73(0.2%) and CD45(0.3%) were suppressed. This is in consistent with the expression profile of DPSCs, CD44 (80%), CD90 (100%), CD 105 (1.1%), CD34 (0.8%), CD73 (2%), CD45 (0.6%). While the expression of hematopoietic markers like CD45 and CD105 was been demonstrated by multiple studies to be absent from the mesenchymal stem cell culture, the existence of CD 34 has been proven to be controversial (Figure 5B). Oct-4 is a reprogramming transcriptional factor closely governing the self-renewal of undifferentiated state of stem cells as well as human malignancies(Takahashi and Yamanaka 2006). It expression was detected and maintained in both DPTEs and DPSCs (Figure 5C).

The cardinal concept of stem cell characteristics is multi-differentiation potency. In order to assess such capacity of DPTEs and compared that to the DPSCs, we evaluated the osteo/odontogenic differentiation capacities by alkaline phosphtase (ALP) activities

under differentiating conditions. Both DPTEs and DPSCs expressed ALP activities when induced with β-glycerophosphate, dexamethasone, and vitamin C, according to the standard protocol. However, the level of ALP activity was notably higher in DPTEs compared with those of DPSCs; ALP activity was detected in DPTEs even in the basal medium without induction (Figure 6A and B). Additionally, we collected the cell extracts of cells in calcifying condition at different days of induction, and the protein expression level of ALP also reinforced our findings (Figure 6C).

The evaluation of mineralization capacity of DPSCs and DPTEs is crucial in that it demonstrates the functional stage of osteo/odontal differentiation. Both cell lines were maintained in calcifying condition up to three weeks after reaching confluency; Alizarin S Red staining was performed periodically. Both DPTEs and DPSCs exhibited mineral accumulation in the culture dish in relation to time maintained in the culture (Figure 7).

Furthermore, in the aim of establishing multi-potency of DPSCs and DPTEs, we also assessed adipogenic differentiation capacities of both cell lines along with bone marrow-derived mesenchymal stem cells (BMSCs) according to method described in our prior study. BMSCs demonstrated potent adipogenic capacity under certain induction. In this experiment, it served as a positive control. After 21 days in the adipogenic induction media, DPTEs as well as DPSCs and BMSCs, clearly showed positive staining for the oil droplets within the cytoplasm. Therefore, DPTEs demonstrate multi-lineage differentiation capacity at least in osteo/odontogenic and adipogenic pathways (Figure 8).

Direct grafting of dental pulp tissue *ex vivo* tissue engineering

Although DPTEs demonstrated stem characteristics *in vitro*, success of pulp tissue transplantation for pulp-dentin regeneration depends in part on the explantation of MSCs

in vivo when transplanted onto dentin slice scaffolds. To test whether minced pulp tissues can be the source of migrating DPTEs *in vivo*, we utilized the dentin-slice model in which minced pulp tissue was placed inside the lumen of dentin disc along with either poly-L-lactic acid (PLLA) or PuraMatrix hydrogel.

DPSC transplantation utilizing the PLLA-dentin disc scaffold yielded excellent outcome *in vivo*. PLLA is a biocompatible synthetic polymer mainly consumed in 3D printing. It is highly soluble in chloroform and insoluble in water, which provided us the foundation for casting the scaffolds. It would also gradually degrade *in vivo*, only serving as a temporary attachment for transplanted tissue. In our *ex vivo* tissue engineering experiment results, DPTEs and DPSCs seeded on PLLA scaffold inside the dentin disc were able to attach on the scaffold and maintain the viability. Also, when minced pulp tissue was inserted inside the dentin disc with PLLA, cells migrated from the tissue explants and were attached on the scaffold. These cells formed mineralized nodules inside the dentin disc when cultured with mineralizing medium for 21 days. These data indicate that minced pulp explants on 3D scaffold can provide migrating DPTEs capable of osteo/odontogenic differentiation *in situ*. (Figure 9)

PuraMatrix Hydrogel is a collection of synthetic natural peptides, resembling 3D ECM microenvironment(Zhang, Ellis-behnke, and Zhao). It is capable of fast self-assembling in culture medium. Hydrogel is injectable with a quick setting time, which would undoubtedly be beneficial if we are to mix it with cells/tissue grafts and place it into the root canal. Both DPSCs and DPTEs were able to grow and proliferate in the 3D hydrogel scaffold. Notably, cells were able to explant out from the minced tissue and came in contact with the dentin wall in the hydrogel scaffold. After 21 days of culture in

osteo/odontal inductive media, Alizarin S Red demonstrated strong mineral deposit site at the inner wall of the dentin with scattered mineralization in the inside of the wall in both DPTE and DPSC cultures. (Figure 10)

DISCUSSION

Tertiary dentin is deposited by functional dental pulp as a protective mechanism for the tissue to fend off stimuli such as mechanical trauma or dental hard tissue infection disease.(Stanley et al. 1983) It is commonly recognized that progenitor cells harbored in the dental pulp are capable of initiating such physiological response (Gronthos et al. 2000). Consequently, multitude investigations have been launched to identify such cell population. Gronthos et al demonstrated the existence of postnatal mesenchymal stem cell (MSC) population in the dental pulp for the first time. By utilizing similar methodology for bone marrow mesenchaymal stem cell (BMMSC) isolation, their research group was able to identify a highly proliferative cell population within healthy dental pulp, namely dental pulp stem cells (DPSC), which exhibited multi-potent property of stem cells by differentiating into both odontoblast-functioning cells as well as chondrocyte-like cells.

As proposed by Gronthos, DPSC primary culture requires enzymatic digestion of pulp tissue to free the individual cell from the connective extracellular matrix. In 1982, Prime reported that transplanting intact human dental pulp beneath the kidney capsule of SCID mice failed to generate calcified dentin matrix or odontoblast-like cells. As such, they concluded that the failure of intact tissue to deposit dentin was due to the sequestration of putative stem cell population within the tissue. By enzymatic digestion, the DPSC constrained in the stem cell niche would be mobile to readily interact with local microenviroment.

The promising utilization of postnatal MSCs in regenerative medicine prompts heated investigations for more MSC sources as well as simplified protocols for MSC

isolation. Numerous studies have investigated the characteristics of pulp cells harvested from tissue explanting culture in the hope of elucidating an untroublesome way for DPSC isolation. Couble discovered that the cells from dental pulp explant culture (DPTE) exhibited features of actively functioning odontoblasts such as the synthesis of an organized dentin-like collagenous mineralizing matrix (Couble et al. 2000). More importantly, they also discovered that DPTE showed a polarized cell morphology and high expression of DSPP *in situ*, which has been associated with the initiation of odontogenic mineralization and considered a terminal phenotypic marker of mature odontoblasts. Couble's study is one of the preliminary investigations validating the feasibility of stem cell isolation by dental pulp tissue explant culture.

Utilizing both primary culture methods, our study indicates that DPTE and DPSC exhibits indistinguishable morphological change from highly proliferative to replicative senescence. However, DPTE replicates faster than DPSC in the exponentially proliferating stage. Additionally, our study suggests that in the odontogenic condition, DPTE shows a more rapid differentiation rate than DPSC by exhibiting much higher ALP expression after the same time period of induction. This data is in consistence to Spath study, in which they suggested that explant-derived human dental pulp stem cells demonstrated enhanced differentiation and proliferation potential (Spath et al. 2010). The differences in the DPTE and DPSC behavior should prompt further investigation of interaction between cells. More is needed to identify if such crosstalk among pulpal MSCs in fact exists and how it is conducted. Co-culture of already established DPSC with the medium extract of DPTE could shed some light on this matter, as growth factors

(protein) as well as exosome (microRNA) can both serve as molecular cues that could be released into the culture media (Valadi et al. 2007).

Additionally, our study investigated the replicative senescence mechanism of DPTE and DPSC from the same donor by subsequent passage culture of cell lines to its terminal stage. Erosion of the repetitive telomeric conservative DNA sequence, namely telomere, is associated with aging and senescence (Blackburn 1991). Both cultures of DPTE and DPSC exhibit progressive shortening of telomeric DNA. However, according to our study, the rate of telomeric deterioration in DPTEs seems to be faster than that of the DPSCs. Consequently in the terminal stage DPTE telomere is significantly shorter than that of the DPSC, even though DPSC has undergone more population doublings. These findings, taken together, suggests that the higher proliferative capacity of DPTE at the early stage of lifespan as well as higher differentiation potential may be correlated to the faster telomeric erosion during replication, as a result, DPTE enters the replicative senescence earlier by exhibiting higher percentage of SA β-gal positive cells and ceases dividing earlier. In addition, we also demonstrate that both DPTE and DPSC possess telomerase activity that is more potent that terminally differentiated somatic cell line, suggesting their limited protective mechanism of elongating their telomeric DNA sequence, which is an indication of highly proliferative stem cell characteristics.

MSC lacks specific surface markers to distinguish them from the general cell population. In recent years, Grothos proposed that the expression of STRO-1 and CD146 might be concomitantly associated with MSC population in dental pulp. Consistent to their proposal, our study suggests that both DPTE and DPSC exhibited STRO-1 and CD146 expression at early stage of in vitro expansion. In their study, Grosthos concluded

that the in situ STRO-1+/CD146+ cells were located in close proximity to blood vessels, suggesting an association of dental pulp MSC population to the vascular structure, namely a perivascular niche. Moreover, Spath utilized the tissue explant cell culture method to isolate DPTE and investigated its reaction to endothelin (ET-1). Produced by endothelium, ET-1 functions as maintaining vascular homeostasis as well as nociception and local inflammation. Their study demonstrated the DPTE was highly reactive to ET-1 challenge with more cytoplasmic calcium release whereas the enzymatic digested DPSC was significantly less reactive, reinforces the hypothesis of a MSC perivascular niche in dental pulp and further proposed that enzymatic digestion of tissue might have a negative effect on cell vitality by damage such a MSC niche in the dental pulp. Souza also utilized tissue outgrowth method to isolate stem cell from the pulp, and concluded that the major advantage of outgrowth method was its convenience and low cost, and suggesting enzyme might be used to completely dissociate cells from the tissue but risking cell damage and cell loss (Souza et al. 2010).

The immunophenotypic profiling in our study demonstrates different protocols of stem cell isolation do not influence the surface marker profiles, which is line with previous findings. However, while the high expression for neural crest cell precursor marker CD117 and low hematopoietic CD45 in MSC population from dental pulp have been widely acknowledged, the expression of CD34, a hematopoietic stem cell marker, however remains controversial. Our finding that CD34 has minimal expression in both DPTE and DPSC is in accordance to (Gronthos et al. 2000)(Gronthos et al. 2002)(George T.-J. Huang, Shagramanova, and Chan 2006)(Kerkis et al. 2006). While (Laino et al. 2006)(Pierdomenico et al. 2005)(De Francesco et al. 2009) argued that CD34 is highly

expressed in dental pulp MSCs. It is believed that all stem cells are considered to be a collection of heterogeneous mixture of cells that exhibit different proliferation and differentiation potentials. More research is needed to elucidating the obstacles of identifying a definitive cell marker for MSC isolation due to their heterogeneity.

The core property of MSC is multi-potency. Our investigation demonstrates that under certain condition, both DPSC and DPTE are capable of at least committing to odontogenic and adipogenic differentiation pathways by mineralization in culture dish and accumulation of cytoplasmic oil lipid droplets respectively. Multiple studies have demonstrated the odontogenic differentiation potential of stem cell from dental pulp. Specifically, Grothos et al showed that stem cell from enzymatic digestion can different in to chondrocytes as well as odonblast-like cells they failed to induce DPSC adipogenesis. Nevertheless Batouli 2003 reported that DPSC were capable of differentiating into adipocytes (Batouli et al. 2003), while Couble 2000 for the first time demonstrated stem cell isolated by tissue explant culture can different into mineral depositing cells. However, as a descriptive study, our results suggest the potency multipotent phenotype of DPTE and DPSC. In order to compare the differences in this matter, expression of osteo/odontogenic markers i.e. BSP as well as adipogenic markers i.e. PPAR-gamma could be further quantified by qPCR and western blotting for a complete understanding of the stem cell properties (Spiegelman 1998; Yu et al. 2007).

In conclusion, both enzymatic digestion and tissue outgrowth methods are reliable for isolation of stem cell from dental pulp, while the cell populations resembles different subpopulations of multipotent cells. As suggested by Kerkis, the outgrowth method might promote the highly proliferative immature dental pulp stem cells while Alleman

concluded that enzymatic digestion might produce heterogeneous populations while the isolation of outgrowth from tissue explants might give rise to large homogeneous populations of stem cell from pulp tissue(Alleman et al. 2000). Above all, all these findings from our study demonstrates that the pulp outgrowth method, as the putative cell proliferation mode in dental pulp tissue grafting approach, is capable of generating highly proliferative and multipotent stem cell population to serve as the cell source for the pulp tissue engineering.

The choice of suitable three-dimensional scaffolds is one of the three major components of tissue engineering. According to, Bottino, such scaffolds should be biocompatible and biodegradable porous material to support the mechanical property of cell growth, while mimicking ECM preferably with biomolecular cues for proliferation/differentiation (Bottino et al. 2013). Cordeiro utilized poly-L-Lactic acid-dentin slice model for SHED in vitro transplantation in a pulp regenerative procedure and attained promising result by successfully generating tissue with an architecture closely resembling that of the normal dental pulp. In their study, they hypothesized that the bioactive molecular cues for stem cell differentiation sequestrated in the dentin matrix would be release upon the localized low pH generated by degradation of PLLA. Such mobilization of growth factors promoted the proliferation and differentiation of DPSC for the their study. Multiple other studies have also applied PLLA-dentin slice model (Lovelace et al. 2011; George T.-J. Huang, Shagramanova, and Chan 2006). Identical scaffold is used in our approach. However, besides DPSC transplantation, minced dental pulp tissue was seeded on to the PLLA scaffold as well in our ex vivo tissue engineering. Our findings suggest that in addition to the survival and attachment of pulp tissue on the

scaffold, cells migration out from the tissue was also observed, while maintained in the odontogenic medium, DPTE is also capable of odontogenic differentiation and mineralization in the 3D culture. Nevertheless, the extrapolation of the degradation of PLLA in vivo to an ex vivo situation is questionable, as the degradation of PLLA, *in vivo or in vitro* would be too slow to actually effect the local microenvironment (Bos et al. 1991). More investigation is desired to elucidate how the proliferative and differentiation-promoting factors effects the behavior of the cells in the scaffold.

However, the rigidity of PLLA scaffold serves as the major disadvantage for its clinic application. Nevertheless, PLLA by itself is hydrophobic, which is not beneficial to the attachment of cells. Pre-treating the PLLA surface with collagen coating might promote a better microenviroment for cell anchoration (Xiao et al. 2006). PLLA requires customized casting and molding to fit individual canal (Cavalcanti et al. 2012). We also investigated the feasibility of an injectable and fast-setting PuraMatrix Hydrogel. Hydrogel is a self-assembly peptide hydrogel, which will polymerize and form a fibrous matrix in the presence of free ions such as the PBS or regular culture media. In addition, such scaffold is capable of being readily manipulated to add in growth factors. In our study, by mixing cells/tissue pieces with the hydrogel before initiating setting with culture medium, the cells were able to maintain its 3D architecture and proliferate by anchoring its self in the matrix. More importantly, cells are also able to explant out from the tissue and came in contact with the inner surface of dentin slices with the ability to undergo odontogenic pathway with mineral deposition onto the culture and the dentin surfaces.

The therapeutic effect of postnatal MSC depends not only on their differentiation ability to repair damaged tissue but also their potency to modulate local microenvironment. However, the detailed mechanism of these remained unclear. Scientific evidence of safety and efficacy of stem cells transplantation must be well established in appropriate animal models before clinical application to eliminate the potential side effects linked to genomic instability. As a consequence, MSC based therapy is under intense regulation. The translation of pre-clinic study of DPSC transplantation into day-to-day clinic practice requires a Good Manufacturing Practice (GMP) facility for the cell culture and expansion. It is essential to manufacture clinical grade stem cells according to GMP condition for the FDA approval of this autologous stem cell transplantation method. Murakami proposed an effective and safe protocol for GMP grade DPSC isolation and expansion by utilizing granulocyte-colony stimulation factor to mobilize the highly proliferative and multi-potent cell population from whole pulp cells. Such subpopulation of DPSC demonstrated successful result from dog model *in vivo* pulp regeneration. However, GMP has demanding and specific requirement for its facility construction. Paramount investment would be anticipated prior to establishment of such a facility in order to satisfy such strict regulation. As such, GMP facility is far from realistic to be incorporated into the initial investment of a regular endodontic clinic. Nevertheless, our approach for regenerative endodontics circumvents the entire process of laboratory isolation and expansion of MSC from dental pulp. Additionally, utilizing healthy dental pulp from developing third molars and/or from exfoliating primary teeth that is readily retrievable, direct tissue grafting could be practical with much less chair time for tissue regeneration.

Our study project has established the fundamental rationale for the utilization of direct pulp tissue grafting in regenerative endodontics. This is a preliminary study of an unprecedented approach to pulp-dentin tissue engineering. By demonstrating the stem-cell characteristics and multi-potency of dental pulp cells from tissue explants (DPTE) in vitro as well as successful three dimensional ex vivo tissue engineering with direct dental pulp grafting, this study has charted out a blueprint for subsequent investigations of a brand-new technique for regenerative endodontics.

Figure 1. Phase-contrast and immunofluorescent microscopy of minced pulp tissue, healthy pulp tissues removed from extracted third molars were minced finely using microscissors and viewed under phase-contrast micrograph at different magnifications. To reveal the cellularity within the pulp tissues, in the right panel we labeled the nuclei with DAPI and viewed under fluorescence microscope.

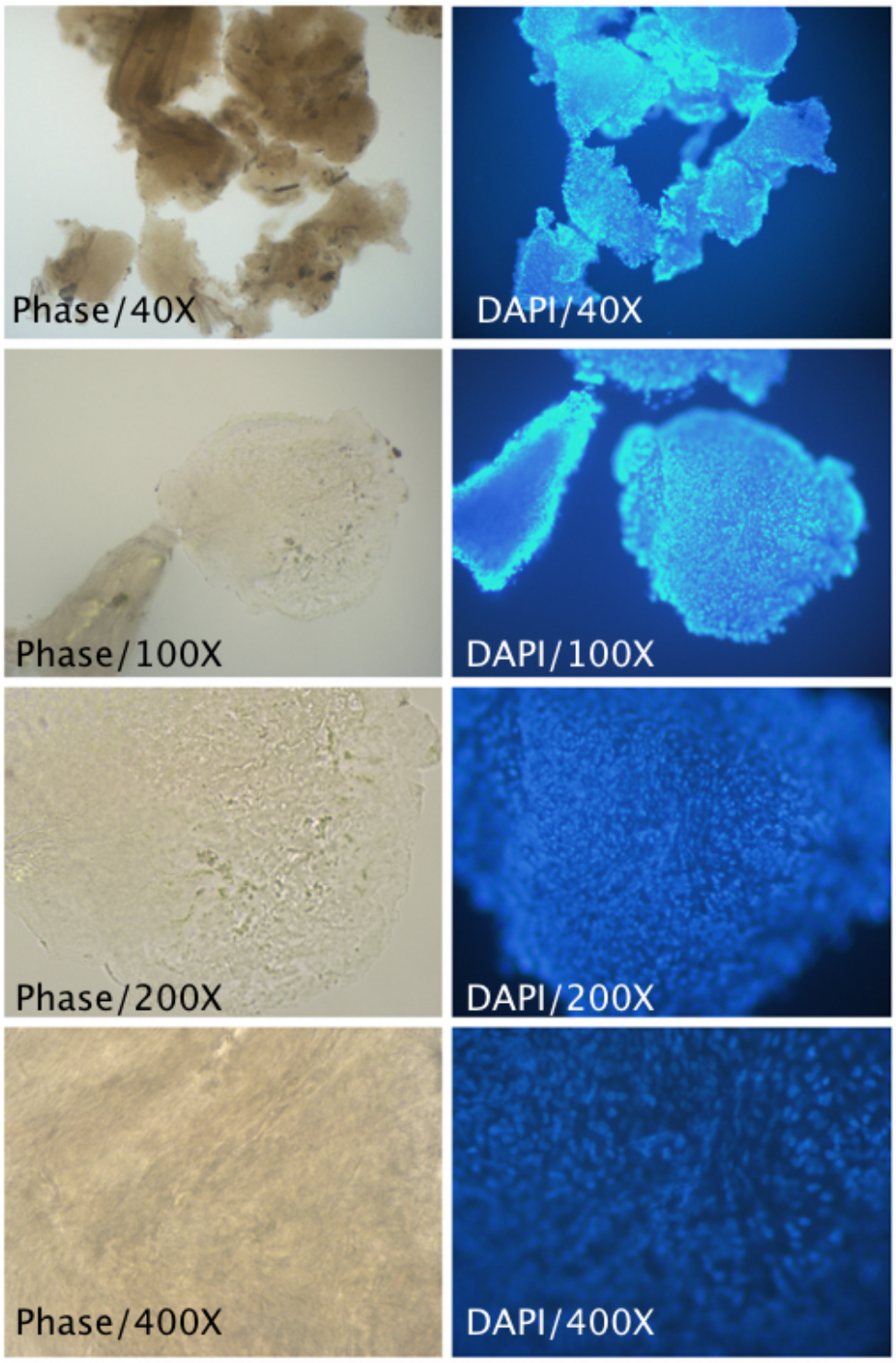

Phase/40X
DAPI/40X
Phase/100X
DAPI/100X
Phase/200X
DAPI/200X
Phase/400X
DAPI/400X

Figure 2. Pulp explantation establishes replicating culture of mesenchymal cells. After the minced pulp tissues were explanted onto cell culture dish, we observed outgrowth of rapidly proliferating cells from the tissue explant, namely DPTE. DPSC and DPTE were viewed under 100x magnifications under phase contrast microscopy at different stages of their lifespan. Figure 2 shows DPTE are morphologically indistinguishable from the DPSC.

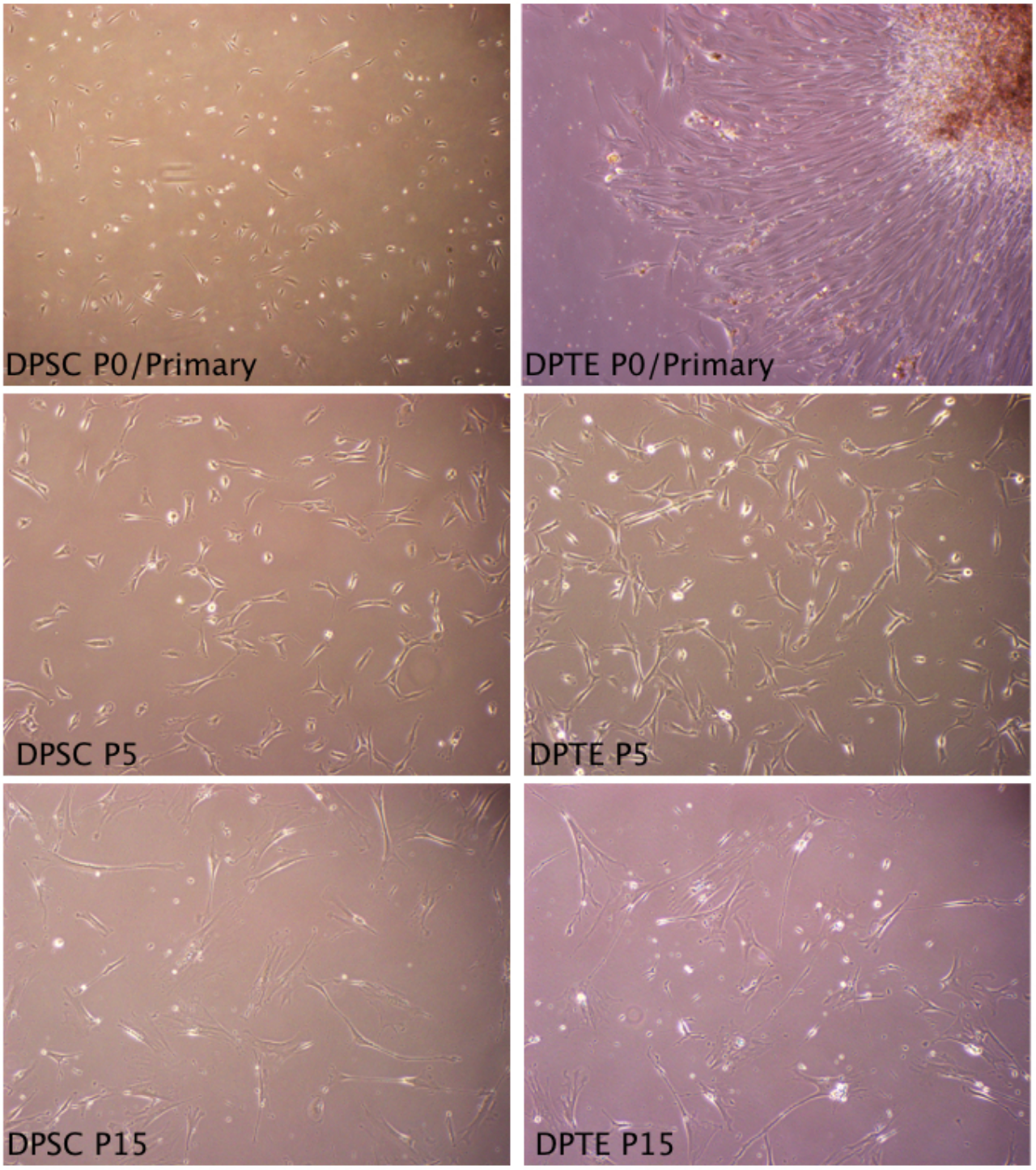
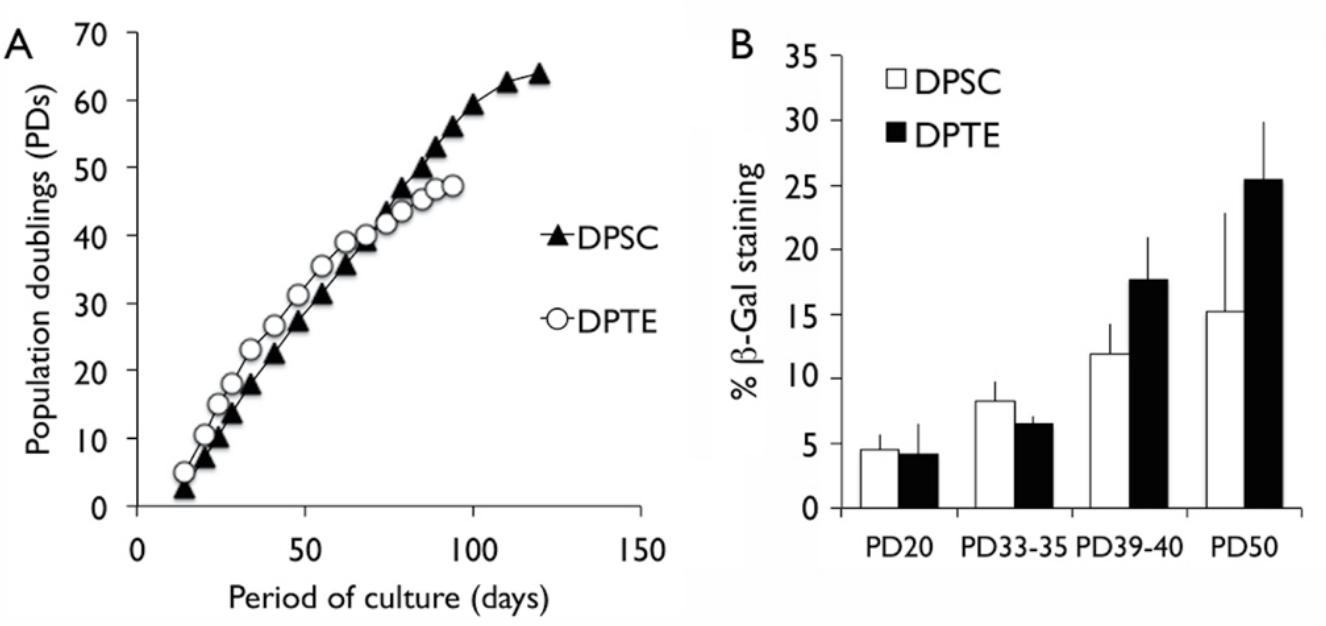

DPSC P0/Primary
DPTE P0/Primary
DPSC P5
DPTE P5
DPSC P15
DPTE P15

Figure 3. DPTE and DPSC undergo limited lifespan and replicative senescence on continuous subculture. (A) Replication kinetics of DPTE and DPSC were plotted against time in culture for the population doublings. (B) Quantification of SA-β positive cells in DPTE and DPSC. (C) Different PD of DPTE and DPSC demonstrated SA-β presented in the cytoplasm.


A
Population doublings (PDs)
70
60
50
40
30
20
10
0
0
50
100
150
Period of culture (days)
DPSC
DPTE
B
% β-Gal staining
35
30
25
20
15
10
5
0
DPSC
DPTE
PD20
PD33-35
PD39-40
PD50

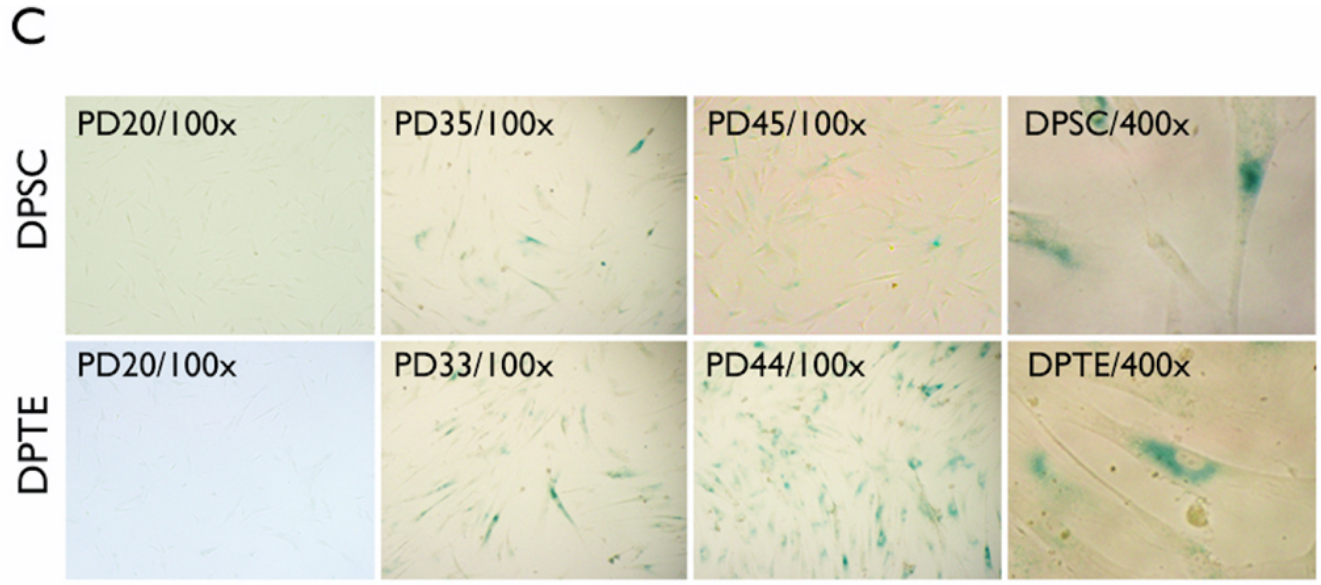
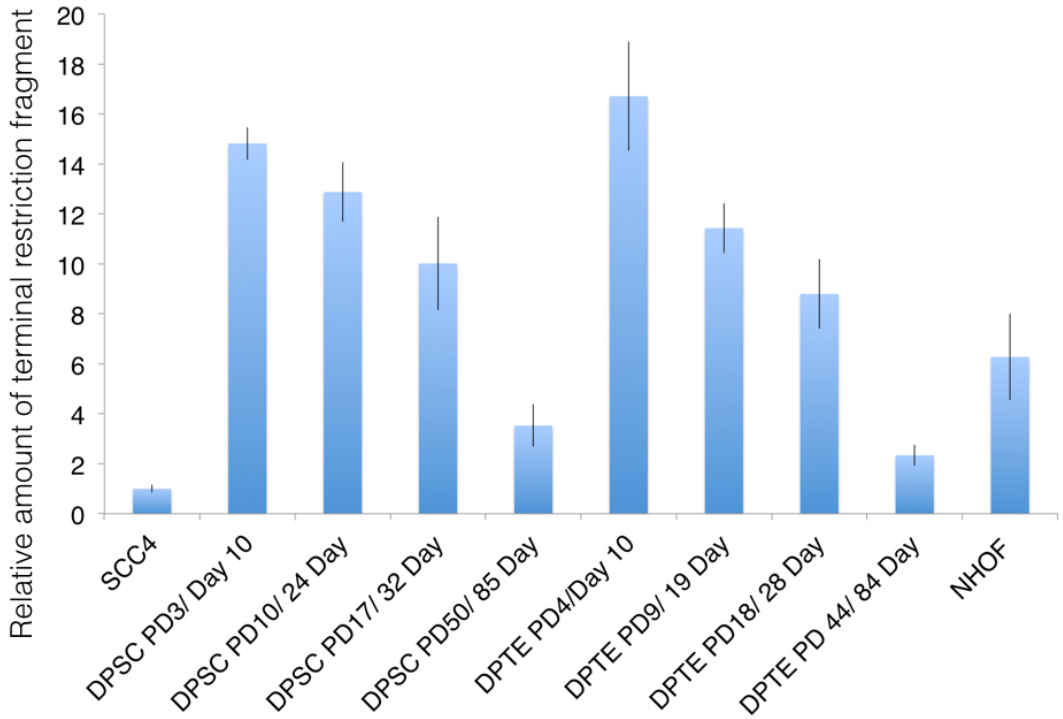
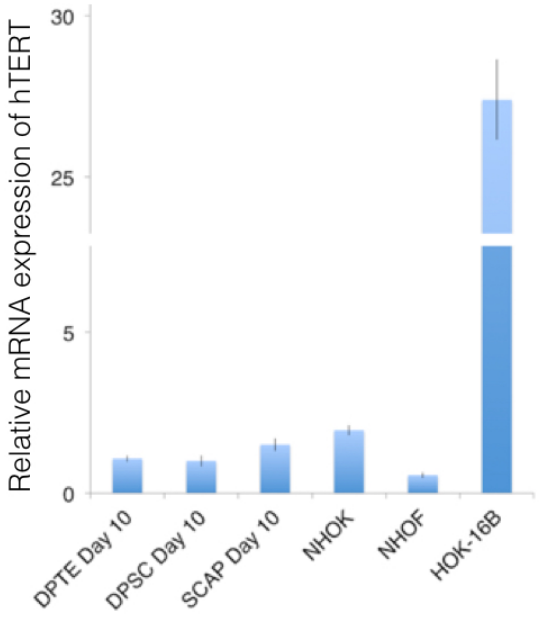

C
DPSC
PD20/100x
PD35/100x
PD45/100x
DPSC/400x
DPTE
PD20/100x
PD33/100x
PD44/100x
DPTE/400x

Figure 4. DPTE and DPSC demonstrate progressive telomere length shortening in continuous subculture, as well as limited telomerase activity. (A) Repetitive terminal DNA sequence (Telomere) was determined by qPCR analysis of genomic DNA of DPSC and DPTE compared to SCC4 and NHOF at different population doublings. (B) Real-time qRT-PCR to quantify the hTERT mRNA in DPTE, DPSC, SCAP from the same donor in compareson to NHOF and HOK-16B; (C) Telomere repeat amplification protocol (TRAP) assay for detection of telomerase activity in DPTE and DPSC.

(A)


Relative amount of terminal restriction fragment
20
18
16
14
12
10
8
6
4
2
0
SCC4
DPSC PD3/ Day 10
DPSC PD10/ 24 Day
DPSC PD17/ 32 Day
DPSC PD50/ 85 Day
DPTE PD4/Day 10
DPTE PD9/ 19 Day
DPTE PD18/ 28 Day
DPTE PD 44/ 84 Day
NHOF

(B)


Relative mRNA expression of hTERT
30
25
5
0
DPTE Day 10
DPSC Day 10
SCAP Day 10
NHOK
NHOF
HOK-16B

(C)

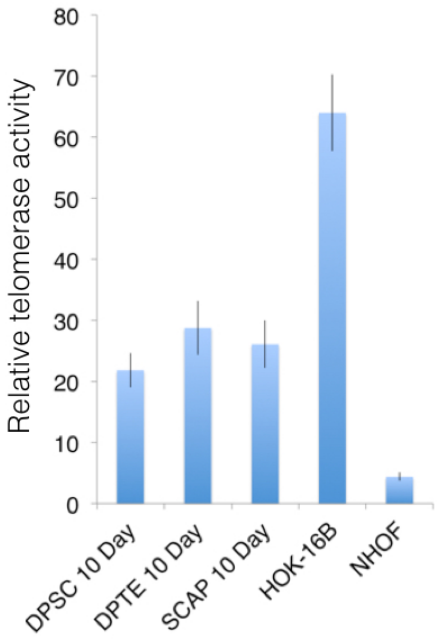
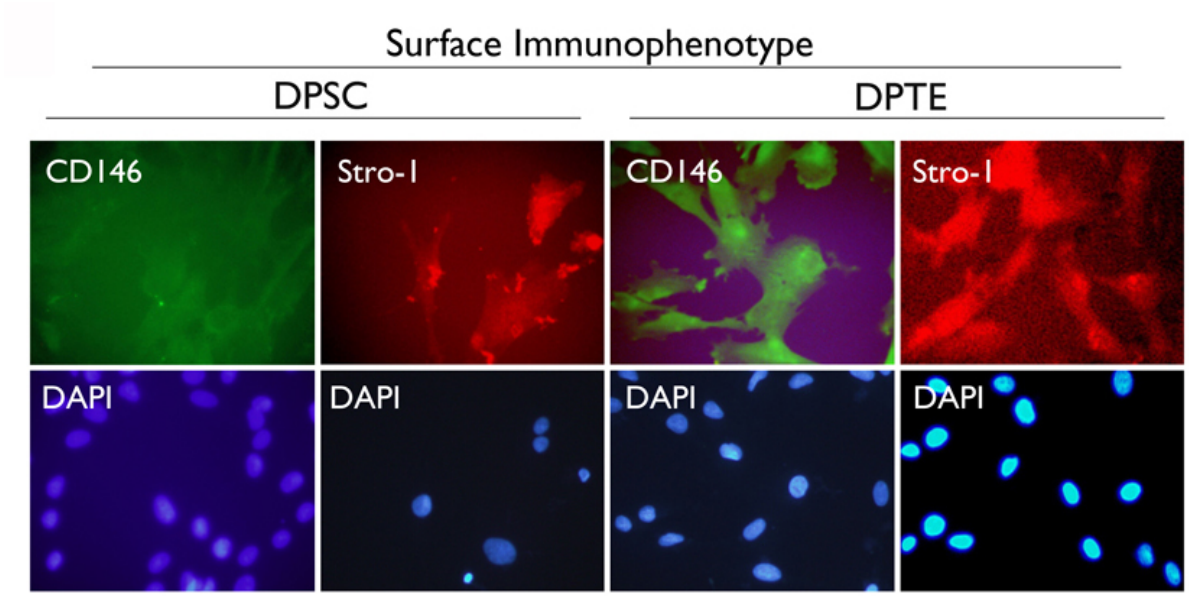
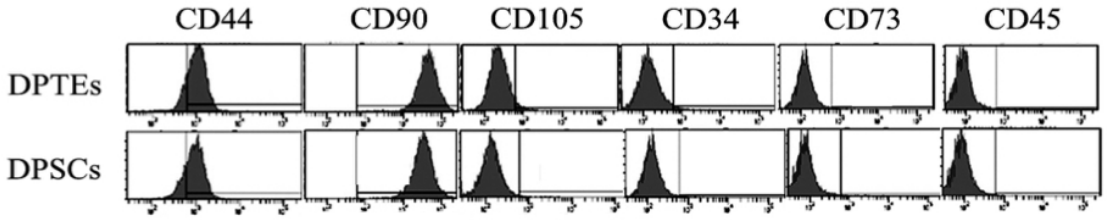

Relative telomerase activity
80
70
60
50
40
30
20
10
0
DPSC 10 Day
DPTE 10 Day
SCAP 10 Day
HOK-16B
NHOF

Figure 5. DPTE express mesenchymal stem cell immunophenotypic profile similar to DPSC as well as maintaining reprogramming factor OCT-4 expression in vitro. (A) Both DPTEs and DPSCs show positive signal for CD146 and STRO-1 as surface markers with immunofluorescent staining (upper panel). Cells were also co-stained for DAPI (lower panel) to reveal the nuclei. Original magnification, 100x. (B) DPTEs exhibited consistent surface marker expression profile for MSCs Upper panel, Quantification for DPTEs CD44 (83%) and CD90 (99%) were highly expressed while CD105 (1%), CD34 (1.1%), CD73 (0.2%), and CD45 (0.3%) were negative. Lower panel, surface marker expression profile for DPSCs, CD44 (80%), CD90 (100%), CD105 (1.1%), CD34 (0.8%), CD73 (2%), CD45 (0.6%). (C) Western blotting of OCT-4 in different passages of DPTEs and DPSCs, with normal human oral fibroblasts (NHOFs) as a negative control, GAPDH as loading control.


Surface Immunophenotype
DPSC
DPTE
CD146
Stro-1
CD146
Stro-1
DAPI
DAPI
DAPI
DAPI


CD44
CD90
CD105
CD34
CD73
CD45
DPTEs
DPSCs

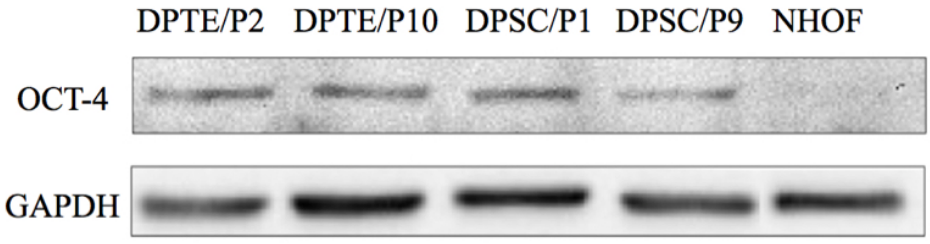
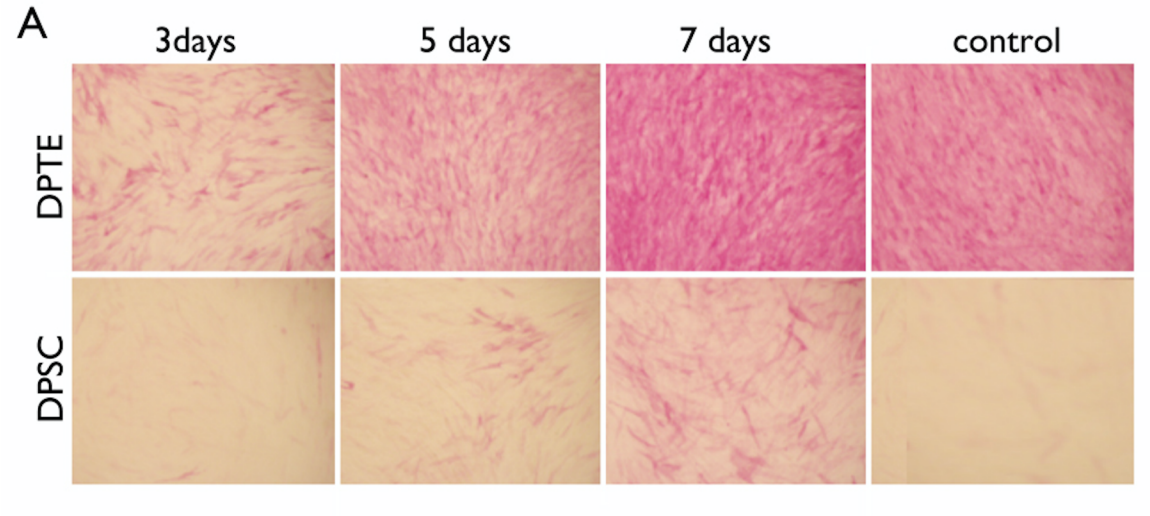
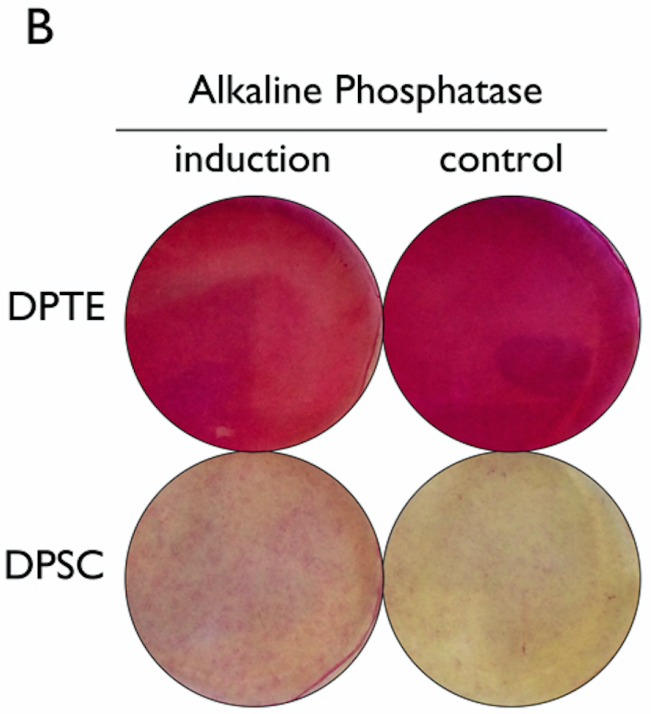

DPTE/P2
DPTE/P10
DPSC/P1
DPSC/P9
NHOF
OCT-4
GAPDH

Figure 6. Osteo/odontogenic differentiation potencial of DPTEs compared to DPSCs. (A) ALP activity of DPTEs and DPSCs in a time course with osteognic induction medium containing β-glycerophosphate, dexamethasone, and vitamin C. Control group represents normal growth medium without these factors. (B) DPTE and DPSC ALP activity was shown in the whole culture plate after 7 days induction along with the control group. (C) Western blotting was performed for ALP in DPTEs and DPSCs after 0, 3, 5, and 7 days post-induction. GAPDH was used as loading control.


A
3days
5 days
7 days
control
DPTE
DPSC


B
Alkaline Phosphatase
induction
control
DPTE
DPSC

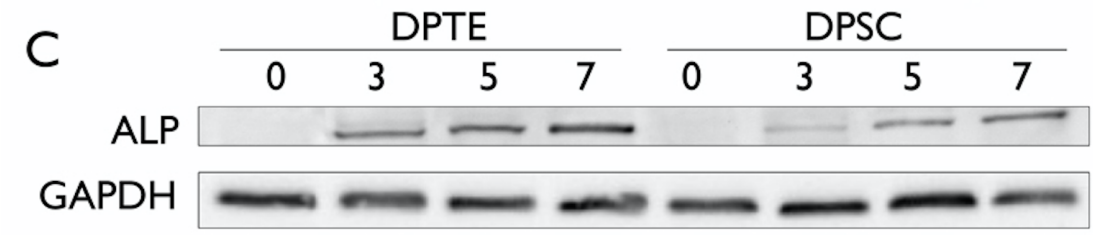

C
DPTE
DPSC
0
3
5
7
0
3
5
7
ALP
GAPDH

Figure 7.DPTEs demonstrated mineralization capacity similar to that of the DPSCs. Each upper panel of DPTE and DPSC, Alizarin S Red staining for mineral deposits in cell culture petri dish with light microscopy observation (each lower rectangular panel, magnification 40X).

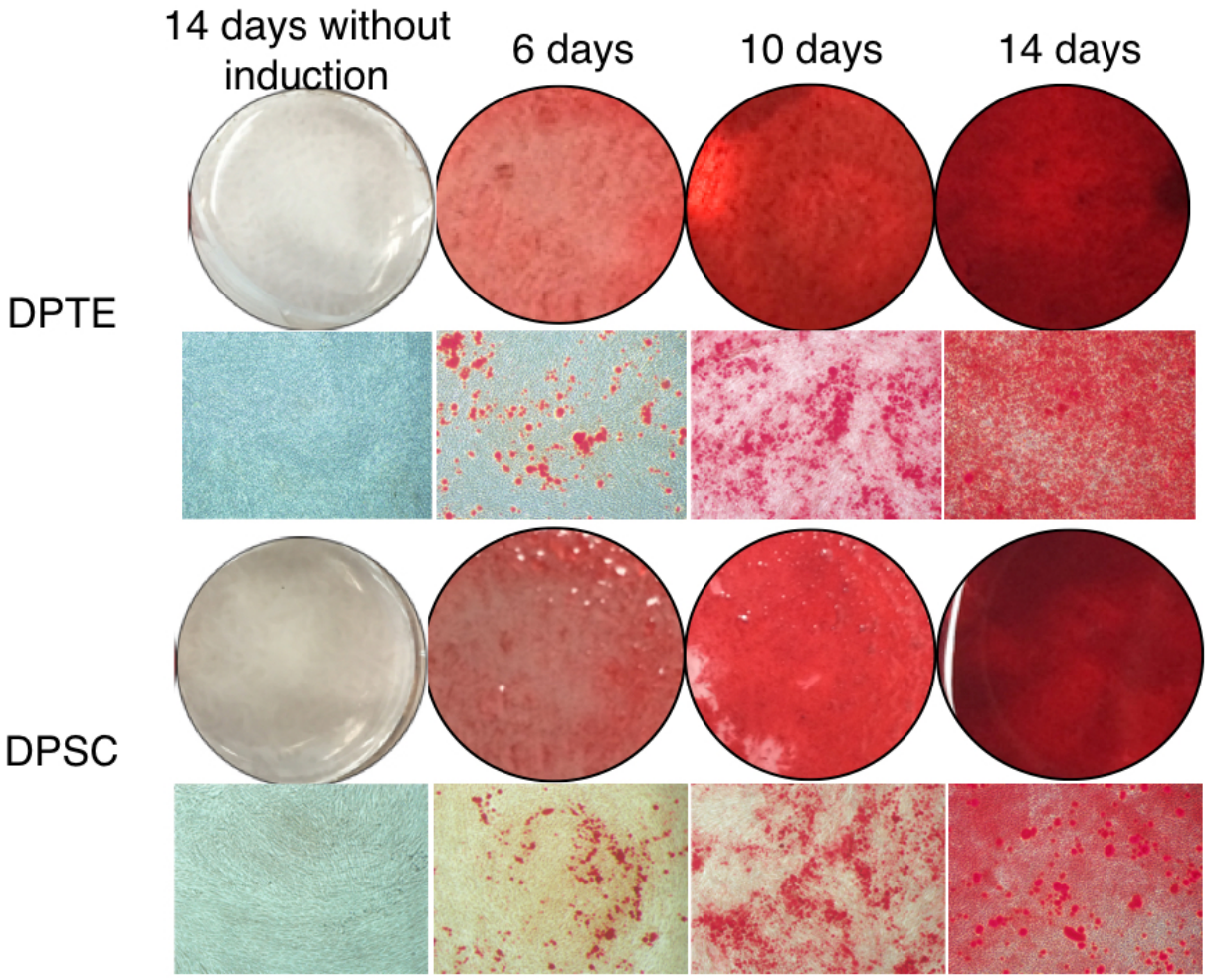
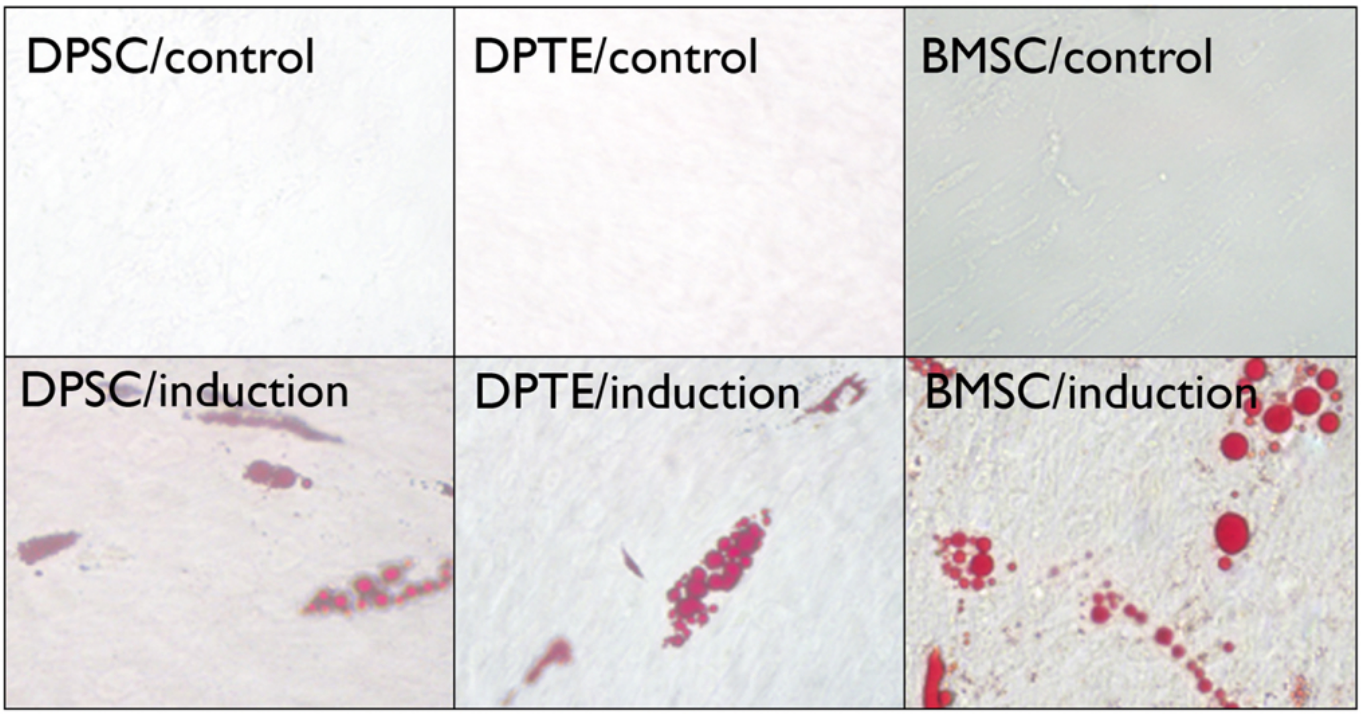

14 days without induction
6 days
10 days
14 days
DPTE
DPSC

Figure 8. DPTEs demonstrate adipogenic differentiation in vitro. DPSCs, DPTEs, and BMSCs were cultured in control or adipogenic differentiation medium for 21 days and stained with Oil O Red for cytoplasmic lipid droplet formation. Original mag., 400x.

Adipogenic Differentiation



400x

Figure 9. Minced pulp explants yield migrating DPTE on 3D PLLA-dentin slice scaffold. (A) Using dentin disc model, we transplanted DPSC or DPTE onto PLLA scaffold inside the lumen of disc. (B) After 7 days of culture, the cells attached onto the scaffold were visible (arrows). Also, minced pulp tissues were added onto PLLA scaffold, from which migrating DPTE were visible (arrows). (C) When the DPTE were maintained on PLLA scaffold in the mineralizing condition, cells formed mineral nodule stained positive for Alizarin S. Red.

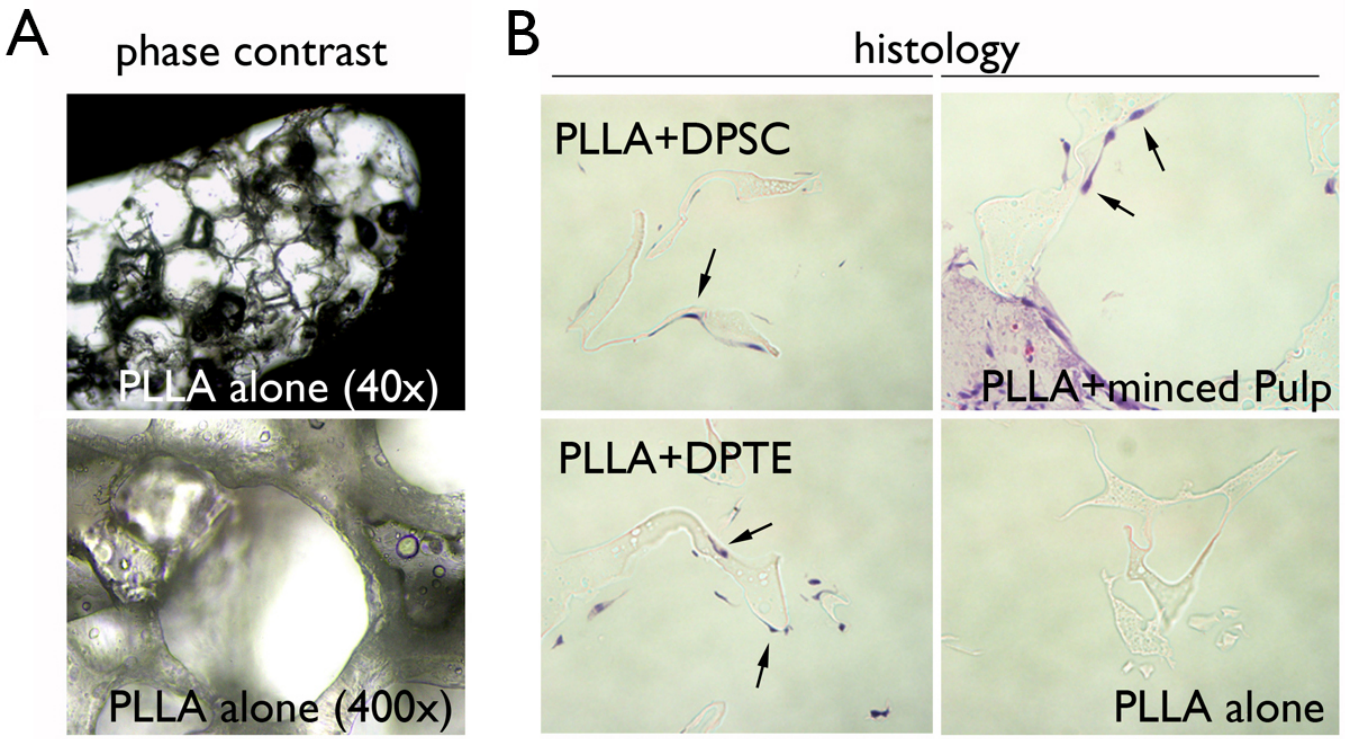
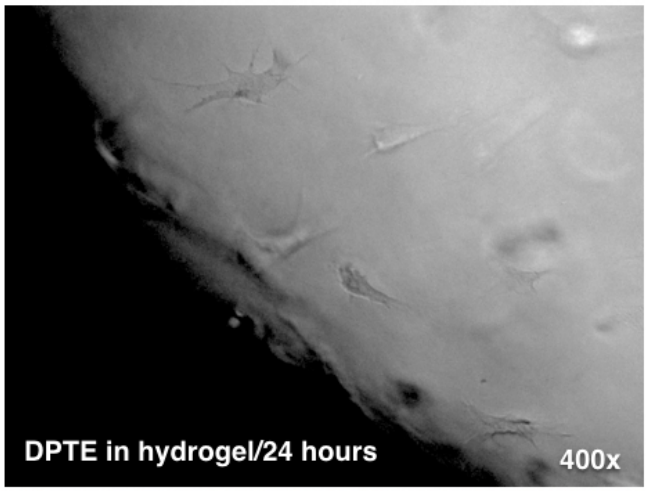
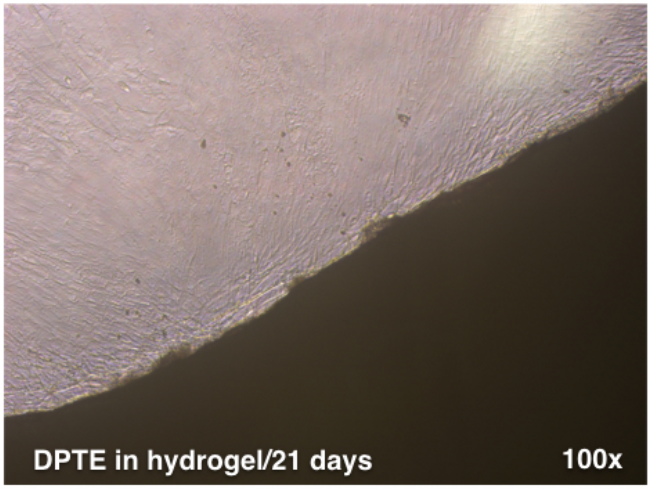

A
phase contrast
PLLA alone (40x)
PLLA alone (400x)
B
histology
PLLA+DPSC
PLLA+minced Pulp
PLLA+DPTE
PLLA alone
C
PLLA+ DPTE
phase contrast
Alizarin S Red

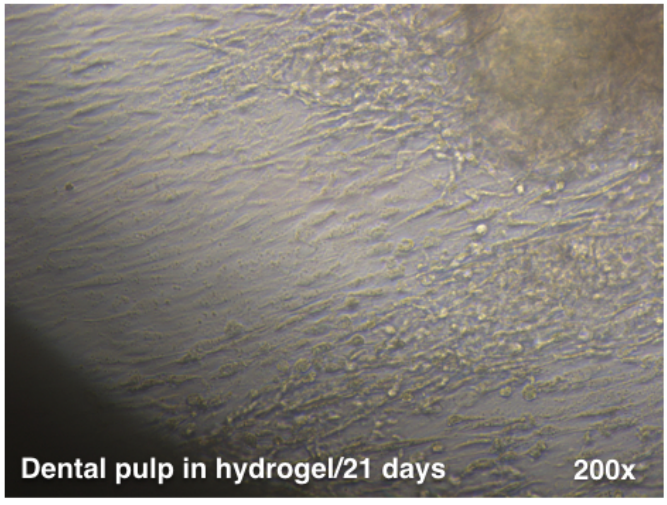
Figure 10. Dental pulp tissue exhibiting enhanced mineralization adjacent to dentin in injectable PuraMatrix hydrogel scaffold. (A) DPTE exhibited normal morphology in 3D hydrogel culture 24 hours after seeding and came to confluency and tight contact with dentin wall in 21 days; (B) Cells were able to migrate out from the dental pulp tissue and proliferated into confluency in 21 days. (C) When the DPTE were maintained in the mineralizing condition with injectable scaffold, cells formed mineral nodule stained positive for Alizarin S. Red with an aggregation around the dentin wall.

A

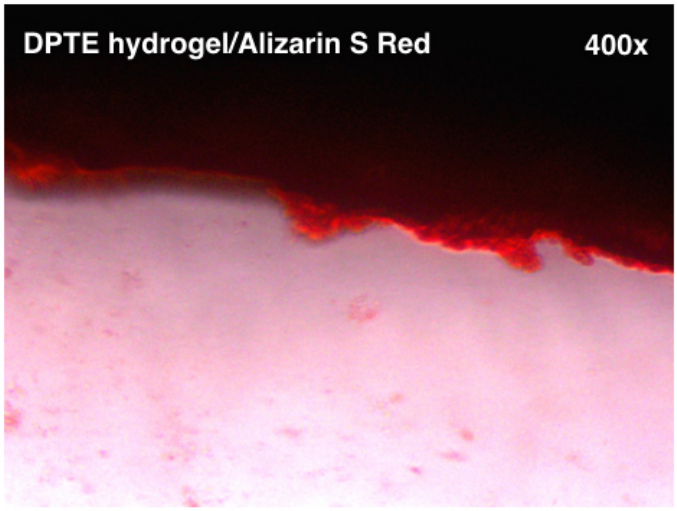

DPTE in hydrogel/24 hours
400x


DPTE in hydrogel/21 days
100x

B


Dental pulp in hydrogel/21 days
200x

C


DPTE hydrogel/Alizarin S Red
400x

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