



<b>Title</b>	<b>The interplay of dental pulp stem cells and endothelial cells in an injectable peptide hydrogel on angiogenesis and pulp regeneration in vivo</b>
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# The Interplay of Dental Pulp Stem Cells and Endothelial Cells in an Injectable Peptide Hydrogel on Angiogenesis and Pulp Regeneration *In Vivo*

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Securing an adequate blood supply for the survival of cell transplants is critical for a successful outcome in tissue engineering. Interactions between endothelial and progenitor/stem cells are important for vascularization of regenerating tissue. Recently, self-assembling peptide nanofibers were described as a promising environment for pulp regeneration due to their synthetic nature and controlled physicochemical properties. In this study, the peptide hydrogel PuraMatrix™ was used as a scaffold system to investigate the role of dental pulp stem cells (DPSCs) in triggering angiogenesis and the potential for regenerating vascularized pulp *in vivo*. Human umbilical vein endothelial cells (HUVECs), DPSCs, or cocultures of both cell types were encapsulated in three-dimensional PuraMatrix. The peptide nanofiber microenvironment supported cell survival, cell migration, and capillary network formation in the absence of exogenous growth factors. DPSCs increased early vascular network formation by facilitating the migration of HUVECs and by increasing vascular endothelial growth factor (VEGF) expression. Both the DPSC-monoculture and coculture groups exhibited vascularized pulp-like tissue with patches of osteodentin after transplantation in mice. The cocultured groups exhibited more extracellular matrix, vascularization, and mineralization than the DPSC-monocultures *in vivo*. The DPSCs play a critical role in initial angiogenesis, whereas coordinated efforts by the HUVECs and DPSCs are required to achieve a balance between extracellular matrix deposition and mineralization. The findings of this study also highlighted the importance of a microenvironment that supports cell–cell interactions and cell migration, which contribute to successful dental pulp regeneration.

## Introduction

**R**APID VASCULARIZATION of a bioengineered tissue/scaffold transplant following implantation *in vivo* is critical for a successful outcome in tissue engineering. The human tooth has a unique architecture with an internal pulp space encased in a thick dentin wall, and the dental pulp only consists of a microcirculatory system originating from a very small opening at the apex of the root. This anatomical configuration hinders the development of an adequate vascular supply during pulp regeneration. Consequently, on *in vivo* transplantation of teeth the tissue constructs must depend solely on the oxygen supply that is delivered via diffusion from the nearest capillary.<sup>1</sup> Oxygen diffusion is effective up to a distance of ~200 μm from a blood vessel<sup>2</sup> and the majority of cells undergo hypoxia when located further from a blood vessel.<sup>3</sup> The survival of a larger implanted tissue construct requires the formation of an intrinsic

capillary network to exchange gases, nutrients, and wastes between the cells and the vascular system. Although host blood vessels invade the implanted tissue construct, partially in response to the angiogenic factors secreted by hypoxic cells, this process occurs very slowly, with vessels growing only a few tenths of a micrometer per day.<sup>1</sup> Therefore, the development of methods to promote rapid vascularization of a tissue construct following *in vivo* implantation is critical for improved functional integration.

To develop novel strategies to promote vascularization of engineered pulp constructs, it is necessary to understand the interactions between the major cell types within the pulp. Dental pulp stem cells (DPSCs), which have been identified as a promising progenitor cell population for pulp repair and regeneration, are known to occupy a perivascular niche. Therefore, DPSCs and endothelial cells (ECs) are physically poised to regulate their respective functions. We recently demonstrated that a coculture of human umbilical vein

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endothelial cells (HUVECs) and DPSCs synergized osteo/odontogenic and angiogenic differentiation in monolayer cultures.<sup>4</sup> However, less information is available regarding the interactions between DPSCs and ECs/HUVECs in a three-dimensional (3D) microenvironment, which may significantly influence their behavior and functionality.

Prevascularization of the cell/scaffold construct by ECs is considered a promising approach to achieve rapid anastomosis between the bioengineered cellular/tissue construct and the host vasculature.<sup>1,5</sup> However, this is a challenging task because ECs are sensitive to their microenvironment and tend to undergo apoptosis after encapsulation in most scaffold materials.<sup>6</sup> External growth factors often have to be supplied to promote *in vitro* angiogenesis. Therefore, a 3D culture system that mimics the natural cell milieu while uncoupling the scaffold-triggered signaling from cell–cell interactions may provide vital insights into the role of EC-DPSC interactions during angiogenesis.

The exceptional anatomy of root canals also poses difficulties for using rigid scaffold systems in pulp regeneration because it is difficult to insert them into the narrow, tapering root canals. Therefore, being able to inject a scaffold system into root canals would be an added advantage when selecting a scaffold for dental pulp regeneration. Using a scaffold with specific fibrous structures that ideally mimic the extracellular matrix of the target tissue is critical in a scaffold-based regenerative approach.<sup>7</sup> Additionally, in terms of dental pulp regeneration, the scaffold should be capable of promoting odontoblastic differentiation of stem/progenitor cells. Therefore, an ideal scaffold for dental pulp regeneration should be injectable and EC friendly and should facilitate odontoblastic differentiation.

Emerging data suggest that the self-assembling peptide hydrogel PuraMatrix™ may fulfill several of these requirements. PuraMatrix is a repeating polymer of the amino acid sequence R-A-D-A and is formulated as an injectable scaffold that assembles into nanofibers when exposed to physiologic concentrations of salts. This scaffold has been used in several studies and has yielded promising results for cardiac,<sup>8–11</sup> neural,<sup>12,13</sup> hepatic,<sup>14</sup> and bone<sup>15</sup> tissue regeneration. Furthermore, PuraMatrix has been shown to be capable of maintaining the viability and odontogenic differentiation capacity of DPSCs.<sup>16</sup> A recent study found that stem cells from exfoliated deciduous teeth that were encapsulated in PuraMatrix and injected into full-length root canals gave rise to functional odontoblasts and pulp-like tissue after transplantation into immunodeficient mice.<sup>17</sup>

This study aimed to elucidate the DPSC-HUVEC interactions during angiogenesis and odonto/osteogenic differentiation in a 3D peptide-hydrogel system. We investigated cell viability, vascular network formation, and the differentiation capacity of mono- or cocultured DPSCs and HUVECs seeded in PuraMatrix and examined the potential of prevascularized PuraMatrix-encapsulating DPSCs to regenerate dental pulp-like tissues *in vivo*.

## Materials and Methods

### *Isolation, culturing, and characterization of cells*

After obtaining informed consent, DPSCs were isolated from freshly extracted sound third molars from human subjects aged 18 to 25 years, as described previously.<sup>18</sup> Briefly, the surfaces of the freshly extracted teeth were cleaned and cut at the cemento-enamel junction using a

sterile fissure bur to reveal the pulp chamber. After gently separating the pulp tissue from the crown and root, it was digested in a solution containing 3 mg/mL collagenase type I (GIBCO-Invitrogen, Carlsbad, CA) and 4 mg/mL dispase (GIBCO-Invitrogen) for 1 h at 37°C. Then, the cells were passed through a 70- $\mu$ m strainer (BD Biosciences, Franklin Lakes, NJ) to obtain single-cell suspensions. These cells were seeded in 75-cm<sup>2</sup> culture flasks containing  $\alpha$ -minimum essential medium ( $\alpha$ -MEM) supplemented with 15% fetal bovine serum, L-ascorbic acid-2-phosphate, 100 U/mL penicillin-G, 100 mg/mL streptomycin, and 0.25 mg/mL fungizone (Gemini Bio-Products, Woodland, CA) and cultured under 5% CO<sub>2</sub> at 37°C. DPSCs isolated from three human donors were characterized and used individually in triplicate experiments. HUVECs that were obtained commercially (ScienCell Research Laboratories, San Diego, CA) were cultured in endothelial cell medium (ECM; ScienCell Research Laboratories) at 37°C with 5% CO<sub>2</sub>. Cells from passages 3 to 6 of each cell type were used in all experiments according to the parameters described below.

### *Characterization of DPSCs*

Before using the DPSCs for experiments, the “stemness” of the freshly isolated cells was assessed by flow cytometric analysis of the expression of mesenchymal stem cell markers CD73, CD90, CD105, STRO-1, and hematopoietic marker CD45. In addition, the multilineage differentiation capacity of the DPSCs was confirmed using osteo-/odontogenic, adipogenic, and neurogenic induction media as described previously.<sup>4,19,20</sup>

### *Cell encapsulation within PuraMatrix*

Monocultures and cocultures of different ratios (3:1, 1:1, or 1:3) of DPSCs and HUVECs were encapsulated in different concentrations (0.5%, 0.25%, or 0.15%) of PuraMatrix (BD-Biosciences, Bedford, MA). Briefly, the viscosity of the BD PuraMatrix stock solution (1% w/v) was reduced by vortexing, and air bubbles were removed by centrifuging. The necessary volume of the desired concentration of PuraMatrix was prepared by diluting the stock with sterile 20% sucrose to generate a 2 $\times$  concentration of PuraMatrix in 10% sucrose (except for 0.5% PuraMatrix). The cells were trypsinized and centrifuged at a concentration of 1 $\times$ 10<sup>6</sup> cells/mL. The medium was removed from the cell pellet, and the cells were resuspended at the desired density in sterile 10% sucrose. The cells were centrifuged again to remove any remaining salts and resuspended in 10% sucrose at twice the final desired cell concentration. Equal volumes of 2 $\times$  PuraMatrix and a 2 $\times$  cell/sucrose mixture were mixed and then carefully added to the center of the well without introducing air bubbles. The ratios of different solutions mixed to prepare 2 mL working concentrations of cell/PuraMatrix constructs were summarized in Table 1. Gelation of the PuraMatrix was initiated by gently running culture media down the side of the well on top of the hydrogel. The medium was gently changed twice over the next hour to equilibrate the pH of the hydrogel.

### *Transwell assay*

The Transwell culture model was used to indirectly coculture DPSCs and HUVECs encapsulated in PuraMatrix. In

TABLE 1. PREPARATION OF DIFFERENT CONCENTRATIONS OF PURAMATRIX™

Final PuraMatrix concentration (%)	1% PuraMatrix (stock) (μL)	20% Sucrose (μL)	DI water (μL)	2×cell suspension in 10% sucrose (μL)
0.5	1000	—	—	1000
0.25	500	500	—	1000
0.15	300	500	200	1000

DI, deionized.

this model, the DPSCs were maintained as a monolayer in the culture wells, and HUVECs encapsulated in PuraMatrix were layered onto the culture insert of a 24-well Transwell plate. The DPSCs were cultured overnight before the HUVEC/PuraMatrix was placed on the inserts. Alternatively, HUVEC/PuraMatrix was cultured using the normal medium or medium supplemented with recombinant human vascular endothelial growth factor (VEGF) 165 (50 ng/mL; R&D Systems, Inc., Minneapolis, MN) on cell culture inserts with no DPSC monolayer in the lower compartment.

#### Live/Dead viability assay

Cell viability in the cell/scaffold constructs was assessed at 24 h, 4 days, and 2 weeks after encapsulation using a Live/Dead/Viability/Cytotoxicity kit (Molecular Probes, Inc., Eugene, OR). As recommended by the manufacturer, the cell/scaffold constructs were rinsed with PBS and then treated with a PBS solution containing 2 mM ethidium homodimer-1 and 4 mM calcein AM for 30 min. The samples were then washed with PBS and observed using confocal microscopy. Live cells with green fluorescence were quantified using CellProfiler, cell image analysis software.

#### Quantification of VEGF by ELISA

VEGF<sub>165</sub> released by the monocultures and the cocultures of different ratios were detected using a specific ELISA kit (Quantikine; R&D Systems, Inc., Minneapolis, MN). Supernatants from independent cultures of DPSCs, HUVECs, and cocultures at 24 h, 48 h, and 4 days were collected, stored at -80°C, and then submitted to the immunoenzymatic detection, according to the protocol described by the manufacturer. The results were expressed as pg VEGF/μg protein.

#### Transfection of cells with green fluorescent protein and red fluorescent protein constructs

DPSCs and HUVECs were transfected for the expression of green fluorescent protein (GFP) and red fluorescent protein (RFP), respectively, using premade GFP and RFP lentiviral particles (GenTarget, Inc., San Diego, CA). The transfection procedure was carried out as described by the manufacturer and stably expressing cell lines were selected using G418 antibiotic.

#### In vitro prevascularization of PuraMatrix

Monocultured or cocultured DPSCs and HUVECs that were expressing GFP and RFP, respectively, were encapsulated in PuraMatrix as described above. The cellular morphologies and

3D organization of the cells within the scaffold were monitored using confocal microscopy for 2 weeks. In addition, DPSC:HUVEC co-encapsulated PuraMatrix was injected into the root canals of root segments and observed whether the vascular structures are formed within the root canal environment.

#### Odonto/osteogenic differentiation assays

Odonto/osteogenic differentiation of cultures was induced in the cocultured encapsulated cells using coculture medium supplemented with 10 nM dexamethasone, 10 mM β-glycerophosphate, 50 μg/mL L-ascorbic acid phosphate, and 10 nM 1,25 dihydroxyvitamin D<sub>3</sub> for 21 days.<sup>4,19,20</sup> The differentiation of DPSC-only cultures was induced using DPSC medium supplemented with the above-mentioned odonto/osteogenic factors.

#### ALP activity

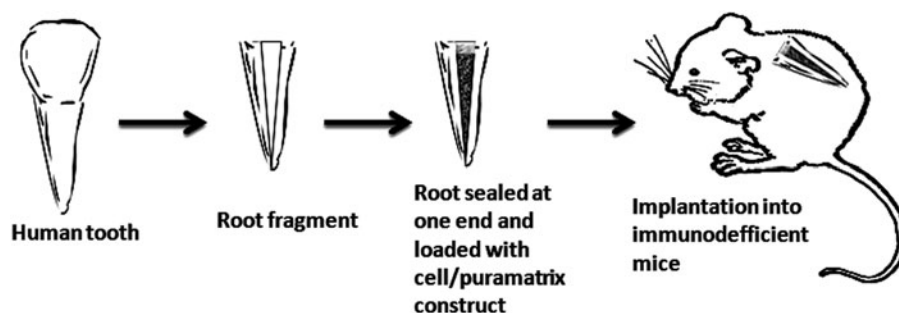
The cultures were rinsed twice with ice-cold phosphate-buffered saline, solubilized using Tris/glycine/Triton buffer (pH 10.5, 50 mM Tris, 100 mM glycine, and 0.1% Triton X-100), and sonicated on ice using two 15-s pulses. The supernatants were collected and used to determine the total protein and ALP levels. The total protein levels were determined using a BCA protein assay kit (Thermo Scientific, Pierce Biotechnology, Rockford, IL). A 100 μL aliquot of a freshly prepared p-nitrophenyl-phosphate (PNPP) solution was added to 200 μL of the supernatant mixed with Tris/glycine/Triton buffer and incubated for 30 min at 37°C. The optical density of p-nitrophenol at 405 nm was measured using spectrophotometry. The ALP activity was expressed as nanomoles of PNP per milligram of protein per minute.

#### von Kossa staining for mineralization

The PuraMatrix/cell constructs were fixed using 4% paraformaldehyde, dehydrated, and embedded in paraffin. Five-micrometer sections were cut, deparaffinized, and rehydrated to water. The sections were incubated with 1% silver nitrate solution placed under ultraviolet light for 20 min. After rinsing with distilled water, the unreacted silver was removed by soaking in 5% sodium thiosulfate for 5 min. The sections were counterstained with nuclear fast red dye for 5 min.

#### Pulp regeneration in vivo in a SCID mouse model

The roots of freshly extracted human teeth were horizontally sectioned into 10 mm-long segments. The root canals were cleaned and shaped using rotary instruments (ProTaper and ProFile, Dentsply Tulsa Dental). The root canals were smoothed using the following sequences of files: SX, S1, S2, F1, F2, and F3. These root fragments were soaked at room temperature in 17% ethylenediamine tetraacetic acid (EDTA) for 10 min and then in 19% citric acid for 1 min to remove the smear layer and subsequently treated with betadine for 30 min and 5.25% NaOCl for 10–15 min to remove the organic component, the debris originating from pulp tissue and microorganisms. The fragments were then rinsed with sterile PBS, soaked in PBS, and then incubated at 37°C for 3–7 days to remove the residual disinfection agents and ensure that there was no microbial contamination.



**FIG. 1.** SCID mouse model for the subcutaneous transplantation of human tooth root fragments loaded with cell/Puramatrix™ constructs. The canal space of the human tooth root fragments (10- to 11-mm long) was enlarged to ~0.5–1.5 mm in diameter. One end of the canal opening was sealed using MTA cement.

The following five experimental groups were created: (1) empty root segments; (2) root segments with PuraMatrix alone; (3) root segments with PuraMatrix + DPSCs (4) root segments with PuraMatrix + 3:1 DPSCs:HUVECs; and (5) root segments with PuraMatrix + 1:1 DPSCs:HUVECs. Eight root segments from each group were placed in a total of 20 mice. The coronal end of each root segments was sealed with MTA so that only the apical end was open to the external environment, which was 500–1000  $\mu\text{m}$  in diameter. After suspending the cells in PuraMatrix, the mixtures were injected into the root segments and incubated overnight in the normal culture medium. Root segments from the different groups were bilaterally implanted in the subcutaneous space of the dorsum of 5- to 7-week-old female severe combined immunodeficient mice (CB.17 SCID) (Fig. 1). After 4 weeks, the implants were retrieved, fixed in 4% formaldehyde for 24 h, demineralized with EDTA until the dentin offered no resistance to being cut with a blade (4 weeks), and then processed for histology and immunohistochemistry.

#### Histology and immunohistochemistry

Hematoxylin and eosin staining was performed to examine the morphology and structure of the regenerated pulp-like tissues. Immunohistochemistry using human-specific mitochondrial [Abcam (Hong Kong) Ltd. HKSP, N.T. Hong Kong] and human-specific CD31 (Santa Cruz Biotechnology, Inc., Dallas, TX) antibodies was conducted to confirm the contribution of the transplanted DPSCs and HUVECs, respectively, to the regenerated tissues. Human specific anti-*nestin* antibody [Abcam (Hong Kong) Ltd. HKSP] and anti-dentin sialoprotein (DSP) antibody (Santa Cruz Biotechnology, Inc.) was used to detect the odontoblastic lineage of the cells in newly formed pulp-like tissues.

#### Quantification of blood vessels

To quantify the blood vessels, three sections per root-slice that were stained with a human-specific anti-CD31 antibody and counterstained with hematoxylin were analyzed. Microscopic images were taken at 20 $\times$  magnification in three random areas per section. EC-lined lumens were counted as total vascular lumens. To determine the density of the human microvessels (mean number of capillaries per square millimeter), the number of structures with lumens surrounded by human-CD31<sup>+</sup> cells was counted manually. EC-lined vessels containing intraluminal red blood cells were considered perfused lumens, whereas lumens without red blood cells were considered nonperfused lumens. The values are given as the mean value  $\pm$  standard deviation.

#### Statistical analysis

The ALP concentration, relative expression levels of the genes, and number of vessels were expressed as a mean  $\pm$  standard deviation and were statistically analyzed using nonparametric statistical test Kruskal–Wallis one-way analysis of variance.  $p < 0.05$  was considered statistically significant.

#### Results

##### In vitro characterization of DPSCs

Freshly isolated DPSCs showed odonto/osteogenic, neurogenic, and adipogenic differentiation after being induced by relevant induction media for a period of 3 weeks (Fig. 2A–C). The results of the flow cytometric analysis showed positivity for STRO-1, CD73, CD105, and CD90, which confirmed the mesenchymal stem cell lineage of the DPSCs (Fig. 2D). Further, the DPSCs were negative for hematopoietic marker CD45 (Fig. 2D).

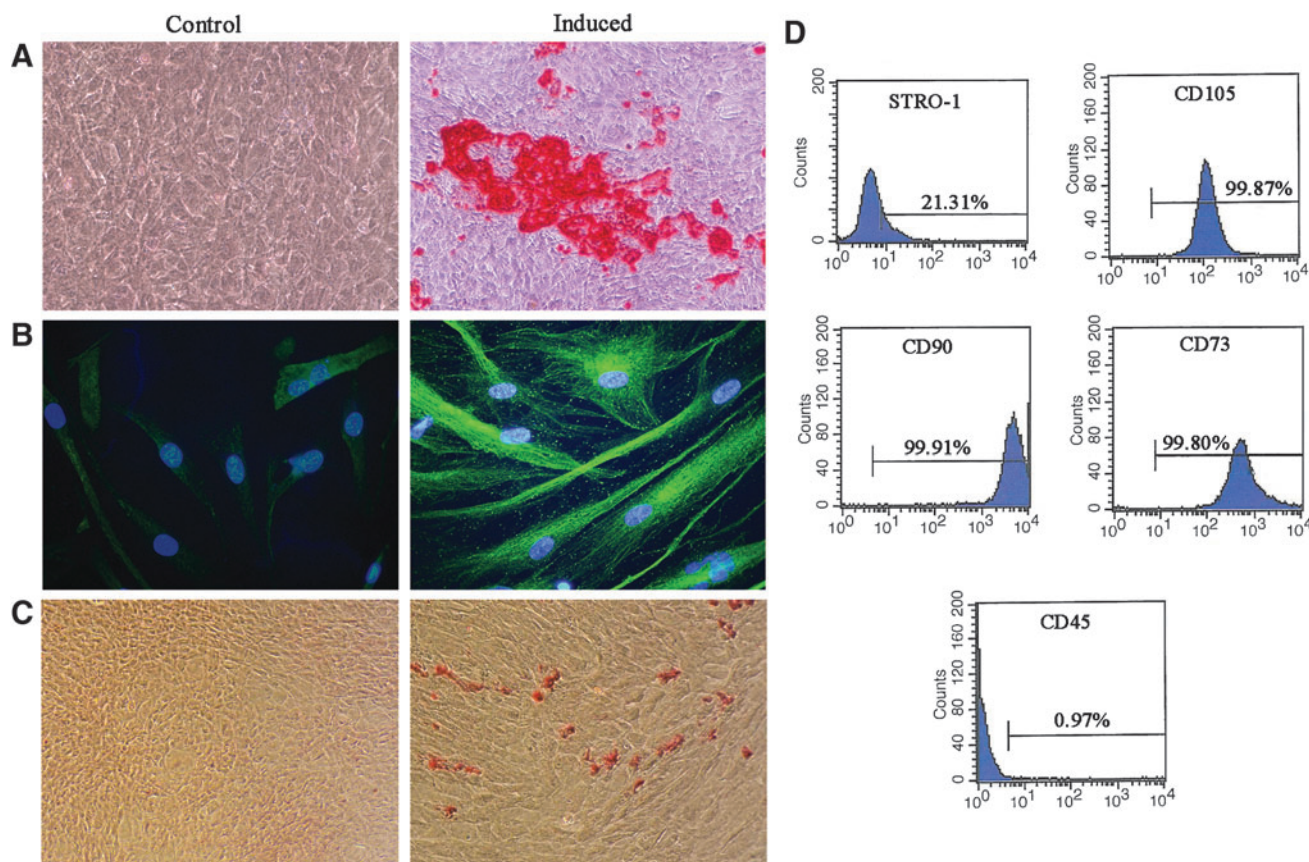
Cell viability, survival, and proliferation within PuraMatrix The Live/Dead assay revealed that 0.15% was the optimal PuraMatrix concentration for HUVECs compared to 0.5% and 0.25% PuraMatrix, in which the HUVECs did not survive after encapsulation. In contrast, the DPSCs survived in Pura Matrix at all three concentrations. Thus, PuraMatrix was used at a concentration of 0.15% in all subsequent experiments described in this study.

When DPSCs and HUVECs were seeded as monocultures in 0.15% PuraMatrix, the DPSCs survived and grew faster than did the HUVECs (Fig. 3). The HUVECs did not survive longer than 2 weeks in monocultures (Fig. 3C, F, I). Cell survival was significantly higher (Fig. 3J) in cocultures than in either DPSC or HUVEC monocultures (Fig. 3B, E, F). Furthermore, vessel-like structures formed near the DPSCs in the cocultures (Fig. 3E). These findings suggested that coculturing DPSCs and HUVECs in PuraMatrix promoted HUVEC survival and the secretion of angiogenic factors that induced vessel-like structure formation.

##### DPSCs promote endothelial tube formation via soluble factors

Unlike the cells in the HUVEC/PuraMatrix monocultures (i.e., without a DPSC monolayer in the lower compartment) (Fig. 4A–C), the cells in the HUVEC/PuraMatrix wells cultured for 48 h over a DPSC monolayer had round to spindle-shaped morphologies and were organized in a tubal network (Fig. 4G–I). Similar morphological changes were observed in the cells when HUVEC/PuraMatrix wells were





**FIG. 2.** Characterization of DPSCs isolated from freshly extracted human third molars. Osteo/odontogenic differentiation—mineralized nodule formation as shown by Alizarin Red staining after 3 weeks of induction (A). Neurogenic differentiation— $\beta$ III-tubulin expression with long cellular processes while maintaining the spindle-shaped fibroblastic cell bodies after 3 weeks of induction as shown by immunofluorescence imaging (B). The green fluorescence shows the positivity for  $\beta$ III-tubulin. Adipogenic differentiation—lipid droplet formation as shown by Oil Red O staining after 3 weeks of induction (C). Expression of mesenchymal stem cell markers STRO-1, CD90, CD105, CD73, and hematopoietic marker CD45 as analyzed by flow cytometry (D). DPSCs, dental pulp stem cells.

cultured in medium supplemented with VEGF (at 50 ng/mL) (Fig. 4D–F). In contrast, the cells in the HUVEC/PuraMatrix wells that were cultured in normal medium did not arrange into a tubal network (Fig. 4A–C). These results suggested that DPSCs secrete pro-angiogenic growth factors that inhibit EC apoptosis and promote tube formation.

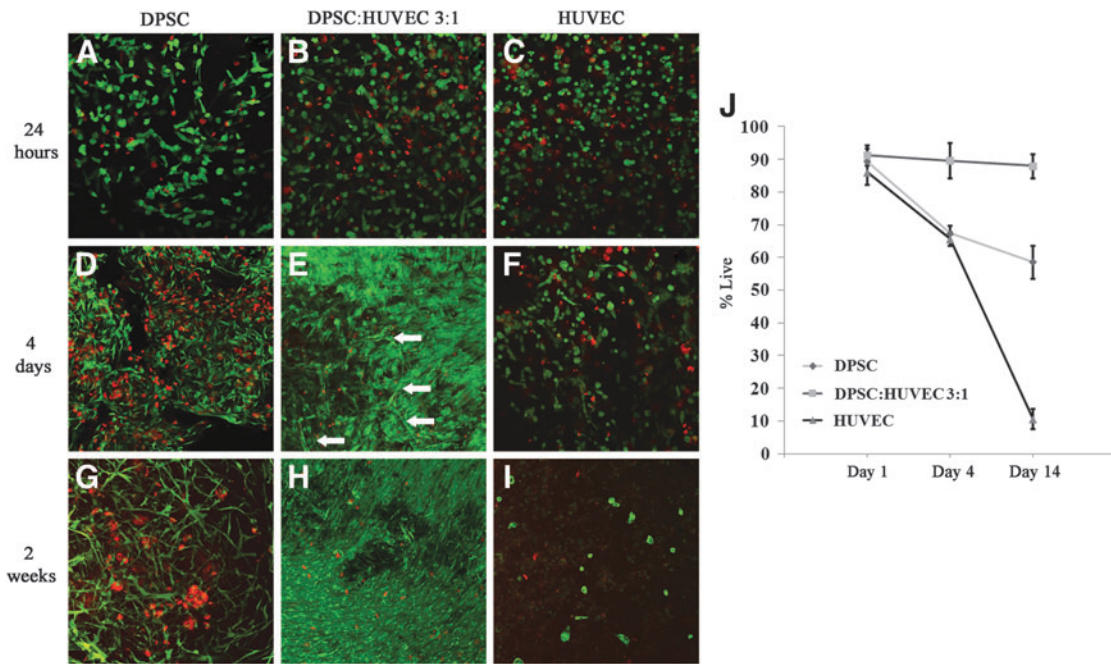
#### DPSCs secrete VEGF

Quantitative determination of VEGF<sub>165</sub> indicated that the VEGF levels in the medium were largely determined by the DPSCs. Significantly higher VEGF<sub>165</sub> contents were detected in the DPSC monocultures (Fig. 4J) than in the cocultures at all the time points. The results also indicated that the decrease in the VEGF level in cocultures was proportional to the number of HUVECs that were incorporated. This finding suggests that in cocultures, the VEGF that is secreted by the DPSCs might be immediately utilized by the HUVECs to activate signaling for migration and vascular structure formation. At day 4, a significantly higher level of VEGF was detected in the cocultures (DPSC:HUVEC 3:1 and 1:1) compared to days 1 and 2, which correlated with the presence of a relatively well-developed vascular network by day 4.

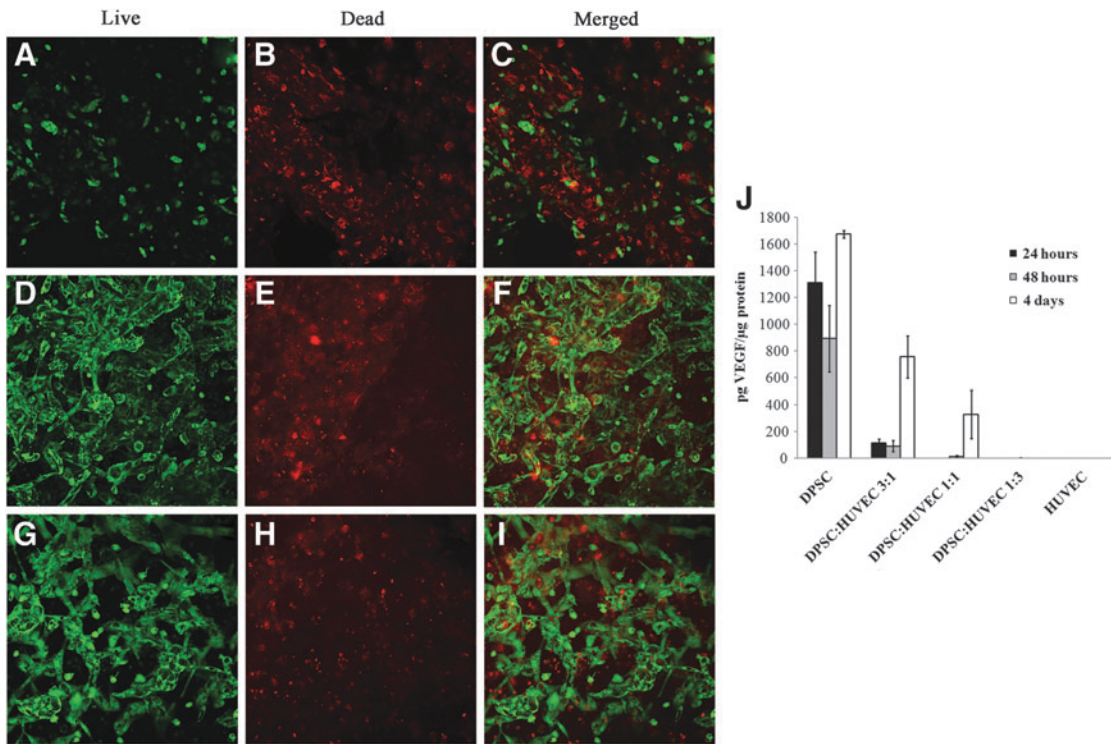
#### Prevascularization of DPSC/PuraMatrix constructs by HUVECs

Immediately after seeding was completed, both the HUVECs and DPSCs attached and uniformly spread across the peptide hydrogel (Fig. 3A–C). Coordinated migration of DPSCs and HUVECs was observed after 48 h (Fig. 5A–C), with DPSCs being near the nascent HUVEC networks and a distinct lack of these cells outside the networks. By day 3, an extensive vessel-like network was observed throughout the cocultured PuraMatrix scaffold (Fig. 5D–F), whereas the HUVEC monocultures failed to form any vessel-like structures (Fig. 5G–I). The vessel structures that formed within the cocultures were three-dimensionally organized and significantly larger (Fig. 5D–F) than the tubular structures observed in the HUVEC monocultures in the presence of DPSC-conditioned medium (Fig. 4G–I) or VEGF (Fig. 4D–F). A low density of vessel-like structures was observed when the EC number was higher (not shown). A 3:1 ratio of DPSC:HUVEC was identified as optimal for vessel structure formation *in vitro*. The vessel-like network continued to remodel and remained in a stable condition for 2 weeks *in vitro* (Fig. 5J–O).

When the DPSCs and HUVECs were co-encapsulated in PuraMatrix and injected into root canals of root

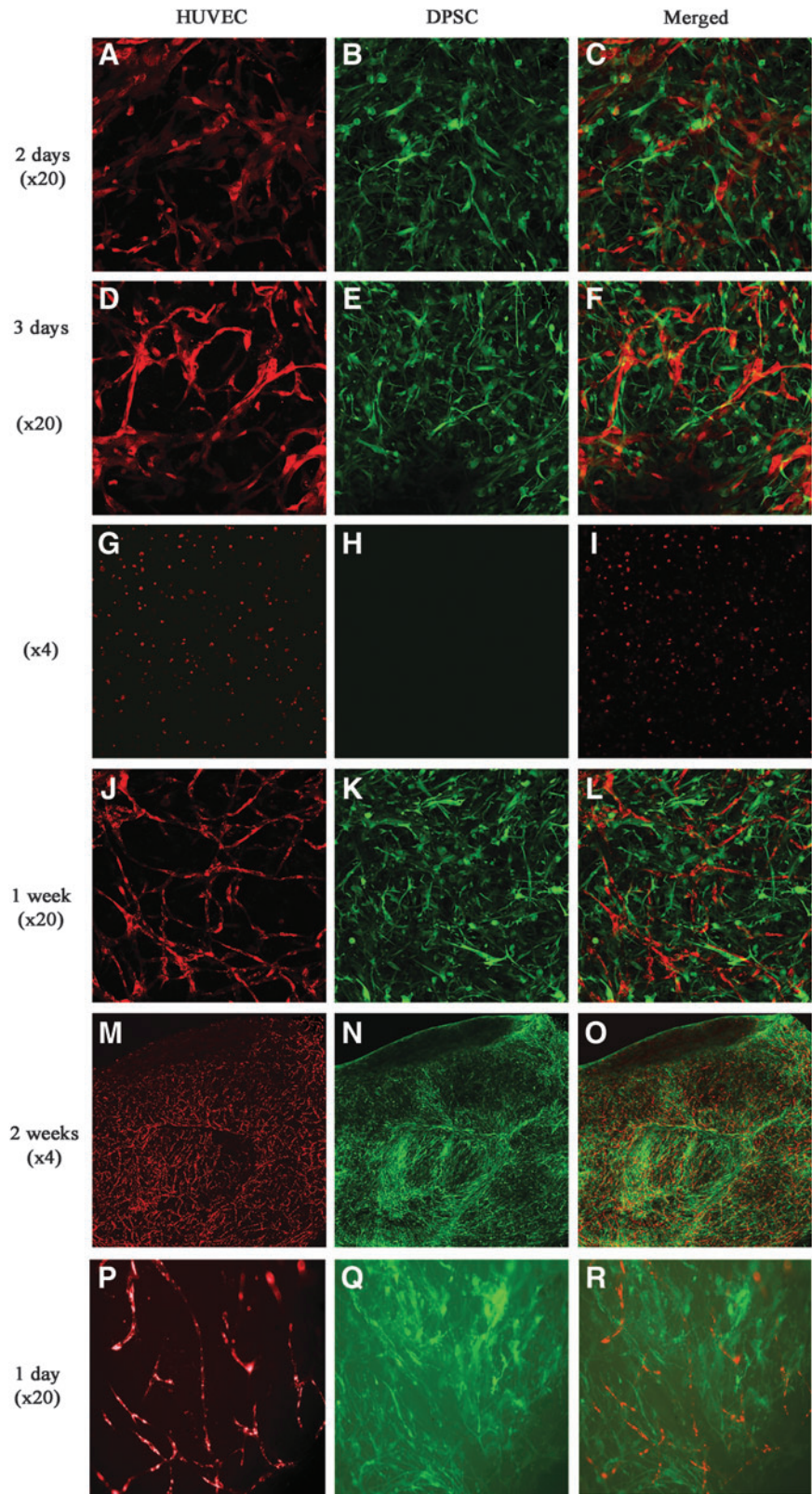


**FIG. 3.** Live/Dead assays were performed on the mono- and cocultures at different time points after the cells were encapsulated in 0.15% PuraMatrix, with cell number quantification by Cell Profiler, cell image analysis software (**J**). The DPSC:HUVEC 3:1 coculture (**B, E, H**) exhibited a significantly higher rate (**J**) of cell survival than DPSC (**A, D, G**) and HUVEC monocultures (**C, F, I**). Vessel-like structures were observed in the cocultures (*White arrows in E*). HUVEC, human umbilical vein endothelial cell.



**FIG. 4.** The DPSCs supported vascular morphogenesis by the HUVECs. HUVECs/PuraMatrix cultured in normal medium did not develop any tubular, vessel-like structures (**A–C**). HUVECs/PuraMatrix cultured in medium supplemented with VEGF 165 (50 ng/mL) initiated the formation of vessel-like structures (**D–F**). HUVECs/PuraMatrix cultured on a cell culture insert with a DPSC monolayer in the lower compartment developed tubular vessel-like networks (**G–I**). **A–I**: Live/Dead staining at 48 h. (**J**) Quantitative determination of the VEGF<sub>165</sub> content of the supernatants of mono- and cocultures of DPSCs and HUVECs using ELISA, expressed as pg VEGF/μg protein at 24 h, 48 h, and 4 days. The DPSC monocultures had significantly higher VEGF levels in their supernatants than the cocultures and HUVEC monocultures at all three time points. The decrease in the VEGF level in cocultures was proportional to the number of HUVECs that were incorporated into the cocultures. The VEGF levels began to increase in the supernatants of the cocultures after 48 h of cultivation. VEGF, vascular endothelial growth factor.





**FIG. 5.** Vessel-like structure formation by HUVECs that were co-cultured as PuraMatrix constructs (*Green*: green fluorescent protein-expressing cells, *Red*: red fluorescent protein-expressing cells). (**A–C**) Coordinated cell migration was observed in the cocultures 48 h after seeding. (**D–F**) Vessel-like structure formation by HUVECs was observed in the cocultures 3 days after seeding, with DPSCs surrounding the nascent HUVEC networks. (**G–I**) HUVEC monocultures did not exhibit cell migration or vessel structure formation. (**J–O**) Vessel-like networks continued to be remodeled and to stabilize for 2 weeks. (**P–R**) Vascular structure formation was observed after 24 h when cocultured PuraMatrix was injected into root canals of root segments.

segments, vessel structure formation was observed after 24 h of initial encapsulation (Fig. 5P–R). This finding suggested that the prevascularized PuraMatrix/cell constructs can be transplanted *in vivo* after incubation for 24 h *in vitro*.

#### ALP activity

To investigate whether PuraMatrix provides a permissible environment for the odonto/osteogenic differentiation of DPSCs, we quantified the ALP activity in 3D mono- and



cocultures of DPSCs and HUVECs. At 7 days in coculture with HUVECs, the DPSCs displayed a significantly higher level of ALP activity than the DPSCs in monocultures ( $p < 0.05$ ) (Fig. 6E). This result confirmed that coculturing DPSCs can enhance their odonto/osteogenic differentiation in 3D and that PuraMatrix is a suitable scaffold for dental pulp regeneration.

#### *von Kossa staining to assess mineralization*

Mineralization in the cultures that were treated with odonto/osteogenic differentiation inducers was examined using von Kossa staining, which revealed a larger amount of mineralization in the cocultures compared with the monocultures (Fig. 6A–D).

#### *Pulp regeneration in vivo*

**Analysis of pulp-like tissue.** Empty root segments and root segments with PuraMatrix alone (i.e., no cells) did not contain any pulp-like tissue (Fig. 7A, B) at 4 weeks after transplantation. Interestingly, in these two groups, not even the ingrowth of mouse subcutaneous fat tissue was evident within the root canals. This result confirmed that PuraMatrix does not possess an intrinsic capability to attract endogenous cells to populate the scaffold structure.

In contrast, pulp-like tissue regeneration occurred in all three of the cell-transplantation groups (DPSC-alone, DPSC:HUVEC 1:1, and DPSC:HUVEC 1:3) within the root canal (Fig. 7C–H). *In vitro* suspended PuraMatrix/cell constructs of all three groups were successfully integrated into the host tissues and vasculature. Importantly, PuraMatrix/cell constructs that were located near the coronal end of the root, where the opening was sealed, could not survive (Fig. 7C–E). Therefore, pulp-like tissue regenerated only up to the middle (5 mm) to lower third (3.3 mm) of the root canals. In the root fragments in which the apical root opening was larger than average, ingrowing host tissue was observed to push the PuraMatrix/cell transplant toward the coronal end (Fig. 7E, H).

Osteodentin/pretentin formation was observed adjacent to the existing dentin surface in the cocultured groups (Fig. 8A, B). An odontoblast-like cell layer was observed adjacent to the existing dentin (Fig. 8C) in the regenerated pulp-like tissue. Positive immunohistochemical staining of these cells to odontoblast markers, DSP (Fig. 8E) and nestin (Fig. 8F) suggested their odontoblastic lineage. Increased vasculature was observed at the border between the transplanted tissue and host tissue (Fig. 8D).

The DPSC:HUVEC cocultured groups exhibited enhanced extracellular matrix formation with high amounts of collagen deposition compared with the DPSC-alone group, which displayed loosely arranged tissue with less cellularity and matrix material (Fig. 9A, C, H).

Positive staining with antibodies against human mitochondria confirmed that the transplanted cells contributed to the regenerated pulp-like tissue in all three groups (Fig. 9B, D, F).

**Analysis of neovascularization.** Enhanced vasculature was observed in the cocultured groups (DPSC:HUVEC 3:1 and 1:1) compared to the DPSC-only group (Fig. 9B, D, F). Quantification of the number of vascular lumens revealed

significantly more ( $p < 0.05$ ) vessels in the cocultured groups compared with the DPSC-alone group (Fig. 10D).

Immunohistochemical staining of human CD31 demonstrated HUVEC-lined vascular lumens in the cocultured groups, and as expected, none of those structures were detected in the DPSC-only group (Fig. 10A–C). The majority of the perfused vessels found in the cocultured groups were CD31<sup>+</sup>, suggesting that the HUVEC prevascular structures that had formed *in vitro* had integrated into the host vasculature (Fig. 10A, C). However, most of the vascular lumens that were located toward the periphery of the transplant and distant from the host tissues were not vascularized (Fig. 10B).

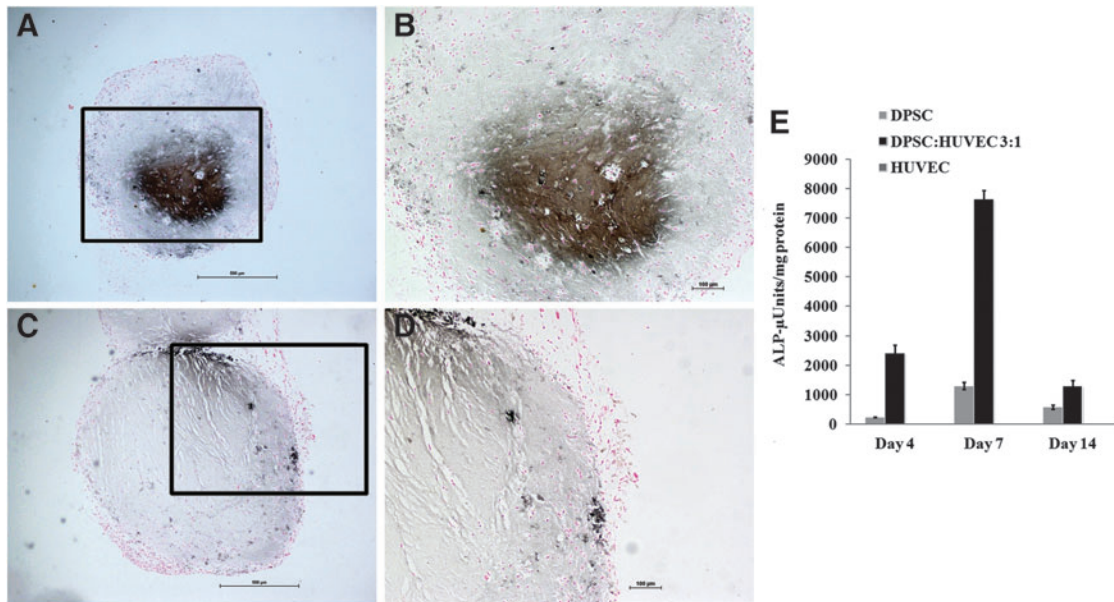
## Discussion

Scaffold materials play an important role in tissue engineering because they provide structural support for progenitor/stem cells and integrate cell–cell and cell–extracellular matrix cross-talk.<sup>7</sup> Although pulp regeneration studies have described several types of scaffolds, most of this work has focused on the odonto/osteogenic differentiation capacity of progenitor/stem cells.<sup>21,22</sup> An increasing number of studies have suggested that odonto/osteogenic and angiogenic processes are interdependent through the close interaction of stem/progenitor cells and ECs or their progenitor cells.<sup>23,24</sup> We describe here, for the first time, the feasibility of using a peptide hydrogel scaffold to regulate the functions of DPSCs and HUVECs in angiogenesis and odontogenic differentiation in mono- and coculture systems. In this model, the interactions between DPSCs and HUVECs occurred via direct cell–cell contacts and paracrine signaling. In addition, the extracellular matrix produced by the DPSCs may have also promoted the stabilization and remodeling of capillary-like structures formed by the HUVECs.

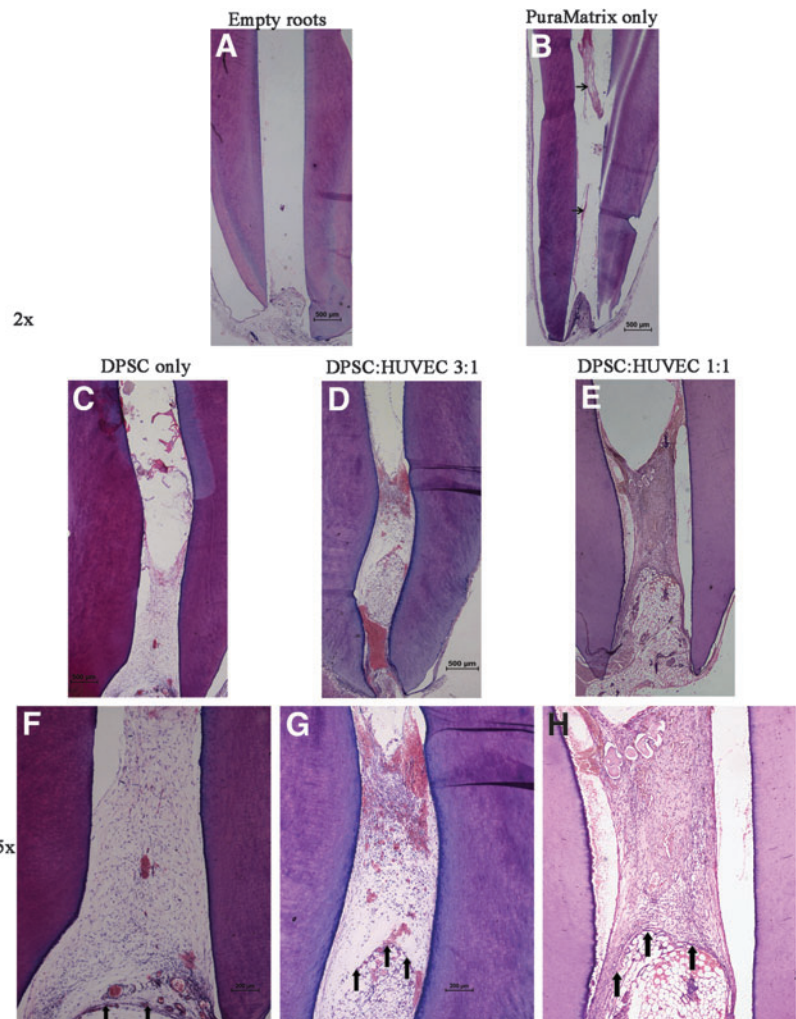
The DPSC isolation protocol used in this study was identical to that previously reported for isolation of DPSCs from human teeth.<sup>4,18,19</sup> Whole populations of low passage cells were used for all experiments rather than sorted subpopulations as these are the cells that would likely be used clinically. Therefore, the term DPSCs used in this study and in many other recently published studies does not refer to pure stem cells.<sup>4,18–20</sup> This population of cells contains immature stem/progenitor cells together with fibroblasts, ECs, and pericytes released from the pulp tissue.<sup>18</sup>

The process of angio/vasculogenesis plays a major role in the integration and engraftment of transplanted bioengineered cellular/tissue constructs. Prevascularization of the engineered tissue construct with an engineered ghost capillary network is considered a promising approach; in this approach, the construct can rapidly anastomose with the host vasculature once transplanted *in vivo*. However, it is difficult to fabricate a stable vascular network *in vitro* because ECs require specific environment elements, such as a specific pH range, signaling molecules, and growth factors, for their survival, proliferation, migration, and vascular morphogenesis.<sup>25</sup> The present report described a stable HUVEC vascular network supported by DPSCs within a peptide hydrogel scaffold, PuraMatrix, which supported the survival, proliferation, and functionalization of both types of cells.

The commercially available peptide hydrogel scaffold PuraMatrix, which was used in this study, is a synthetic

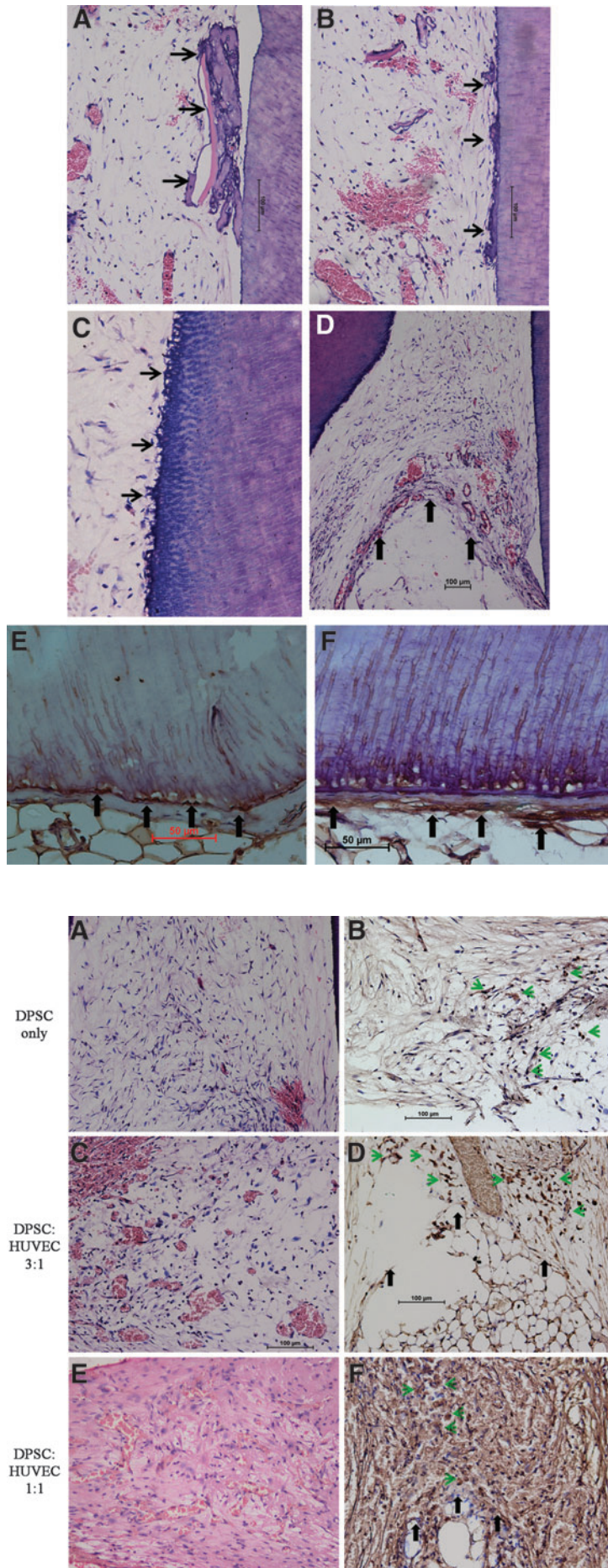


**FIG. 6.** Osteo/odontogenic differentiation in mono- and cocultures within PuraMatrix. (A–D) Mineralization in the mono- and cocultures of PuraMatrix constructs at 3 weeks of induction, as shown by von Kossa staining (*black*: calcium deposits, *pink-red*: nuclei). (A) DPSC:HUVEC 3:1 (4×), (B) magnified view of panel A (10×), (C) DPSC-alone (4×), (D) magnified view of panel C (10×). (E) Quantitative determination of ALP in mono- and cocultures of DPSCs and HUVECs in PuraMatrix constructs, demonstrating a significantly higher ALP level in the DPSC:HUVEC 3:1 culture on day 7 compared with that of the monocultures.



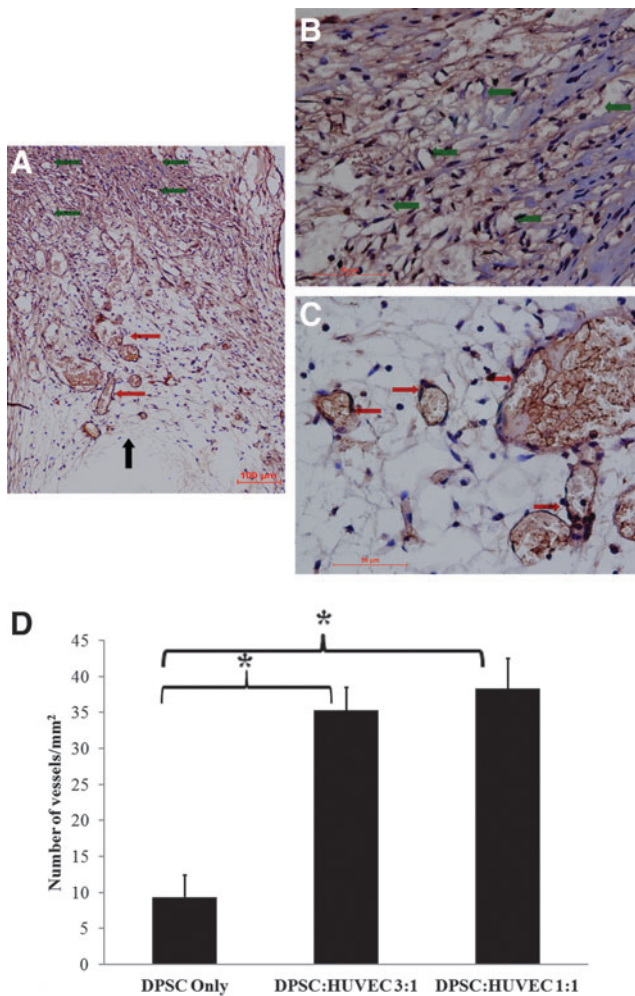
**FIG. 7.** Pulp regeneration in cell/PuraMatrix constructs *in vivo* 4 weeks after transplantation. (A) Empty roots, (B) PuraMatrix-alone in root fragments (*black arrows* indicate the remnants of PuraMatrix), (C, F) DPSC-alone in PuraMatrix, (D, G) DPSC:HUVEC 3:1 in PuraMatrix, (E, H) DPSC:HUVEC 1:1 in PuraMatrix. Vertical *black arrows* indicate the border between the transplanted tissue and host tissue.





**FIG. 8.** Pulp-like tissue within the cell/PuraMatrix transplantation groups. (A, B) Osteodentin (*black arrows*) formation adjacent to the existing dentin in a DPSC:HUVEC 3:1 coculture. (C) Odontoblast-like cells aligned with the existing dentin. (D) Increased vasculature at the border (*vertical black arrows*) between the transplant and host tissue. (E, F) Immunohistochemical staining for human DSP and Nestin respectively. *Black arrows* indicate the positively stained cells. DSP, dentin sialoprotein.

**FIG. 9.** Pulp-like tissue regenerated in the mono- and cocultured constructs *in vivo* (magnification: 20x). (A, C, E) Hematoxylin and eosin staining, (B, D, F) immunohistochemical staining of human mitochondria. The *vertical black arrows* indicate the border between the transplant and host tissue. *Green arrows* indicate the positively stained cells. DPSC-alone in PuraMatrix resulted in loosely arranged connective tissue with comparatively less extracellular matrix and vasculature (A, B). Cocultures of DPSC:HUVEC at 3:1 (C, D) and 1:1 (E, F) in PuraMatrix contained significantly more vascular lumens. Significantly increased extracellular matrix deposition was observed in the DPSC:HUVEC 1:1 group (E, F).



**FIG. 10.** Neovascularization within the regenerated pulp-like tissue. (A–C) Immunohistochemical staining of human-specific CD31 in DPSC:HUVEC 3:1 in PuraMatrix. The CD31<sup>+</sup> vascular lumens located near the host tissues were perfused (A, C). The majority of the vascular lumens near the periphery of the transplant (distant from the host tissues) were not perfused (A, B). Black arrow: border between the host tissues and transplant, Red arrows: CD31<sup>+</sup> perfused vessels, Green arrows: CD31<sup>+</sup> nonperfused vessels. Magnifications—(A) 10×, (B) 50×, (C) 50×. (D) Quantification of the number of perfused vessels within the regenerated pulp-like tissues of the different transplantation groups (\* $p < 0.05$ ).

matrix comprising a repeated polymer of four amino acids (R-A-D-A) and water. In the presence of physiologic salt concentrations, the peptide component self-assembles into a hydrogel that exhibits a nanofibrous structure. PuraMatrix forms a relatively soft fibrous matrix once 1% stock solution is diluted to achieve working concentrations. However, at concentrations lower than 0.15%, it becomes very fragile and impossible to handle and inject into root canals. Therefore, it was not possible to test concentrations lower than 0.15%. The results of the present study demonstrated that HUVECs remained viable only in the 0.15% peptide hydrogel micro-environment and contributed to the development of a capillary network when cocultured with DPSCs. The appearance of the endothelial networks that

developed in the DPSC:HUVEC cocultures was similar to that of the capillaries that formed in this material when HUVECs were cultured with 50 ng/mL VEGF, as reported previously.<sup>26,27</sup> The rationale for using 50 ng/mL VEGF is that it is the physiologically achievable level of VEGF sufficient for EC survival.<sup>28,29</sup> We fabricated the HUVEC vascular network by coculturing HUVECs with DPSCs without adding VEGF. This accomplishment indicated that DPSCs can be used as a replacement for exogenous VEGF in fabricating a HUVEC capillary network.

Interestingly, VEGF secretion by the DPSCs in the cocultures appeared to be precisely orchestrated with parallel stages of the initial angiogenic process. The angiogenic process appears to begin with the VEGF-induced activation of ECs and capillary sprouting.<sup>30</sup> The levels of the VEGF that is utilized by the ECs gradually decrease when sprouting is replaced with capillary stabilization. Consistent with this phenomenon, the results of this study indicated that the VEGF levels in the supernatants of DPSC-HUVEC cocultures were lowest during the first 48 h (i.e., when the VEGF that is secreted by the DPSCs is used by the HUVECs) and then began to increase.

It was shown that cell migration and the corresponding scaffold disruption promote capillary morphogenesis and stabilization during the early stages of vasculogenesis.<sup>31</sup> In line with this finding, our results demonstrated a coordinated migration of DPSCs and HUVECs in PuraMatrix during the first 48 h until the HUVECs became organized in a capillary network. Significantly larger and more three-dimensionally organized vessel-like structures were observed within the cocultures, in contrast to the tubular structures that were observed in the HUVEC monocultures supplied with DPSC-conditioned medium or VEGF. These findings confirmed that DPSCs facilitated vessel formation via cell migration and the corresponding scaffold disruption in addition to secreting angiogenic factors. Furthermore, DPSCs may have a regulatory function in vascular morphogenesis by changing the extracellular microenvironment via matrix deposition and metalloproteinase-mediated ECM remodeling, particularly at the later stages.<sup>32,33</sup>

Interestingly, vessel structure formation was observed after 24 h of encapsulation when the cocultures were injected into root canals of root segments. This observation may be due to the hypoxic environment within the root canals, which can be further explained based on prior studies demonstrating that hypoxia-derived elements increase VEGF transcription and subsequent vascular morphogenesis.<sup>34</sup>

Following *in vivo* transplantation, a significantly higher number of vascular lumens was observed in the cocultured groups compared with the DPSC-alone control group. Immunohistochemical labeling of human CD31 indicated that the majority of the perfused vessels in the cocultured groups were positively stained for CD31, suggesting that the HUVEC prevascular structures that had formed *in vitro* had integrated with the host vasculature. Furthermore, increased vasculature was observed at the borders of the transplant and mouse tissue, suggesting that the transplanted cells exerted a paracrine effect on the host tissues.

The levels of ALP protein and mineralization were higher in the coculture groups than in the monocultures *in vitro*. The current results also indicate that coculturing leads to increased mineralization and ECM formation



within the pulp-like tissue that regenerated *in vivo*. Furthermore, more vascular lumens were observed near the mineralizing sites within the regenerated pulp-like tissue than elsewhere. This finding indicated that the HUVECs had an inducing effect on mineralization by the DPSCs. Previous reports have demonstrated that in addition to a paracrine effect, direct cell–cell contact of HUVECs with osteoblasts stimulates ALP activity.<sup>35–37</sup> Furthermore, HUVECs were shown to secrete BMP-2, which is a mineralization/bone inducing factor.<sup>38</sup> These data explain the mineralization-promoting effects of HUVECs on DPSCs.

The period of *in vivo* transplantation used in this study was 4 weeks unlike some other studies that chose longer periods.<sup>20</sup> One of the main objectives of this study was to examine the anastomosis of *in vitro* formed prevascular structures of HUVECs with the host vasculature upon transplantation compared to the DPSC-alone group. This aspect requires to be examined at relatively early time points that is, prior to achieve vascular homeostasis within the regenerated tissue.<sup>28</sup> Furthermore, there are previous studies that used transplantation periods of 2–4 weeks and showed pulp-like tissue regeneration in similar models.<sup>17,39</sup> Therefore, 4 weeks was identified as the appropriate transplantation period to achieve the objectives of this study. Although HUVECs are a readily available source and were used in this study, HUVECs might not be an option in the clinical setting due to immune incompatibility. Potential alternatives include microvascular ECs and endothelial progenitor cells,<sup>40</sup> which have already been investigated in vasculogenic studies.

*In vivo* transplantation of PuraMatrix/cell constructs in root fragments resulted in the partial regeneration of pulp-like tissue within the root canals. The pulp-like tissue regenerated only up to the middle (5 mm) to lower third (3.3 mm) of the root canals. Because the coronal openings of the root fragments were sealed with MTA prior to transplantation, only the apical regions could attain nutrients and a vascular supply. This result suggested that although prevascularization can enhance vascularization within a cell construct, this strategy does not allow for the regeneration of full-length pulps when only the apical region is available for vascular connection. However, in the root segments in which the apical opening was larger than average, ingrowing host tissue appeared to push the PuraMatrix/cell transplant toward the coronal end. This result implies the importance of the size of the apical opening for pulp regeneration.

Taken together, the foregoing suggests the need to define a critical defect size and a critical apical opening size for pulp regeneration. This knowledge would allow for standardization in studies of regenerative therapy and a predictable outcome. For example, prevascularized PuraMatrix/DPSC constructs can be successfully used to regenerate pulp of up to 5 mm in length and can be applied for pulp regeneration in teeth with an open apex. In cases in which full-length pulp regeneration is required in a tooth with a closed root apex, we propose a multiple transplantation approach, in which 3- to 4-mm segments of pulp are regenerated at once and multiple transplantations are performed at intervals to allow vascularization of the transplanted tissues.

## Conclusion

In summary, this study described the novel approach of utilizing prevascularized PuraMatrix constructs of DPSCs for pulp regeneration. The results demonstrated that DPSCs mediated the angiogenic process *in vitro*, largely via the expression of the angiogenic factor VEGF. The results also suggested that EC behavior could be regulated by additional factors, such as the composition, and remodeling of the scaffold, all of which are in turn affected by the presence of DPSCs. Therefore, our results provide insight into the complex intercellular cross-talk between HUVECs and DPSCs in relation to angiogenesis and pulp regeneration. The results of the present study, together with those of recent *in vivo* studies of pulp regeneration<sup>17</sup> and cardiac regeneration,<sup>10,11</sup> suggest that a peptide nanofibrous environment may be uniquely appropriate as a tissue engineering scaffold for the regeneration of vascular tissues. Our findings demonstrate the significance of creating a microenvironment that supports cellular cross-talk and cell migration, which contribute to the creation of an optimal environment for dental pulp regeneration.

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## Disclosure Statement

No competing financial interests exist.

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