Injectable Highly Tunable Oligomeric Collagen Matrices for Dental Tissue Regeneration

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Conflicts of interest

There are no conflicts to declare.

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ABSTRACT: Current stem cell transplantation approaches lack efficacy because they limit cell survival and retention, and more importantly lack a suitable cellular niche to modulate lineage-specific differentiation. Here, we evaluate the intrinsic ability of type I oligomeric collagen matrices to modulate dental pulp stem cells (DPSCs) endothelial and odontogenic differentiation as a potential stem cell-based therapy for regenerative endodontics. DPSCs were encapsulated in low (235Pa) and high (800Pa) stiffness oligomeric collagen matrices and then evaluated for long-term cell survival, as well as, endothelial and odontogenic differentiation following *in vitro* cell culture. Moreover, the effect of growth factor incorporation, i.e., vascular endothelial growth factor (VEGF) into 235Pa oligomeric collagen or bone morphogenetic protein (BMP2) into the 800Pa oligomeric collagen counterpart on endothelial or odontogenic differentiation of encapsulated DPSCs was investigated. DPSCs-laden oligomeric collagen matrices allowed long-term cell survival. RT-PCR data showed that the DPSCs cultured in 235Pa matrices demonstrated increased expression of endothelial markers after 28 days and the effect was enhanced upon VEGF-incorporation. There was a significant increase in ALP activity at Day 14 in the 800Pa DPSCs-laden oligomeric collagen matrices regardless of BMP2 incorporation. However, Alizarin S data demonstrated higher mineralization by Day 21 and the effect was amplified in BMP2-modified matrices. Herein, we present key data that strongly support future research aiming at the clinical translation of an injectable oligomeric collagen system for delivery and fate regulation of DPSCs to enable pulp and dentin regeneration at specific locations of the root canal system.

KEYWORDS: injectable, hydrogel, oligomeric collagen, regeneration, dental pulp, angiogenesis

INTRODUCTION

In dentistry, the treatment of pulpal necrosis due to caries or trauma[1], especially in immature permanent tooth, remains a clinical challenge as the dental roots are thin and short, which consequently increase the risk of tooth fracture upon secondary trauma[2]. Besides playing a key role in tooth development, the dental pulp functions as a protective organ that can detect variations in temperature, pressure, or trauma[3]. For these reasons, numerous investigators are exploring novel matrices and/or strategies for pulp-dentin regeneration aiming to extend the lifetime of permanent teeth[4-10].

The development of injectable matrices for use in full length root canals that supports cell growth, vascularization and dentin formation is a key step towards clinically feasible pulp regeneration. Natural and synthetic hydrogels, nanofibers, nano-assemblies and microspheres are being evaluated by investigators for pulp regeneration. Customized self-assembling peptide hydrogels consisting of "multidomain peptides" (MDPs) incorporating growth factors and dental pulp stem cells (DPSCs) induced formation of vascularized soft connective tissue resembling dental pulp[9]. Prevascularized constructs containing co-cultured DPSCs and endothelial cells embedded within PuraMatrix, a self-assembling peptide hydrogel, demonstrated enhanced *in vivo* neovascularization and osteodentin formation[4]. Gelatin methacryloyl (GelMA) hydrogels with tunable physical and mechanical properties and ability for prevascularization is another recent strategy adopted for pulp regeneration[5]. Scaffolds made of alginates and chitosan have also demonstrated promising results for their odontogenic differentiation capabilities. Alginate and nano-hydroxyapatite composite scaffolds induced mineralization and differentiation of human DPSCs *in vitro*[11]. Porous chitosan-calcium-aluminate scaffold in combination with a bioactive dosage of 1α,25-dihydroxyvitamin D3 enhanced odontogenic potential of dental pulp cells *in vitro*[12].

Meanwhile, nanostructured multilayered nano-assemblies and microspheres are other promising approaches to promote dental pulp tissue regeneration. Nanostructured and functionalized multilayered polyelectrolyte films of poly-l-lysine dendrigraft, containing poly-glutamic acid-α-melanocyte stimulating hormone were proposed to decrease inflammation and promote tissue regeneration in the pulpal space[13].

Type I collagen-surface modified PLGA microspheres enhanced cell proliferation and odontogenic differentiation of dental pulp cells *in vitro* [14].

Collagen is one of the most widely used injectable natural biomaterial because of its excellent biocompatibility and easy tissue integration following degradation, and by their ability to act as a vehicle for growth factors[15]. DPSC-encapsulated commercially available rhCollagen hydrogel transplanted to full length root canals demonstrated improved organization of the newly formed pulp tissue[10]. Collagen scaffolds incorporated with chemotactic factors, stromal cell derived factor 1α and bFGF, led to recruitment of stem/progenitor cells from adult dental pulp[16]. Collagen gels have also seen to support vasculogenesis and cell survival of endothelial colony forming cells in the presence of platelet releasate[17]. However, a major limitation of present-day commercial polymerizable monomeric collagens is their inconsistent fibril density because of the monomeric nature that varies with every batch, thus making it difficult to predict their mechanical behavior and overall cell function [18, 19]. To that end, the fabrication of collagen-fibril matrices with tunable and highly reproducible fibril microstructures and biophysical properties (e.g., stiffness) is critical to guiding cell-matrix interactions. Recent research has focused on tailoring the stiffness and microstructure of collagen matrices by modulating collagen concentration (fibril density) and the oligomer:monomer ratio (extent of interfibril connectivity). Indeed, the cell-instructive properties of these matrices were unveiled when endothelial progenitor cells cultured on low stiffness oligomeric collagens induced more extensive and persistent endothelial vessel networks compared to monomeric matrices with identical stiffness[20].

In the current study, we hypothesized that the collagen matrix with low stiffness (235Pa) would induce endothelial differentiation of DPSCs and the presence of vascular endothelial growth factor (VEGF) would enhance this differentiation, and the collagen matrix with higher stiffness (800Pa) would induce osteogenic differentiation and the presence of bone morphogenetic protein-2 (BMP2) would enhance the odontogenic/osteogenic differentiation of DPSCs. VEGF is a prototypic pro-angiogenic factor and BMP2 is a osteoinductive protein which has respectively proven to play a vital role in inducing endothelial[21] and

osteogenic differentiation[22] of mesenchymal stem cells. Thus, herein, we first determined the initial cell adhesion and spreading, long-term survival, and differentiation ability of DPSCs within unique oligomeric collagen matrices of precise stiffness. Additionally, we investigated whether the incorporation of vascular endothelial growth factor (VEGF) into the low stiffness oligomeric collagen matrix or bone morphogenetic protein (BMP2) into the high stiffness counterpart would further amplify endothelial or odontogenic differentiation of encapsulated DPSCs, respectively. The uniqueness of our innovative strategy, i.e., DPSCs transplantation using a stiffer matrix along with BMP2 (dentin formation) or within a more compliant matrix containing VEGF (pulp regeneration), when concentrically injected into the root canal (Figure 1), carries a strong translational potential as a single cell population could be used to target both endothelial and odontogenic differentiation as a stem cell-based therapy for regenerative endodontics.

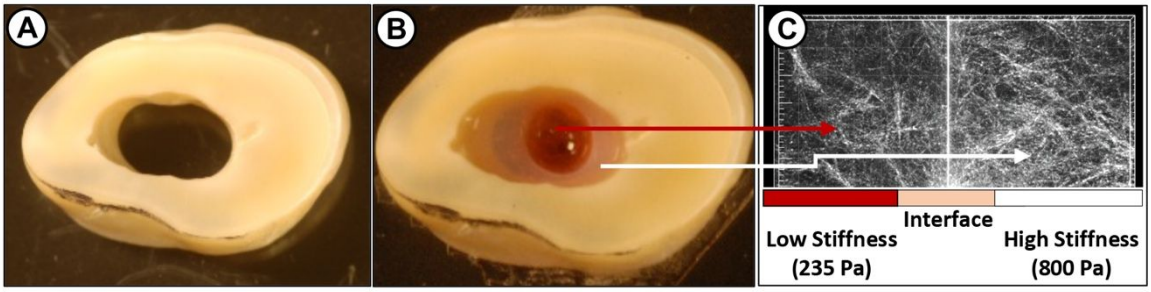


Figure 1. Schematic illustration of the proposed highly tunable injectable oligomeric collagen-fibril matrix system using a well-established tooth slice model: (a) tooth slice, (b) concentrically injected collagen matrices (low stiffness matrix purposely dyed in red), and (c) interface adaptation between the two matrices evidenced by reflectance microscopy. For the preparation of concentric collagen matrices, an endodontic fiber post (1.3 mm in diameter, 3M ESPE, St. Paul, MN) was placed and stabilized inside the tooth slice. The collagen solution of 2.88 mg/ml (800Pa) containing DPSC and BMP2 was injected using a needle to the area surrounding the fiber post. Once the matrix is polymerized, the fiber post was lifted and the lumen was injected with collagen solution of 1.37 mg/ml (235Pa) containing DPSC and VEGF165 and allowed to polymerize. The volume ratio of the collagen solutions 1.37 mg/ml and 2.88 mg/ml was adjusted to 2:1, which compares with the anatomical features of a root canal. The fibril density and interface adaptation between the two collagen matrices of lower and higher stiffness are shown.

▪ MATERIALS AND METHODS

Cell Culture. Human DPSCs isolated from adult third molars (Lonza) were cultured in DPSC complete medium (DPSCM) containing α-MEM (Sigma), 10% FBS (Atlanta Biologicals) and antibiotics (100 U/mL penicillin and 100 µg/ml streptomycin). Cells were maintained in a humidified environment

of 5% CO_2 in air at 37°C and culture medium changed every other day. DPSCs were passaged at confluency and passages 3 through 6 were used for the experiments.

Preparation of DPSC, VEGF, and BMP2 Encapsulated Oligomeric Collagen Matrices. Type I oligomeric collagen was derived from the dermis of market-weight pigs as previously described.[23] Oligomer was dissolved in 0.01 N hydrochloric acid (HCl) and standardized based on molecular composition and polymerization capacity according to ASTM International Standard F3089-14.[24] Here, the polymerization capacity refers to the relationship (polynomial function) between shear storage modulus (G', Pa) of the self-assembled matrix and oligomer concentration. Matrix stiffness values of collagen matrices prepared at oligomer concentrations of 1.37 mg/ml and 2.88 mg/ml are given as shear storage modulus (G', Pa). To achieve matrices of defined fibril density and matrix stiffness, stock oligomer was diluted in 0.01N HCl to a final concentration of 1.37 mg/ml (235 Pa) for endothelial differentiation, and 2.88 mg/ml (800 Pa) for odontogenic differentiation, and neutralized with a proprietary 10X self-assembly reagent prior to DPSCs (1×10^6 cells/mL) encapsulation. To prepare GF-modified oligomeric collagen matrices, VEGF165 (50 ng/ml, Invitrogen) and BMP2 (50 ng/ml, Invitrogen) were added to collagen solutions before polymerization. To induce differentiation, endothelial growth medium (EGM) containing growth supplements and 2% FBS (EGM-2 Bullet Kit, Lonza) and the hMSC osteogenic differentiation medium (Lonza) containing dexamethasone, ascorbate and β-glycerophosphate were used, respectively. The following approaches were evaluated (Figure 2).

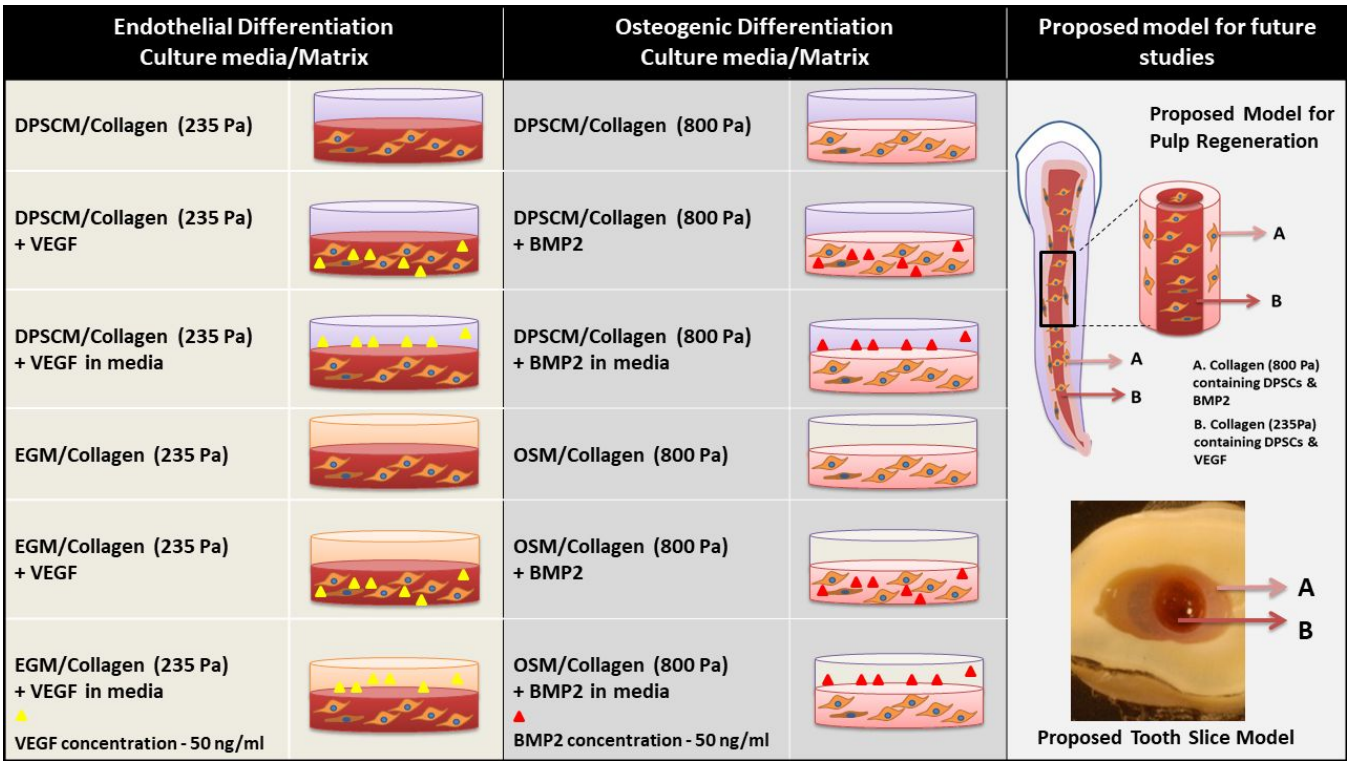


Figure 2. Oligomeric collagen matrices and culture media used for endothelial and osteogenic differentiation. The concentric tooth slice model and the dental pulp regeneration model for the future studies.

Simulated Growth Factor Release from Oligomeric Collagen Matrices. Fluorescein isothiocyanate (FITC)-labeled dextran with molecular weight (M_w) 40,000 and 20,000 were used as model drugs to simulate the M_w of VEGF and BMP2, respectively. The distinct oligomeric collagens (235 Pa and 800 Pa) were prepared (100 µl), modified with 500 µg Dextran (M_w 40,000 or 20,000), and then pipetted into each well of a 96-well plate. Media (200 µl) was then added to each well. The constructs were incubated at 37°C and 5% CO_2 for 14 days and 100 µl of medium was collected from each well at distinct time points up to 14 days, while maintaining the incubation volume constant by replacing the withdrawn amount with fresh medium. Fluorescence intensity was measured (Synergy HTX Microplate Reader, Biotek) using excitation and emission wavelengths of 485 nm and 520 nm, respectively. The results were compared to the number of simulated GFs released when similarly incorporated into a commercially available hydrogel (PuraMatrix™) prepared at concentrations of 0.20% and 0.25% (n= 5).

Cell Morphology. Cell morphology within the oligomeric collagen matrices was evaluated by confocal microscopy after staining the actin cytoskeleton (Rhodamine-phalloidin, Molecular Probes) and

nuclei (SYTO 13, Molecular Probes) 24h and 72h post-culture (n=3). Briefly, 1×10^6 cells were suspended in 1 ml of oligomeric collagen solution and 200 µl of the suspension was allowed to polymerize in each well of an 8-well slide (Lab-Tek Permanox chamber slide; Thermo Scientific). To each well 200 µl of medium was added for cell culture. At each time point, cells were fixed with 3.7% paraformaldehyde in PBS for 30 min at RT and permeabilized for 10 min with 0.1% Triton X-100. F-actin was stained using Rhodamine-phalloidin (1:1000 dilution) for 30 min and nuclei was stained using SYTO 13 (10 min; 1:1000 dilution). Images were captured using a confocal/2-photon Olympus FV1000 MPE system (Olympus America) using a XLUMPLFL20XW objective with 0.95 numerical aperture (NA). The images presented are maximum intensity projections of Z-slices (Depth ~ 300 µm). Rhodamine phalloidin and SYTO 13 have excitation and emission at 540⁄565 and 488⁄509 nm, respectively.

Cell Survival. Cell survival within the oligomeric collagen matrices was investigated using CellTiter 96 AQueous One Solution Reagent (Promega). The cells were encapsulated in the oligomeric collagen matrices at a density of 10^6 cells/ml and 150 µl collagen-DPSC solution was added to each well of a 96-well plate to form constructs and 150 µl of media was used for culture. Cell proliferation was assessed on days 1, 3, 7, 14, and 21. At each time point, 150 µl of the reagent-media mixture (5:1) was added to each well and incubated for 3 h at 37°C and 5% CO_2. After incubation, 100 µl of the mixture was pipetted to each well of a 96-well plate and the absorbance read at 490 nm using a microplate reader (Biotek).

Endothelial Differentiation. DPSCs-laden oligomeric collagen matrices (~10^6 cells/ml) were cultured in 24-well plates containing DPSCM or EGM for 1, 3, 7, 14, 21, and 28 days. Total RNA from constructs was extracted using TRIzol® reagent.[18] Real-time PCR (CFX96, BioRad) was performed to determine the expression of selected endothelial markers, von Willebrand Factor (vWF), platelet endothelial cell adhesion molecule 1 (PECAM-1) and vascular endothelial (VE)-cadherin using primers designed using the Primer quest tool (IDT) (Table 1). RNA yield was quantified using a Take3 plate (Biotek) and cDNA was synthesized using Improm II reverse transcription kit (Promega) with 1 µg total

RNA as template, 5 × reaction buffer, $MgCl_2$, dNTP mix, RNAse inhibitor and Improm-II reverse transcriptase.

Table 1. Primers of target genes in RT-PCR

Gene	Sequence ID	Primer Sequence	Product Length (bp)
vWF	NM_000552.4	For: 5'-ACGTTCTGGTGCAGGATTAC-3'	106
		Rev: 5'-GTGACCCGTTTCTTGCATTTC-3'	
PECAM 1	XM_011524890.1	For: 5'-CCGATGTCAAGCTAGGATCATT-3'	109
		Rev: 5'-GATGTGGAACTTGGGTGTAGAG-3'	
VE-Cadherin	NM_001795.4	For: 5'-ACCCAAGATGTGGCCTTTAG-3'	100
		Rev: 5'-GTGACAACAGCGAGGTGTAA-3'	
GAPDH	NM_002046.5	For: 5'-GGGAAGGTGAAGGTCGGAGT-3'	119
		Rev: 5'-TTGAGGTCAATGAAGGGGTCA-3'	

Real-time PCR was performed using 8 µl cDNA, 10 µl iQSYBR green PCR Master Mix (Bio-Rad Laboratories) and 25 pmol/µl of forward and reverse primers (Integrated DNA Technologies) using a RT-PCR system (n=3). The PCR cycling conditions were 5 min at 95°C for initial denaturation followed by 40 cycles of 30 s at 95°C, 30 s at 53°C (depending upon the primer annealing temperatures) and 30 s at 72°C (Table 1). Specificity of the primers was analyzed by performing a melting curve analysis. Each reaction had three individual samples in duplicates and values of the threshold cycles were averaged. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method by comparing all the Ct values to Day 14 control, Collagen – DPSC cultured in DPSC media. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference gene to normalize target gene expression.

Alkaline Phosphatase Assay. Osteo/odontogenic differentiation of DPSCs on high stiffness (800Pa) oligomeric collagen matrices with and without GF incorporation was quantitatively determined by alkaline phosphatase (ALP) activity on days 7 and 14 using an ALP assay kit (SensoLyte pNPP). The assay is based on the conversion of *p*-nitrophenyl phosphate (pNPP) to *p*-nitrophenol by the enzyme, which is measured spectrophotometrically at 405 nm. Briefly, the cells encapsulated in oligomeric collagen matrices (n=4) at a density of 1×10^6 per ml were cultured in DMEM/osteogenic differentiation medium (Lonza) with or without BMP2 for 14 days. On days 7 and 14, the cells were lysed using lysis buffer containing Triton-X 100 (400 µL), vortexed and the cell-gel suspension was centrifuged for 10 min

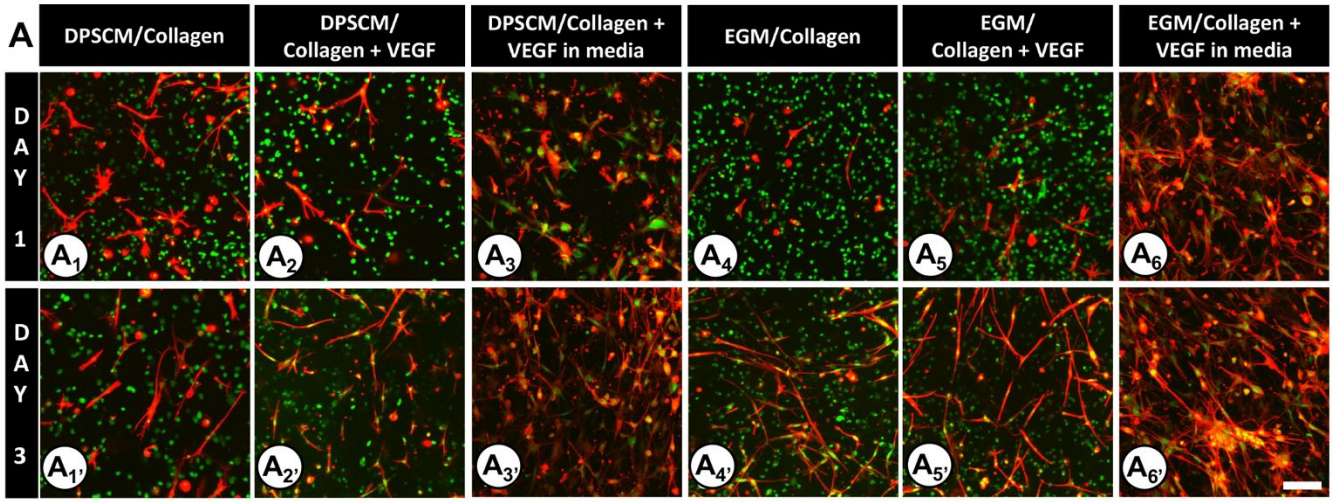
at 4°C. Then, 50 µL of the supernatant was added to each well of a 96-well plate and allowed to react for 15 min with 50 µL of *p*NPP substrate solution at 37°C. *p*-nitrophenol (0–300 ng/mL) was used as the standard. The absorbance of standards and samples was measured at 405 nm. Protein concentrations in the samples were determined using a Pierce™ assay kit (Thermo Scientific).

Alizarin S Red Staining. For Alizarin S red staining and quantification (Sciencell), the DPSCs-laden oligomeric collagen matrices (800Pa) (n=4) were stimulated with DMEM or OSM, with or without BMP2. The cultures were fixed in 3.7% formaldehyde for 30 min, and rinsed with distilled water (3×). Then, alizarin S staining solution was added and incubated at RT for 20 min. Excess dye was removed by rinsing (4×) with distilled water and images were acquired using an inverted bright field contrast microscope (Leica). Alizarin was extracted from each sample using 10% acetic acid, transferred to microtubes, vortexed and incubated at 85°C for 10 min. After cooling in ice, the cells were centrifuged at 20,000g for 15 min and the supernatant (200 µl) was neutralized with 75 µl of NH_4OH. Then, 50 µl of the neutralized solution was transferred to a 96-well plate and the absorbance was measured at 405 nm using a microplate reader.

Statistical Analysis. Two-way analyses of variance (ANOVA) followed by Holm-Sidak's multiple comparisons were used to evaluate differences in simulated growth factor release, cell survival, endothelial marker mRNA expression, ALP activity and Alizarin S red staining between the groups. The significance level was set at $p<0.05$.

▪ RESULTS

Effect of Oligomeric Collagen Stiffness on DPSCs Morphology and Migration. As earlier stated, by modulating collagen concentration, we were able to establish two distinct matrix stiffness (235 Pa and 800 Pa).[19] Here, DPSCs cultured in Oligomer 235Pa demonstrated spreading on day 1 (Figures $3A_1$- A_6). Notably, spreading was enhanced in the presence of VEGF. On day 3 (Figures $3A_{1'}$- $A_{6'}$), DPSCs demonstrated a tubular morphology with profuse spreading when VEGF was supplemented in DPSCM (Figure $3A_{3'}$) and EGM (Figure $3A_{6'}$). DPSCs cultured within Oligomer 800Pa demonstrated decreased spreading (Figures $2B_1$- B_6) compared to low stiffness 235Pa oligomeric collagen matrices. Cytoplasmic projections were found all around the cell periphery (Figures $3B_1$- B_6 and $3B_{1'}$- $B_{6'}$) compared to the tubular morphology of cells grown in low stiffness counterparts. DPSCs cultured in OSM and BMP2 (Figures $3B_5$ and $3B_6$) showed less pseudopodial processes likely due to the combined effect of OSM and BMP2 on day 1. However, by day 3 (Figures $3B_{1'}$- $B_{6'}$) the number of pseudopodial extensions from cells increased and appeared to be similar in all matrices.



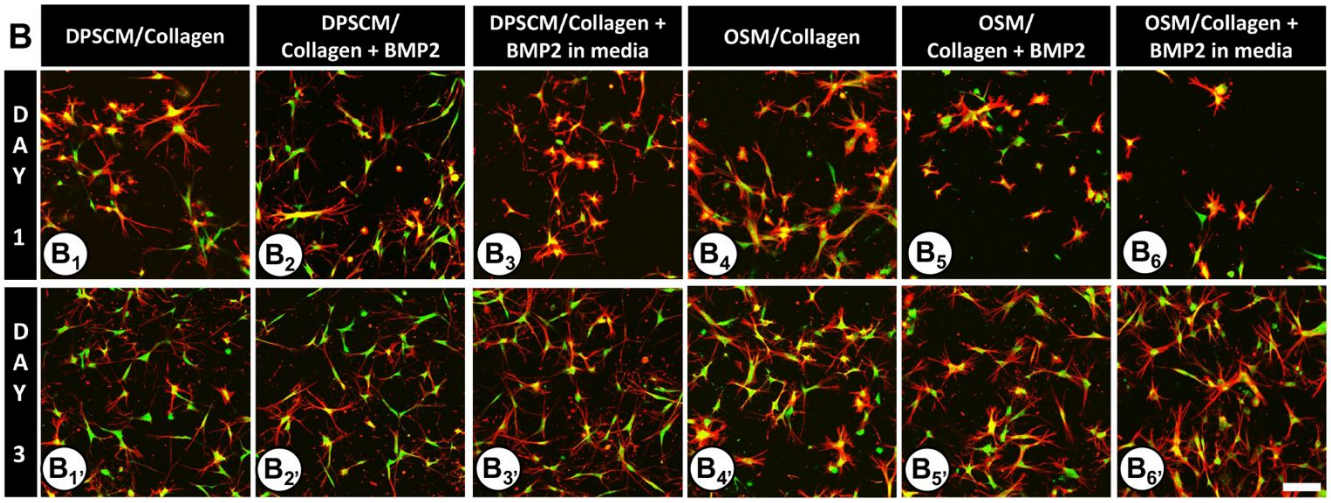


Figure 3. DPSC adhesion and spreading within Oligomer 235Pa collagen matrices under various conditions at days 1 and 3 on the following matrices: (A_1, $A_{1'}$) DPSCM/Collagen; (A_2, $A_{2'}$) DPSCM/Collagen + VEGF; (A_3, $A_{3'}$) DPSCM/Collagen + VEGF in media; (A_4, $A_{4'}$) EGM/Collagen; (A_5, $A_{5'}$) EGM/Collagen + VEGF; (A_6, $A_{6'}$) EGM/Collagen + VEGF in media. DPSC adhesion and spreading within Oligomer 800Pa collagen matrices under various conditions at days 1 and 3 on the following matrices: (B_1, $B_{1'}$) DPSCM/Collagen; (B_2, $B_{2'}$) DPSCM/Collagen + BMP2; (B_3, $B_{3'}$) DPSCM/Collagen + BMP2 in media; (B_4, $B_{4'}$) OSM/Collagen; (B_5, $B_{5'}$) OSM/Collagen + BMP2; (B_6, $B_{6'}$) OSM/Collagen + BMP2 in media. For all images, the actin filaments are stained red with Rhodamine-Phalloidin (excitation and emission at 540/565 nm) and the nucleus is stained green using SYTO 13 (excitation and emission at 488/509 nm). Regions that appear yellow indicate the co-localization of actin and nucleus. Each experiment had three samples. Images are maximum intensity projection of Z-slices (~300 μm depth) captured using a confocal/2-photon Olympus FV1000 MPE system (Olympus America) with a XLUMPLFL20XW objective and 0.95 NA. Scale bar = 100 μm.

DPSCs Proliferation in the Oligomeric Collagen Matrices. DSPCs proliferation was significantly increased for all the 235Pa (Figure 4A) and 800Pa (Figure 4B) oligomeric collagen matrices on day 14. However, cell proliferation was not significantly different between the control and VEGF-modified matrices at any time point (Figure 4A). Cell proliferation decreased on days 14 and 21 in 800Pa matrices containing BMP2 compared to the other two matrices.

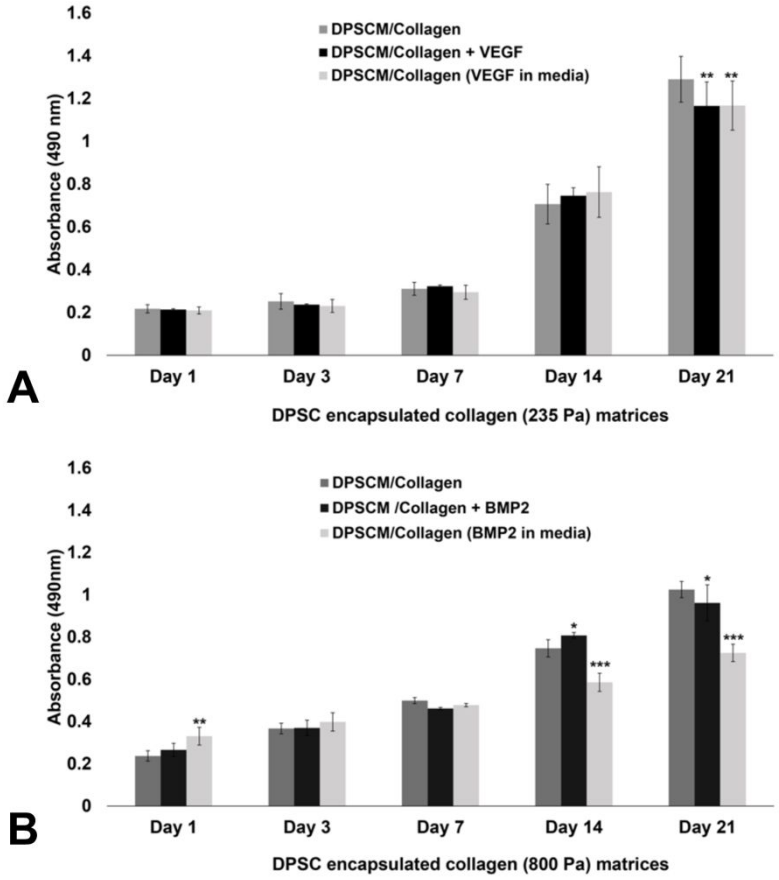


Figure 4. Long-term survival of DPSCs on 235Pa and 800Pa collagen matrices: (A) DPSCs survival on 235Pa collagen matrices on days 1, 3, 7, 14 and 21 post-encapsulation, (B) DPSCs survival on 800Pa collagen matrices on days 1, 3, 7, 14 and 21 post-encapsulation. The absorbance was read at 490nm using a microplate reader (Biotek, Winooski, VT, USA). Values represent mean ± SD (n=3). Statistical difference designations: ***$p<0.001$, **$p<0.01$ and *$p<0.05$.

Simulated Growth Factor Release. The release of FITC-dextran M_w 20,000 and 40,000 from the oligomeric collagen matrices was continuous for 6 days. However, a burst release was seen for PuraMatrix™ where the simulatory drugs were released within the 48 h (Figure 5).

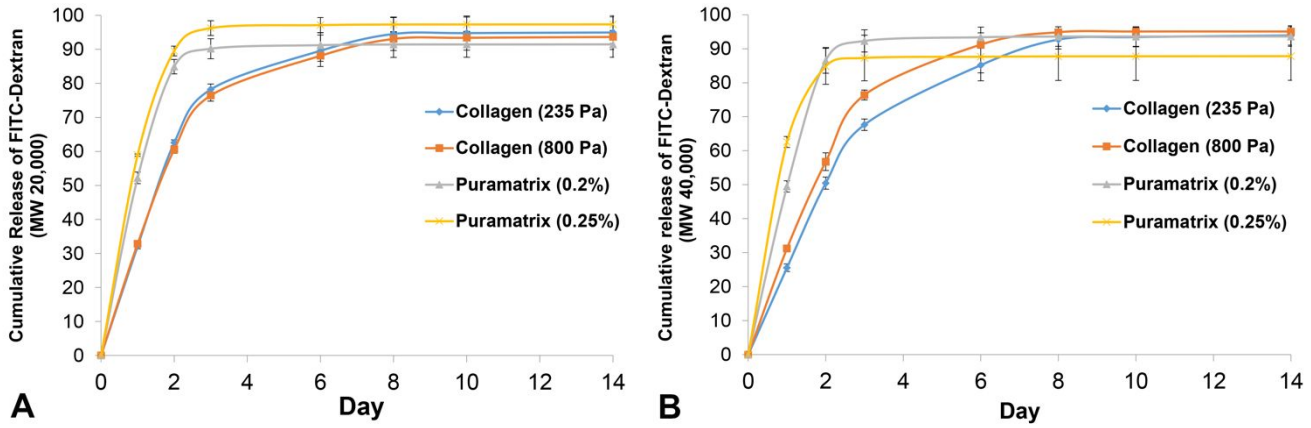


Figure 5. Cumulative release of FITC-Dextran from PuraMatrix™ and oligomeric collagen matrices for 14 days (N=5). FITC-dextran (M_w 20,000 and 40,000) served as model growth factors for (A) BMP2 and (B) VEGF, respectively. Fluorescence intensity was measured (Synergy HTX Microplate Reader, Biotek) using excitation and emission wavelengths of 485 nm and 520 nm, respectively.

Endothelial Differentiation on Low Stiffness (235 Pa) Oligomeric Collagen Matrices. Figure 5 shows the expression of endothelial cell (EC) markers on day 14 even in the absence of GFs. There was an increase in the expression of the EC markers when GFs were added to the media demonstrating their supplementary effect on DPSCs along with the biophysical factors. The mRNA transcripts of vWF, VE-Cadherin and PECAM were observed from day 14 in DPSCs grown in all matrices including the control matrices cultured in DPSCM. Thereafter, a steady increase in the mRNA levels of EC markers was observed until day 28 (Figures 6A-F). The cells in VEGF-modified matrices as well as the control matrices demonstrated a 4- to 5-fold increase in vWF mRNA levels by day 28 (Figure 6A). Matrices cultured in EGM showed a significant increase when VEGF was added to the media (Figure 6B). VE-Cadherin expression significantly increased 10-, 12- and 16-fold by day 28 in control, VEGF-modified and VEGF added (i.e., supplementation of VEGF in the culture media) matrices, respectively (Figure 6C). The effect was increased 14-, 25- and 35-fold, respectively when the same matrices were cultured in differentiation media (Figure 6D). PECAM expression in VEGF-modified matrices in the control and differentiation media showed a 6-fold increase on day 21. Moreover, there were 10- and 12-fold increases in PECAM expression, respectively when cells were grown in VEGF-modified matrices in control and differentiation media (Figures 6E and 6F). The expression levels of VE-Cadherin were higher compared to vWF and

PECAM in matrices cultured in DPSCM and EGM, and the effect of VEGF incorporation was evident. These results demonstrate that the collagen matrices induced endothelial marker expression when cultured in DPSC media. Surprisingly, the level of marker expression increased even in the absence of endothelial differentiation media (Figures 6A, 6C, and 6E).

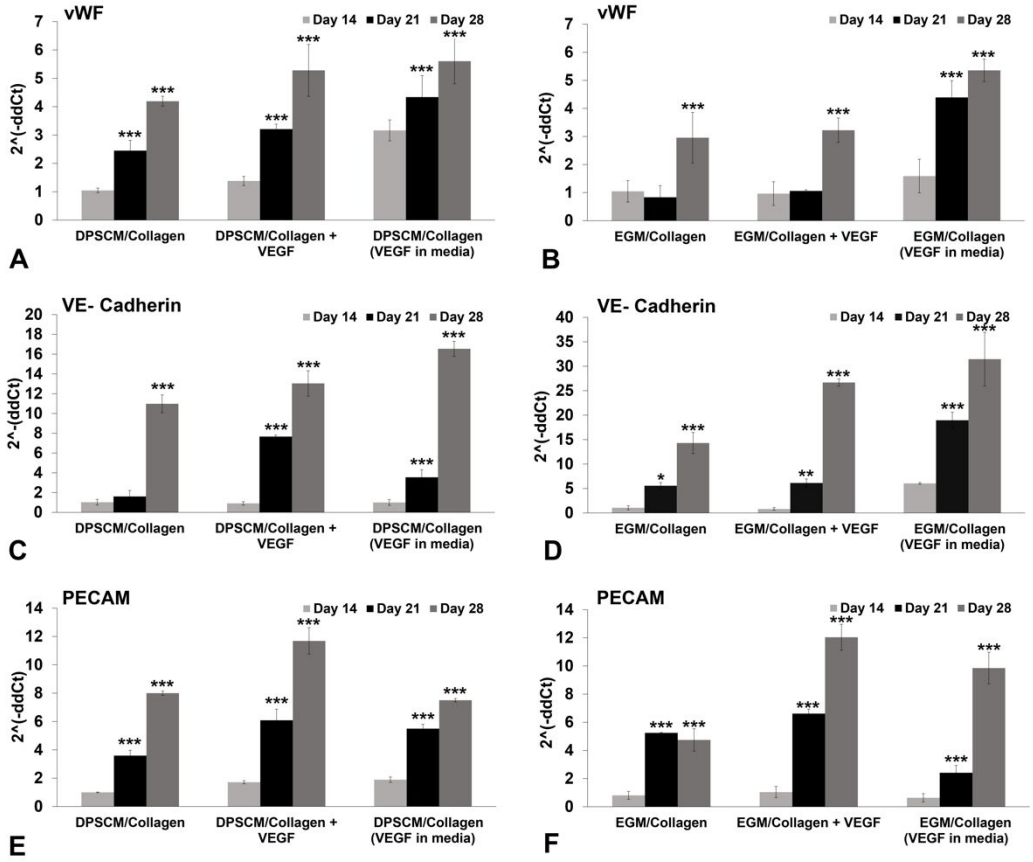


Figure 6.
Endothelial differentiation on collagen (235Pa) matrices. Real-time PCR data (CFX96, Bio-Rad) showing expression levels of endothelial cell markers in DPSCs encapsulated in 235Pa collagen matrices on days 14, 21 and 28. (a) vWF - DPSCM, (b) vWF - EGM, (c) VE-Cadherin - DPSCM, (d) VE-Cadherin - EGM, (e) PECAM - DPSCM, and (f) PECAM - EGM. Relative expression of target genes was performed using the $2^{-\Delta\Delta Ct}$ method. The results were normalized against the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Statistical analysis was performed to compare matrices on days 21 and 28 to day 14. Values represent mean ± SD (n=3). Statistical difference designations: ***p<0.001, **p<0.01 and *p<0.05.

Osteogenic Differentiation on High Stiffness (800 Pa) Oligomeric Collagen Matrices. ALP activity was observed in DPSCs cultured in 800 Pa collagen matrices even in the control media (DPSCM) demonstrating the effect of stiffness on osteogenic differentiation (Figure 7A). While ALP activity significantly increased on day 14 in control matrices, in osteogenic differentiation media ALP activity

increased on day 7 in the presence of BMP2. Alizarin staining also demonstrated the level of mineralization of DPSCs in collagen matrices (Figure 7B). Mineralized nodules were observed on day 7 in control collagen matrices (inset Figure 7A). The presence of BMP2 increased mineralization levels in the collagen matrices cultured in DPSCM (inset Figure 7C) and OSM (inset Figure 7F). Significant increases in Alizarin staining in the OSM and BMP2 matrices on day 21 are shown in Figure 7B.

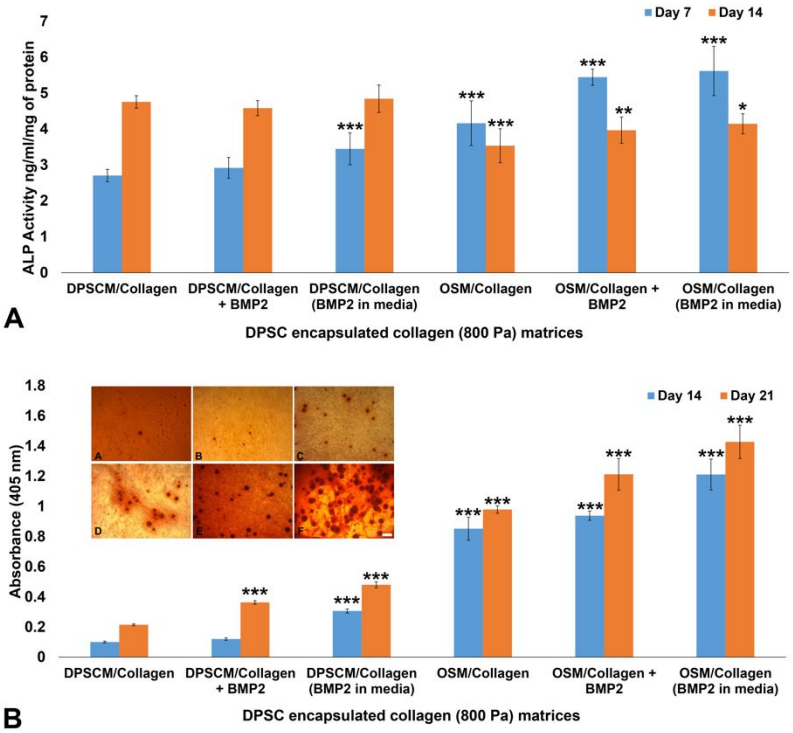


Figure 7. Osteogenic differentiation on collagen (800Pa) matrices: A) ALP activity of DPSCs encapsulated in collagen (800Pa) matrices on days 7 and 14. The results were normalized to the total protein level and expressed as ng of p-nitrophenol produced per mL per µg protein. Statistical analysis was performed to compare samples to DPSCM/Collagen (Control) on days 7 and 14. B) Alizarin staining of DPSCs encapsulated in collagen (800Pa) matrices and cultured in DPSCM and OSM for 14 days. Inset shows the bright field images (Primovert, Zeiss) of Alizarin stained matrices after 7 days in culture; (A) DPSCM/Collagen, (B) DPSCM/Collagen + BMP2, (C) DPSCM/Collagen + BMP2 in media, (D) OSM/Collagen, (E) OSM/Collagen + BMP2, (F) OSM/Collagen + BMP2 in media. Values represent mean ± SD (n=4). Statistical difference designations: ***$p<0.001$, **$p<0.01$ and *$p<0.05$. Scale bar = 50 µm.

▪ DISCUSSION

DPSCs were used as candidate cells in this study because of their proven ability to co-differentiate into odontoblasts and endothelial cells.[7, 10, 22, 25, 26] In detail, stem cells derived from the pulp tissue of exfoliated

deciduous teeth (SHEDs) have been shown to differentiate into functional odontoblasts and endothelial cells when seeded into tooth slices and implanted subcutaneously in immunodeficient mice[27].

An ideal scaffold is expected to provide sufficient cell attachment and support long-term cell survival.[28, 29] In this study, DSPCs proliferation was significantly increased for all the 235Pa (Figure 4A) and 800Pa (Figure 3B) oligomeric collagen matrices on day 14. However, cell proliferation was not significantly different between the control and VEGF-modified matrices at any time point (Figure 4A). Cell proliferation decreased on days 14 and 21 in 800Pa matrices containing BMP2 compared to the other two matrices (Figure 4B). The rate of cell proliferation in the compliant matrix was higher on days 14 and 21 compared to the stiffer matrix, which could be likely due to the longer time taken by the cells to penetrate stiffer matrices. The effect of stiffness on the cytoskeletal organization and cell shape was also evident on the two matrices; stiffer collagen (800Pa) increased the spread area and actin fiber organization, while compliant collagen supported growth of elongated DPSCs. Notably, the addition of VEGF to the culture medium improved the amount of sprouting in 235Pa matrices but the morphology remained tubular because of the compliant nature of the matrix. The present findings corroborate with a previous study in which oligomeric collagen matrices were able to guide three-dimensional lumenized vessel network formation by endothelial colony forming cells *in vitro*[20], and could be made denser to mimic hard tissues.[30]

In the present investigation, we used oligomeric collagen matrices that can be precisely tuned for specific stiffness to instruct the cells to a specific lineage. Furthermore, this study sought to determine whether DPSCs' differentiation ability could be further enhanced by morphogenetic signals that supplement those coming from the host enabling guided stem cell differentiation. Here, BMP2 was added to the matrix to supplement the biophysical factor-induced osteogenic differentiation of DPSCs because dentin-derived BMP2 has been demonstrated to play a key role in inducing differentiation of dental stem cells into odontoblasts[22]. The potent role of VEGF in the angiogenesis of pulp tissue is also well-established.[31] Thus, we incorporated BMP2 and VEGF into the collagen matrices by adding the GFs to the collagen solution before gelation. The GF is retained in the matrices by adsorption and relies on a

physical retention and release process via the natural porosity of the oligomeric collagen matrix. Conversely, Galler and colleagues[9] incorporated fibroblast growth factor, transforming growth factor β1, and VEGF to a self-assembling peptide hydrogel via heparin binding. Although addition of heparin in the current study, could have improved affinity towards the growth factors[9, 32] we did not attempt it because it could potentially interfere with previously optimized matrix stiffnesses. However, even in the absence of heparin the model growth factors used herein demonstrated a slow release until day 6, compared to PuraMatrix™, which released the GFs in 48 h. Moreover, the released GFs also enhanced endothelial and osteogenic differentiation. Based on these previous reports, it is plausible that during regenerative endodontics in clinics, VEGF-incorporated matrices, as well as, VEGF released by DPSCs could enhance vascularization.

As formerly mentioned, matrix stiffness has shown to specify stem cell lineage.[33] We observed the expression of EC markers on day 14 even in the absence of GFs. Compliant substrates robustly improved EC commitment of human induced pluripotent stem cells through VECad, CD31, vWF and eNOS marker expression, without the addition of small molecules.[34] Studies have demonstrated that undifferentiated MSCs grown on polymer gels mimicking the ECM elasticity of a given tissue could induce expression of precursor proteins for the cell type typically present in that tissue in the absence of specific GFs.[35] There was an increase in the expression of the EC markers when GFs were added to the media demonstrating their supplementary effect on DPSCs along with the biophysical factors. Mullane et al.[31] demonstrated that the treatment of dental pulp tissue in tooth slices with 50 ng/mL recombinant human VEGF prior to implantation into immunodeficient mice increased pulp micro-vessel density *in vivo*. VEGF/MEK1/ERK signaling pathway and Wnt/β-catenin signaling were demonstrated to be key regulators of endothelial differentiation in DPSCs.[25, 26] The mRNA transcripts of vWF, VE-Cadherin and PECAM were observed from day 14 in DPSCs grown in all matrices including the control matrices cultured in DPSCM. Thereafter, a steady increase in the mRNA levels of endothelial cell markers was observed until day 28 (Figures 6A-F). The cells in VEGF-modified matrices as well as the control matrices demonstrated a 4-

to 5-fold increase in vWF mRNA levels by day 28 (Figure 6A). Matrices cultured in EGM showed a significant increase when VEGF was added to the media (Figure 6B). VE-Cadherin expression significantly increased 10-, 12- and 16-fold by day 28 in control, VEGF-modified and VEGF added (i.e., supplementation of VEGF in the culture media) matrices, respectively (Figure 6C). The effect was increased 14-, 25- and 35-fold, respectively when the same matrices were cultured in differentiation media (Figure 6D). PECAM expression in VEGF-modified matrices in the control and differentiation media showed a 6-fold increase on day 21. Moreover, there were 10- and 12-fold increases in PECAM expression, respectively when cells were grown in VEGF-modified matrices in control and differentiation media (Figures 6E and 6F). The expression levels of VE-Cadherin were higher compared to vWF and PECAM in matrices cultured in DPSCM and EGM, and the effect of VEGF incorporation was evident. These results demonstrate that the collagen matrices induced endothelial marker expression when cultured in DPSC media. Surprisingly, the level of marker expression increased even in the absence of endothelial differentiation media (Figures 6A, 6C, and 6E).

Over the last decade, several approaches have been adopted to induce vascularization and mineralization for dental tissue regeneration. Investigators have demonstrated that a co-culture of DPSCs and HUVECs enhanced the odonto-/osteogenic potential of DPSCs and vasculogenic capacity of HUVECs in cell-cell contact cultures.[36] Moreover, the co-culture of DPSCs and HUVECs in PuraMatrix™ induced more extracellular matrix, vascularization and mineralization than the DPSC-monocultures *in vivo*.[4] In this study, we observed improved osteogenic activity of DPSCs in the oligomeric collagen (800Pa) matrices following the addition of BMP2. Autogenous transplantation of BMP2-treated pellet culture stimulated reparative dentin formation in canine amputated pulp.[37] Another study demonstrated that blocking BMP2 signaling inhibited the expression of odontoblastic differentiation markers by SHED cultured in tooth slice/scaffolds, thus demonstrating the key role of BMP2 in odontogenic differentiation.[22] The role of p38 mitogen-activated protein kinase-activated canonical Wnt pathway in BMP2-induced DPSC differentiation has also been elucidated.[38] ALP activity was observed

in DPSCs cultured in 800Pa matrices even in the control media (DPSCM) demonstrating the effect of stiffness on osteogenic differentiation of cells (Figure 7A). While ALP activity significantly increased on day 14 in control matrices, in osteogenic differentiation media ALP activity increased on day 7 in the presence of BMP2. Alizarin staining also demonstrated the level of mineralization of DPSCs in collagen matrices (Figure 7B). Mineralized nodules could be observed on day 7 in control collagen matrices (inset Figure 7A). The presence of BMP2 increased mineralization levels in the collagen matrices cultured in DPSCM (inset Figure 7C) and OSM (inset Figure 7F). Significant increases in Alizarin staining in the OSM and BMP2 matrices on day 21 are shown in Figure 7B. These results demonstrate that the high stiffness (800Pa) oligomeric collagen matrices could induce osteogenic differentiation, and that the effect was enhanced when BMP2 was directly incorporated to the matrix.

▪ CONCLUSIONS

In aggregate, the current study shows that the collagen gels of stiffnesses 235Pa and 800 Pa demonstrated slow release of simulated growth factors, supported long-term cell survival, and favored differentiation of cells to specific lineage. The collagen matrices of precise stiffness, 235Pa and 800Pa, respectively induced endothelial and osteogenic differentiation, and these effects could be further enhanced by the addition of specific growth factors. Our long-term goal is to develop and optimize unique injectable collagen-fibril matrices for delivery and fate regulation of DPSCs in a spatially oriented fashion to form dentin and vascularized pulp at the appropriate locations within the root canal system. Based on these data, we expect that DPSCs transplantation within high stiffness matrix containing BMP2 or within a low stiffness matrix containing VEGF, when concentrically injected into the root canal system, will lead to dentin and pulp regeneration in the appropriate locations. We believe that this approach will be an effective strategy for dental pulp regeneration with significant translational outcomes. Future studies involving the assessment of vascularization and dentin formation using clinically relevant tooth slice model *in vivo* injected with collagen gels individually and concentrically are warranted.

▪ ACKNOWLEDGEMENTS

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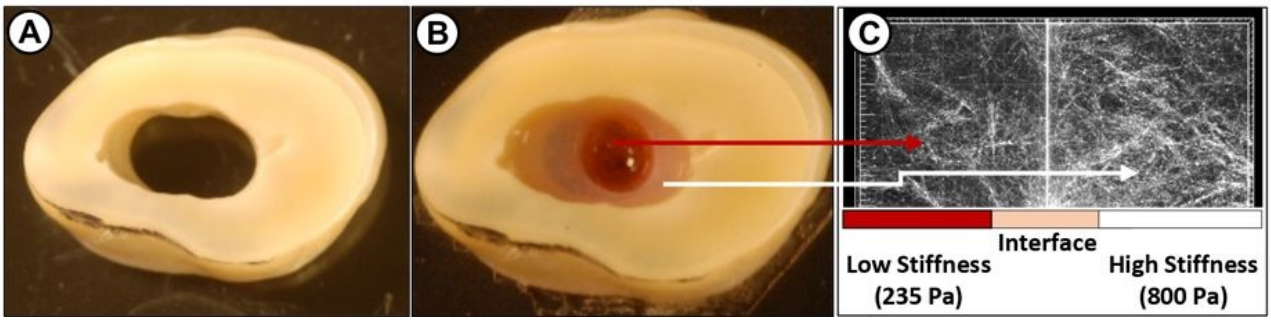
Graphic Abstract



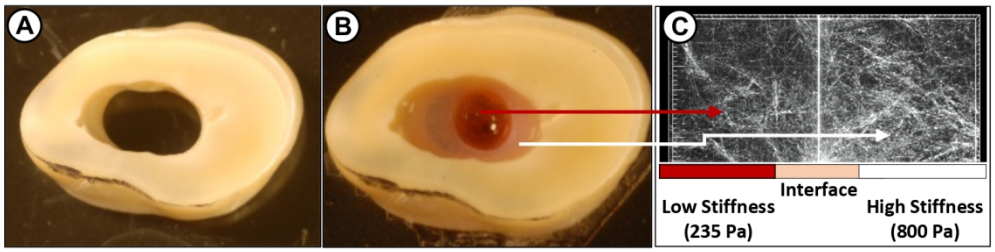


Figure 1. Schematic illustration of the proposed highly tunable injectable oligomeric collagen-fibril matrix system using a well-established tooth slice model: (a) tooth slice, (b) concentrically injected collagen matrices (low stiffness matrix purposely dyed in red), and (c) interface adaptation between the two matrices evidenced by reflectance microscopy. For the preparation of concentric collagen matrices, an endodontic fiber post (1.3 mm in diameter, 3M ESPE, St. Paul, MN) was placed and stabilized inside the tooth slice. The collagen solution of 2.88 mg/ml (800Pa) containing DPSC and BMP2 was injected using a needle to the area surrounding the fiber post. Once the matrix is polymerized, the fiber post was lifted and the lumen was injected with collagen solution of 1.37 mg/ml (235Pa) containing DPSC and VEGF165 and allowed to polymerize. The volume ratio of the collagen solutions 1.37 mg/ml and 2.88 mg/ml was adjusted to 2:1, which compares with the anatomical features of a root canal. The fibril density and interface adaptation between the two collagen matrices of lower and higher stiffness are shown.

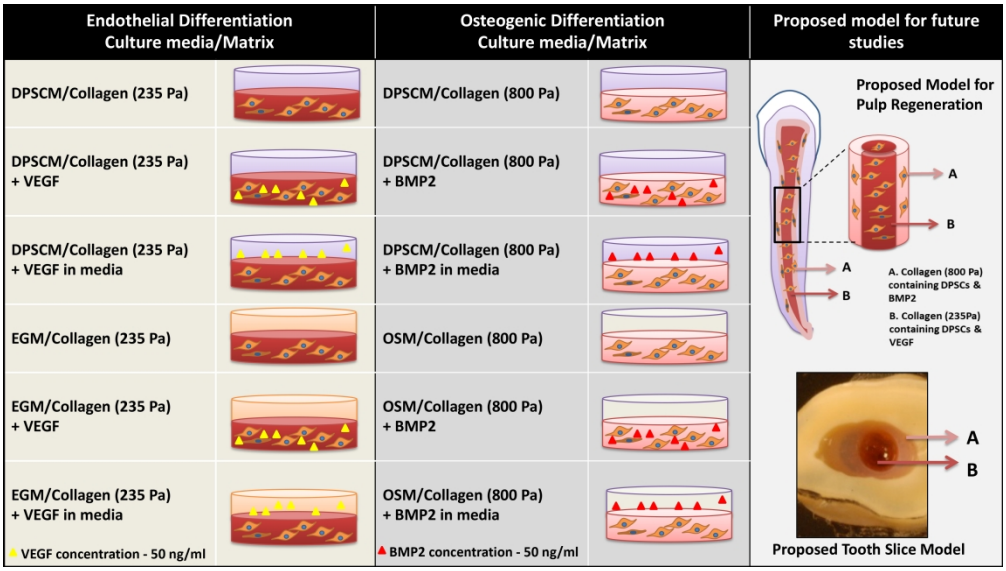


Figure 2. Oligomeric collagen matrices and culture media used for endothelial and osteogenic differentiation. The concentric tooth slice model and the dental pulp regeneration model for the future studies.

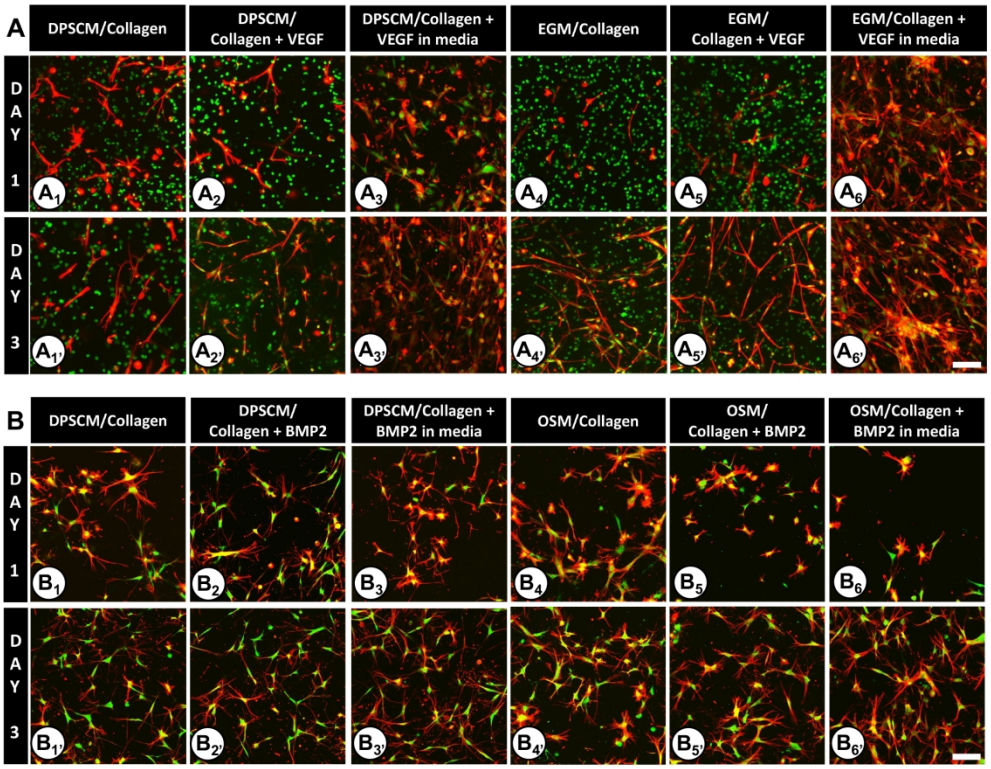


Figure 3. DPSC adhesion and spreading within Oligomer 235Pa collagen matrices under various conditions at days 1 and 3 on the following matrices: (A1, A1') DPSCM/Collagen; (A2, A2') DPSCM/Collagen + VEGF; (A3, A3') DPSCM/Collagen + VEGF in media; (A4, A4') EGM/Collagen; (A5, A5') EGM/Collagen + VEGF; (A6, A6') EGM/Collagen + VEGF in media. DPSC adhesion and spreading within Oligomer 800Pa collagen matrices under various conditions at days 1 and 3 on the following matrices: (B1, B1') DPSCM/Collagen; (B2, B2') DPSCM/Collagen + BMP2; (B3, B3') DPSCM/Collagen + BMP2 in media; (B4, B4') OSM/Collagen; (B5, B5') OSM/Collagen + BMP2; (B6, B6') OSM/Collagen + BMP2 in media. For all images, the actin filaments are stained red with Rhodamine-Phalloidin (excitation and emission at 540/565 nm) and the nucleus is stained green using SYTO 13 (excitation and emission at 488/509 nm). Regions that appear yellow indicate the co-localization of actin and nucleus. Each experiment had three samples. Images are maximum intensity projection of Z-slices (~300 μm depth) captured using a confocal/2-photon Olympus FV1000 MPE system (Olympus America) with a XLUMPLFL20XW objective and 0.95 NA. Scale bar = 100 μm.

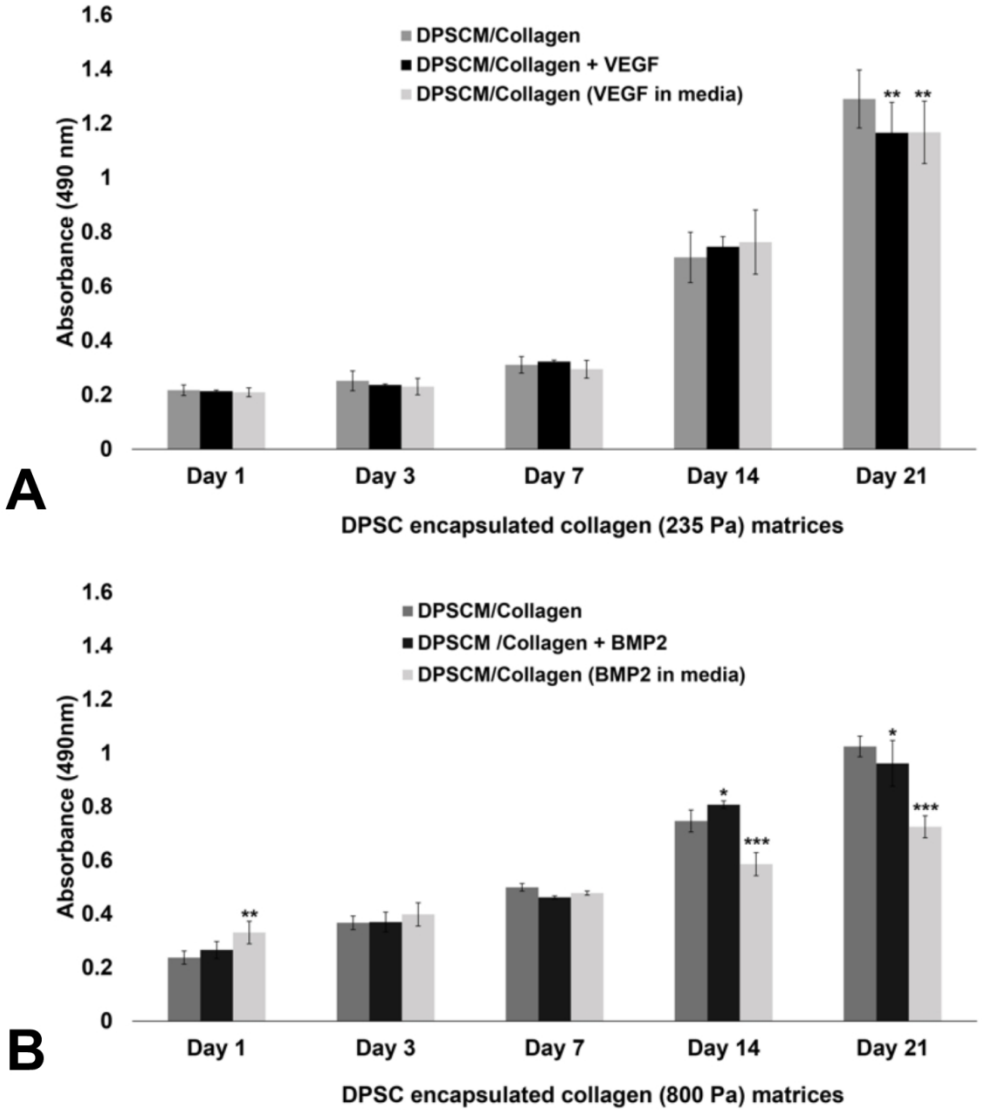


Figure 4. Long-term survival of DPSCs on 235Pa and 800Pa collagen matrices: (A) DPSCs survival on 235Pa collagen matrices on days 1, 3, 7, 14 and 21 post-encapsulation, (B) DPSCs survival on 800Pa collagen matrices on days 1, 3, 7, 14 and 21 post-encapsulation. The absorbance was read at 490nm using a microplate reader (Biotek, Winooski, VT, USA). Values represent mean ± SD (n=3). Statistical difference designations: ***$p<0.001$, **$p<0.01$ and *$p<0.05$.

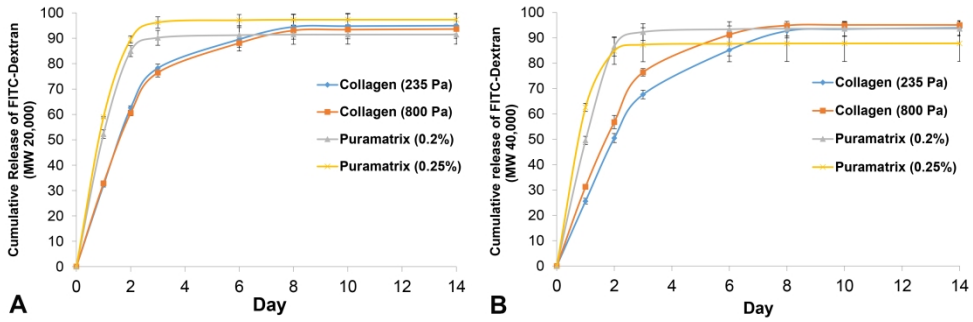


Figure 5. Cumulative release of FITC-Dextran from PuraMatrixTM and oligomeric collagen matrices for 14 days (N=5). FITC-dextran (Mw 20,000 and 40,000) served as model growth factors for (A) BMP2 and (B) VEGF, respectively. Fluorescence intensity was measured (Synergy HTX Microplate Reader, Biotek) using excitation and emission wavelengths of 485 nm and 520 nm, respectively.

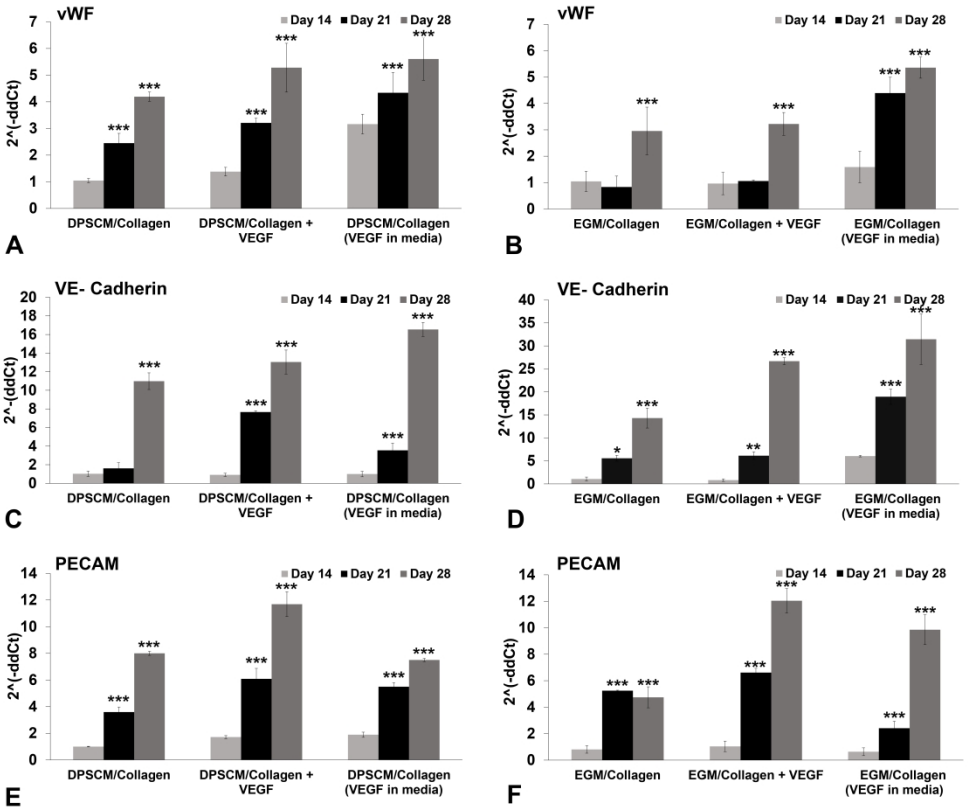


Figure 6. Endothelial differentiation on collagen (235Pa) matrices. Real-time PCR data (CFX96, Bio-Rad) showing expression levels of endothelial cell markers in DPSCs encapsulated in 235Pa collagen matrices on days 14, 21 and 28. (a) vWF - DPSCM, (b) vWF - EGM, (c) VE-Cadherin - DPSCM, (d) VE-Cadherin - EGM, (e) PECAM - DPSCM, and (f) PECAM - EGM. Relative expression of target genes was performed using the 2-ΔΔCt method. The results were normalized against the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Statistical analysis was performed to compare matrices on days 21 and 28 to day 14. Values represent mean ± SD (n=3). Statistical difference designations: ***p<0.001, **p<0.01 and *p<0.05.

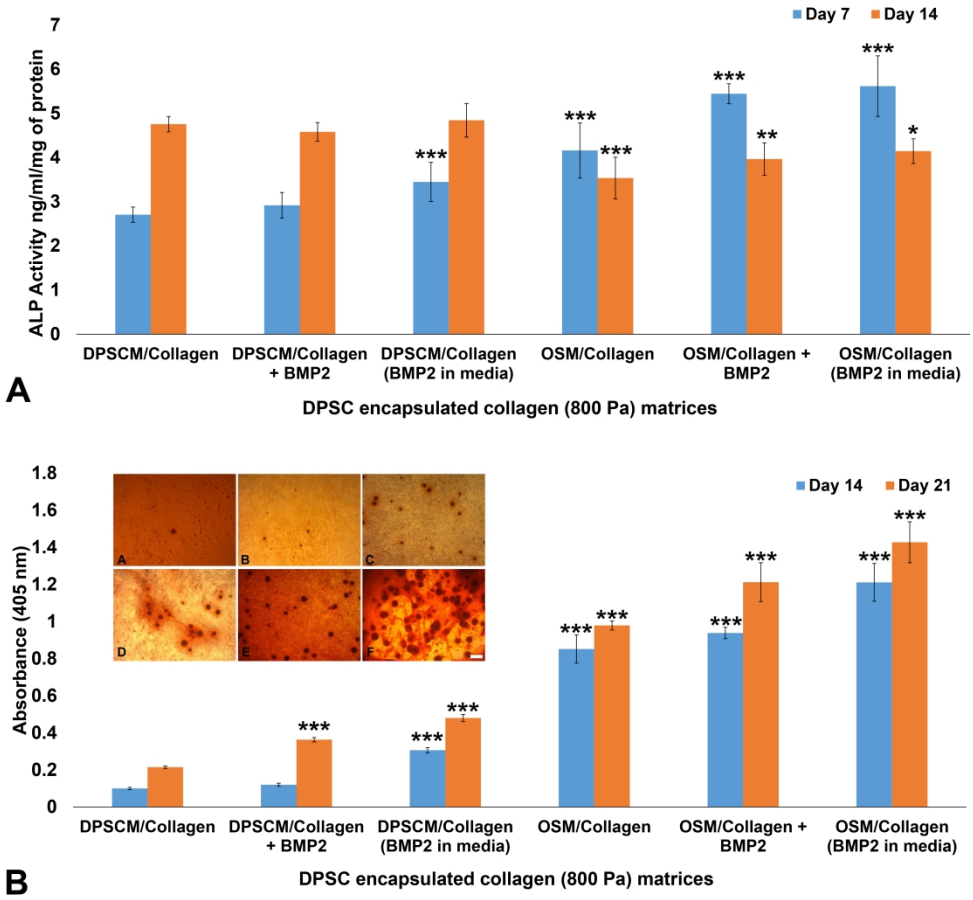


Figure 7. Osteogenic differentiation on collagen (800Pa) matrices: A) ALP activity of DPSCs encapsulated in collagen (800Pa) matrices on days 7 and 14. The results were normalized to the total protein level and expressed as ng of p-nitrophenol produced per mL per µg protein. Statistical analysis was performed to compare samples to DPSCM/Collagen (Control) on days 7 and 14. B) Alizarin staining of DPSCs encapsulated in collagen (800Pa) matrices and cultured in DPSCM and OSM for 14 days. Inset shows the bright field images (Primovert, Zeiss) of Alizarin stained matrices after 7 days in culture; (A) DPSCM/Collagen, (B) DPSCM/Collagen + BMP2, (C) DPSCM/Collagen + BMP2 in media, (D) OSM/Collagen, (E) OSM/Collagen + BMP2, (F) OSM/Collagen + BMP2 in media. Values represent mean ± SD (n=4). Statistical difference designations: ***p<0.001, **p<0.01 and *p<0.05. Scale bar = 50 µm.