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In Vivo Angiogenic Capacity of Stem Cells from Human Exfoliated Deciduous Teeth with Human Umbilical Vein Endothelial Cells

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Dental pulp is a highly vascularized tissue requiring adequate blood supply for successful regeneration. In this study, we investigated the functional role of stem cells from human exfoliated deciduous teeth (SHEDs) as a perivascular source for *in vivo* formation of vessel-like structures. Primarily isolated SHEDs showed mesenchymal stem cell (MSC)-like characteristics including the expression of surface antigens and *in vitro* osteogenic and adipogenic differentiation potentials. Moreover, SHEDs were positive for NG2, α-smooth muscle actin (SMA), platelet-derived growth factor receptor beta (PDGFRβ), and CD146 as pericyte markers. To prove feasibility of SHEDs as perivascular source, SHEDs were transplanted into immunodeficient mouse using Matrigel with or without human umbilical vein endothelial cells (HUVECs). Transplantation of SHEDs alone or HUVECs alone resulted in no formation of vessel-like structures with enough red blood cells. However, when SHEDs and HUVECs were transplanted together, extensive vessel-like structures were formed. The presence of murine erythrocytes within lumens suggested the formation of anastomoses between newly formed vessel-like structures in Matrigel plug and the host circulatory system. To understand underlying mechanisms of *in vivo* angiogenesis, the expression of angiogenic cytokine and chemokine, their receptors, and MMPs was compared between SHEDs and HUVECs. SHEDs showed higher expression of VEGF, SDF-1α, and PDGFRβ than HUVECs. On the contrary, HUVECs showed higher expression of VEGF receptors, CXCR4, and PDGF-BB than SHEDs. This differential expression pattern suggested reciprocal interactions between SHEDs and HUVECs and their involvement during *in vivo* angiogenesis. In conclusion, SHEDs could be a feasible source of perivascular cells for *in vivo* angiogenesis.

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INTRODUCTION

Stem cells reside in most tissues and when tissue is injured by harmful stimuli, resident stem cells proliferate and regenerate their own organs (Rumman et al., 2015). Recently, stem cells derived from human teeth have been reported and stem cells from human exfoliated deciduous teeth (SHEDs) are one of the dental stem cells that are derived from deciduous teeth. SHEDs have bone marrow-derived mesenchymal stem cells (MSCs)-like characteristics and *in vivo* transplantation study showed that SHEDs could make dental pulp-like structures (Gronthos et al., 2000; Miura et al., 2003; Seo et al., 2004). Moreover, the beneficial paracrine effects that are involved in immunomodulation and angiogenesis have been reported. These beneficial paracrine effects will enlarge the potential clinical applications of dental stem cells into various diseases (Huang et al., 2009; Li et al., 2014; 2015; Tomic et al., 2011; Volponi et al., 2010).

Angiogenesis, the formation of capillaries from pre-existing blood vessels, is an important process in tissue engineering, especially thick engineered tissues (Isner and Asahara, 1999; Isner et al., 1996). The formation of vascular network with host circulatory system can supply nutrients and oxygen, and eliminate waste products, which increase success rate of tissue engineering (Jain et al., 2005). The angiogenic properties of MSCs is mediated by soluble angiogenic factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and stromal cell-derived factor-1α (Ishii et al., 2011; Kinnaird et al., 2004). Recently, the angiogenesis by dental pulp stem cells (DPSCs) was also demonstrated (Bronckaers et al., 2013), suggestive of their potential application into vascular diseases.

MSCs may be originated from perivascular region of blood vessel which is enriched with stem cells (Crisan et al., 2008). The location of pericytes, also called as perivascular cells is

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within blood vessels and they have similar characteristics to MSCs, which suggest the pericyte origin of MSCs (Caplan, 2008, Crisan et al., 2012; Feng et al., 2010). The pericyte-like characteristics of MSCs has been proved by the expression of multiple pericyte markers, because there are no specific markers to distinguish between MSCs and pericyte (Caplan, 2008; Crisan et al., 2008). Recently, dental pulp stem cells were reported as derived from perivascular region (Shi and Gronthos, 2003).

Dental pulp is a highly vascularized tissue, which imply the importance of *in vivo* angiogenesis for successful regeneration of dental pulp. In this study, we investigated the perivascular characteristics of stem cells from human exfoliated deciduous teeth (SHEDs) and their potential usage as an alternative source for perivascular cells to form functional microvessels *in vivo*. Primarily isolated SHEDs showed MSC-like characteristics and the expression of pericyte markers was determined. To confirm functional roles of SHEDs as perivascular cells, SHEDs were transplanted *in vivo* with or without human umbilical vein endothelial cells (HUVECs). To understand underlying mechanisms of *in vivo* angiogenesis, the expression of angiogenic cytokines/chemokines and their receptors was determined. Results of this study suggested the potential applicability of SHEDs as a perivascular source, which would contribute to *in vivo* angiogenesis in the regeneration of dental pulp.

MATERIALS AND METHODS

Primary isolation and culture

The experimental protocol was approved by the Institutional Review (S-D20070004). Informed consent was obtained from the patients. Deciduous teeth were delivered in Hank's balanced salt solution (HBSS; Welgene, Korea) supplemented with 3% antibiotic-antimycotic solution (Gibco, USA) at 4°C. Deciduous dental pulps were gently extracted with tweezers and minced in 1 mg/ml of collagenase type I and 2.4 mg/ml of dispase (Gibco) at 37°C for 1 h. Single-cell suspensions were plated and maintained in Minimum Essential Medium Alpha (α-MEM; Hyclone, USA) supplemented with 10% Fetal Bovine Serum (FBS; Hyclone) and 1% antibiotic-antimycotic solution. The medium was changed every 3 days and the cells were sub-cultured at 70% confluency. At each passage, cells were counted and photographed using an inverted microscope (Nikon Eclipse TS 100, Japan). HUVECs were purchased from Lonza and cultured in endothelial basal medium (EBM-2, Lonza) supplemented with SingleQuots (EGM-2, Lonza). All experiments were conducted at passage 6. Human Hertwig's Epithelial Rest Sheath/Epithelial rest of Malassez (HERS/ERM) cells were primarily isolated and cultured according to previous report (Nam et al., 2014).

Acidic β-galactosidase staining

Cellular senescence was analyzed using Cellular Senescence Detection Kit (Cell Biolabs Inc., USA). SHEDs at passage 3, 6, and 9 were cultured to be 70% confluent. HERS/ERM cells at passage 3 were cultured for 7 days to be confluent and used as positive control for β-gal staining. After washing twice with PBS, cells were fixed with fixing solution for 5 min at room temperature. After washing three times with PBS, cells were incubated with staining working solution for 4 h at 37°C in darkness. After washing three times with PBS, cells were observed using an inverted microscope (Nikon Eclipse TE2000-U, Japan).

FACS analysis

For fluorescence-activated cell sorter (FACS) analysis, cells were detached and washed with DPBS supplemented with 2% FBS. The antibodies were listed in Supplementary Table 1. From 100,000 to 500,000 cells were incubated with fluorescent-conjugated antibodies for 30 min on ice. After washing, cells were fixed with 4% paraformaldehyde at 4°C before analysis. Fluorescence intensity was measured on a FACS Calibur (BectonDickinson, USA), and data were analyzed using FlowJo (Tree Star, Inc., USA) software.

In vitro osteogenic and adipogenic differentiation

For osteogenic differentiation, cells were cultured to confluence. Cells were cultured for 21 days in α-MEM supplemented with 5% FBS, 10 mM β-glycerophosphate (Sigma-Aldrich, USA), 50 μg/mL L-ascorbic acid phosphate (Sigma-Aldrich), and 0.1 μM dexamethasone (Sigma-Aldrich). Medium was changed every 3 days. Alzarin red (Sigma-Aldrich) solution was used to stain calcium deposit. For adipogenic differentiation, cells were cultured to confluence. Cells were cultured for 21 days in α-MEM supplemented with 5% FBS, 50 μM indomethacin (Sigma-Aldrich), 0.5 mM isobutylmethaylxanthin (Sigma-Aldrich), 1 μM dexamethasone, and 10 μg/mL insulin (Sigma-Aldrich). Oil red O solution (Sigma-Aldrich) was used to stain lipid droplets.

Quantitative PCR (qPCR)

Total RNA was obtained from three lines of SHEDs and HUVECs using an RNeasy Mini Kit (Qiagen, USA). The total RNA (2 μg) was reverse-transcribed with M-MLV (Invitrogen TM, USA) and oligo dT during a 50 min incubation at 37°C followed by incubation for 15 min at 70°C. The cDNA was amplified in a reaction mixture (20 μl) containing 10 μl of THUNDERBIRD SYBR qPCR Mix (QPS-201, TOYOBO, Japan) and 0.25 μM of each primer (Supplementary Table 2). qPCR was performed using a CFX Connect Real-Time PCR Detection System (Biorad, USA). The copy numbers of the mRNAs were standardized to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

In vivo Matrigel plug assay

All experiments using animals followed protocols approved by the Institutional Animal Care and Use Committee of Seoul National University (SNU-1010046). Animal experiments were conducted in accordance with the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals. A total of 2.0×10^6 cells was resuspended in 200 μl of ice-cold Phenol Red-free Matrigel (BD Bioscience, USA), at ratios of 100:0, 50:50, 0:100 (HUVECs: SHEDs). Implants of Matrigel alone served as controls. The mixture was transplanted subcutaneously into the dorsal surface of 10-week-old immunocompromised beige mice (NIH-bg-nu-xid, Harlan Sprague-Dawley, USA) using a 25-gauge needle. One implant was injected per mouse. Mice were sacrificed at 7 days after injection and Matrigel plug was removed according to the previous report (Melero-Martin et al., 2007; 2008).

Immunofluorescent staining

For immunofluorescent staining, 5-μm-thick sections were deparaffinized in histoclear (National Diagnostics, Somerville, USA) and rehydrated through a series of graded alcohols and distilled water. Endogenous peroxidase activity was quenched with 10% hydrogen peroxide for 10 min, and antigen retrieval was carried out by pepsin for 10 min at 37°C. The sections were blocked for 30 min in 10% normal goat serum and incubated with primary antibodies for 1 h at room temperature. The following primary antibodies were used: rabbit anti-human CD31 (1:50; Santa Cruz Biotechnology) and mouse anti-α-smooth muscle actin (1:500; Sigma-Aldrich). Secondary anti-

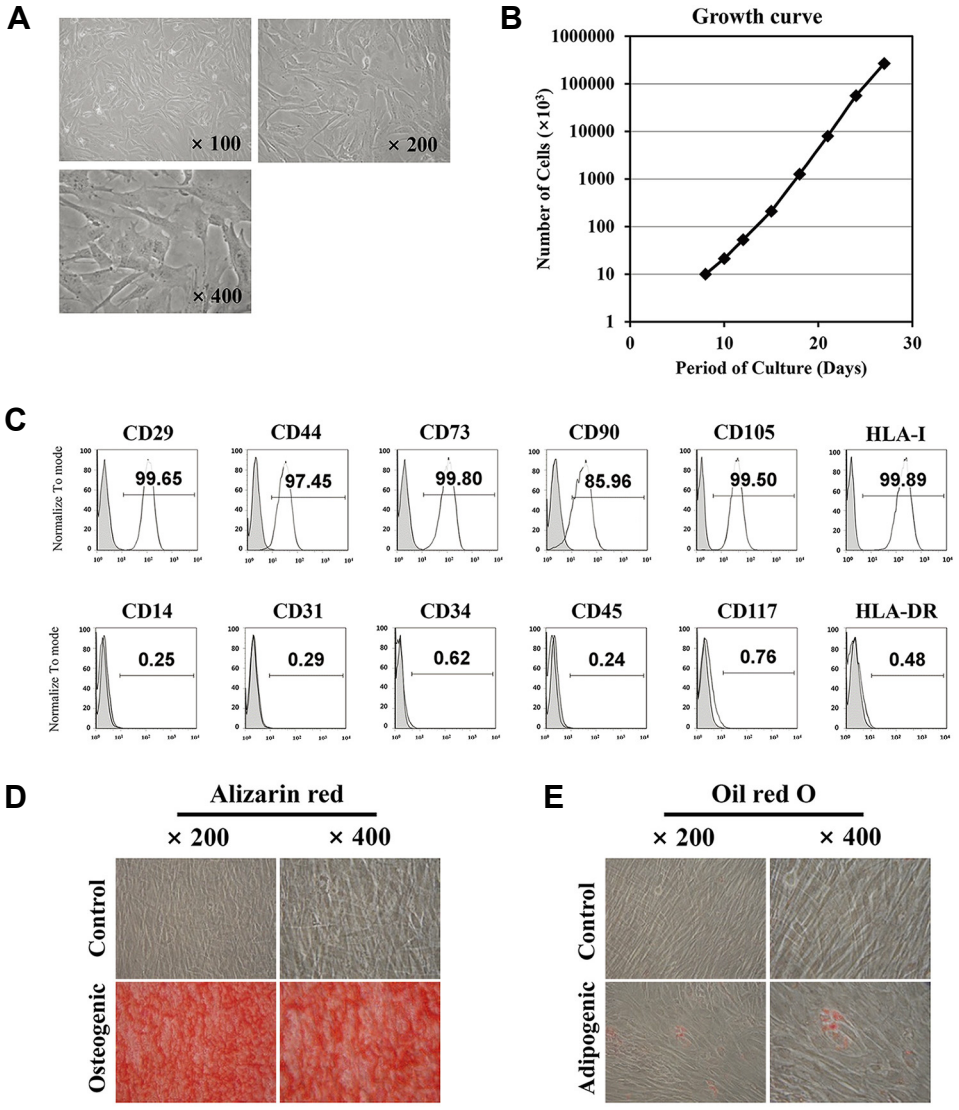


Fig. 1. Characterization of SHEDs and HUVECs. SHEDs were primarily isolated and cultured for more than 8 passages. (A) SHEDs were observed at passage 3. SHEDs showed bi-polar fibroblast morphology. (B) During culture period, the growth of SHEDs was linear. (C) FACS analysis showed that SHEDs were positive for mesenchymal markers (CD29, CD44, CD73, CD90, and CD105) and negative for endothelial marker (CD31) and hematopoietic markers (CD14, CD34, CD45, CD117, and HLA-DR). (D, E) *In vitro* differentiation of SHEDs was conducted in osteogenic and adipogenic culture condition. After 21 days, calcium deposits were stained by Alizarin red (D) and lipid vacuoles were stained by Oil red O (E).

body incubations were carried out for 1 h at room temperature using Alexa 488-conjugated goat-anti rabbit IgG (1:700; Invitrogen) and Alexa 594-conjugated goat-anti mouse IgG (1:700; Invitrogen) antibodies. All the fluorescent-stained sections were counterstained with DAPI (Sigma-Aldrich). Slides were observed using a confocal laser scanning microscope (Fluoview FV 300, Olympus, Japan).

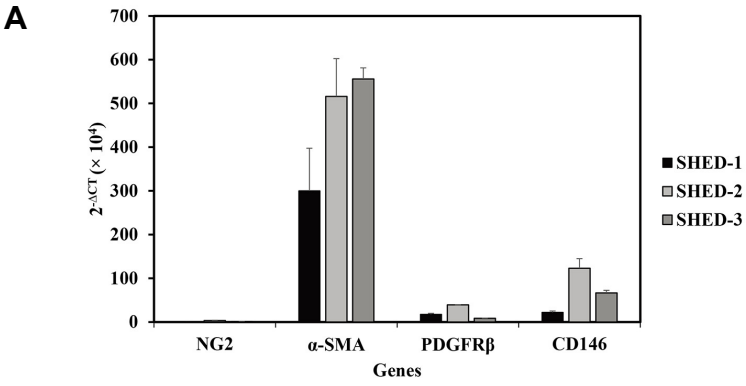
RESULTS

Primary isolation and characterization

Primarily isolated and cultured SHEDs showed typical fibroblast-like morphology (Fig. 1A). SHEDs could be grown for more than 8 passages without senescence during the culture period (Fig. 1B and Supplementary Fig. 1). The expression of surface markers was analyzed by FACS analysis. SHEDs were positive for mesenchymal cell markers (CD10, CD29, CD44, CD73, CD90, and CD105), but negative for hematopoietic cell markers (CD14, CD34) and endothelial cell marker (CD31) (Fig. 1C). When SHEDs were cultured in osteogenic or adipogenic culture conditions, we observed calcium deposits or lipid vacuoles, respectively (Figs. 1D and 1E). These data confirmed MSC-like characteristics of SHEDs.

The perivascular characteristics of SHEDs

For further characterization of SHEDs as perivascular characteristics, the expression of pericyte markers was determined by quantitative PCR (qPCR) and FACS analysis. In Fig. 2A,



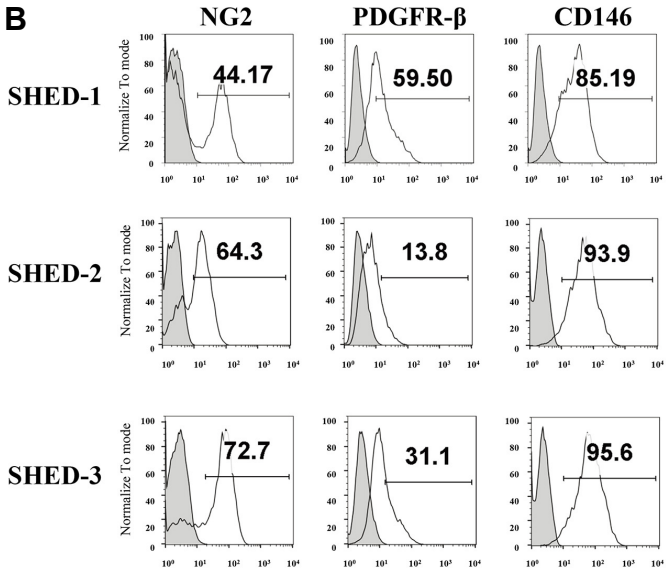


Fig. 2. The expression of pericyte markers in SHEDs. The expression of pericyte markers in three different lines of SHEDs at passage 3 were determined by qPCR and FACS analysis. (A) Three lines of SHEDs expressed NG2, α-SMA, PDGFRβ, and CD146. (B) In the results by FACS analysis, three lines of SHEDs were positive for NG2, PDGFRβ, and CD146 despite of different levels of positive peaks. However, the expression of NG2 and CD146 was subdivided or broadened, respectively.

SHEDs showed higher expression of NG2, α-smooth muscle actin (α-SMA), PDGF receptor beta (PDGFRβ), and CD146 and among them, the expression of α-SMA was highest. We could confirm the expression of NG2, PDGFRβ, and CD146 by FACS analysis (Fig. 2B). SHEDs were positive for PDGFRβ, but showed different expression pattern of NG2 and CD146. In the result of NG2, SHEDs could be subdivided into positive and negative populations. In the result of CD146, we could observe broad range of expression pattern. These data suggested that SHEDs had pericyte-like characteristics, and might be derived from perivascular region. We further characterized their functional roles as perivascular cells during *in vivo* microvessel formation.

In vivo angiogenesis

The *in vivo* angiogenic potential of SHEDs was determined through Matrigel plug assay. SHEDs were mixed with or without HUVECs in Matrigel, and the mixtures were injected subcutaneously into immunodeficient mice. After one week, Hematoxylin and Eosin staining revealed that grafts with SHEDs alone or HUVECs alone did not contain typical vessel-like structures (Fig. 3A). However, when SHEDs and HUVECs were transplanted together, robust vessel-like structures were observed in the grafts. The presence of luminal murine erythrocytes and leukocytes suggested that the newly formed vessel-like structures anastomized with host vasculature and were perfused within one week. To determine the localization of transplanted SHEDs and HUVECs, immunofluorescent staining by α-SMA and CD31 was conducted. Localization of α-SMA-positive SHEDs and CD31-positive HUVECs was surrounding vessel-like structures (Fig. 3B).

Reciprocal interaction between SHEDs and HUVECs

To investigate underlying mechanisms of *in vivo* angiogenesis, the expression of angiogenic factors and their receptors, and MMPs was verified by qPCR. SHEDs showed higher expression of VEGF, SDF-1α, and PDGFRβ than HUVECs (Fig. 4A). On the contrary, the expression of VEGFR1, VEGFR2, CXCR4, and PDGF-BB was higher in HUVECs than SHEDs (Fig. 4B). These selective expressions of angiogenic factors and their receptors suggested the reciprocal interactions between SHEDs and HUVECs. In the results of qPCR by MMPs, MMP-2 was highly expressed in SHEDs (Fig. 4C). HUVECs expressed MMP-1 and -2 than other MMPs (Fig. 4C). The expression of MMPs could be involved in the remodeling of Matrigel and migration of SHEDs and HUVECs.

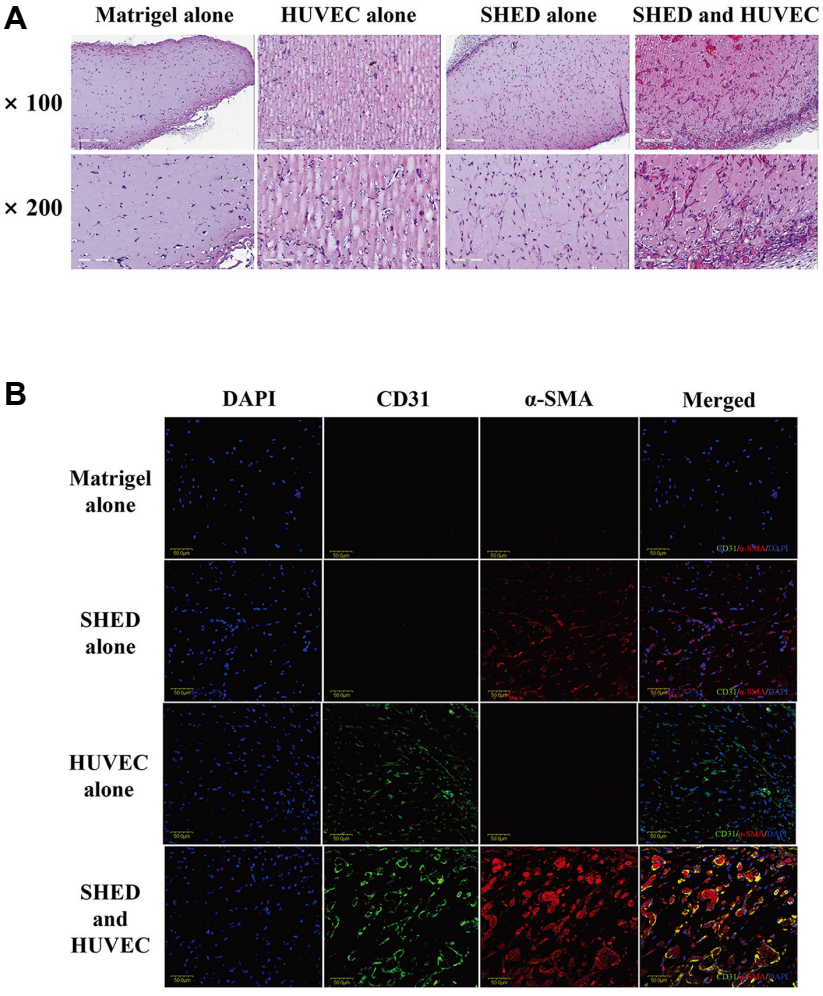


Fig. 3. *In vivo* angiogenesis. To confirm functional role of SHEDs as a perivascular source, *in vivo* Matrigel plug assay was conducted. SHEDs and HUVECs were transplanted into immunodeficient mouse separately or together, and after 7 days of transplantation, Matrigel plug was removed and analyzed. (A) The H&E-staining showed that no functional vessel-like structures in the grafts containing SHEDs alone or HUVECs alone. Functional vessel-like structures were only observed in the case of co-transplantation by SHEDs and HUVECs. The presence of luminal structures with erythrocytes suggested functional anastomoses with the host circulatory system. (B) In the results by immunofluorescent staining by α-SMA and CD31, vessel-like structures within the graft were stained positive for both human CD31 (green) and α-SMA (red). In the case of transplantation by SHEDs alone and HUVECs alone, α-SMA-positive SHEDs and CD31-positive HUVECs were observed, respectively.

DISCUSSION

Dental stem cells that can make dentin-pulp and root-periodontal complex, are emerging as sources for tissue engineering (Beck and D'Amore, 1997; Schmalz and Smith, 2014). Due to the thickness of dentin or root of teeth, blood supply into dental pulp is an utmost prerequisite for the survival of transplanted stem cells. Our results indicated that co-transplantation of SHEDs and HUVECs is a feasible solution for the regeneration of dental pulp and other regeneration processes requiring high vascularization.

Perivascular region of blood vessel is reported as a source for MSCs (Crisan et al., 2008). According to a previous report, dental pulp stem cells are localized in perivascular region and are positive for pericyte markers (Shi and Gronthos, 2003). Although SHEDs are derived from deciduous teeth, the developmental and anatomical similarity between deciduous dental pulp and adult dental pulp suggest that SHEDs also may be originated from perivascular region and have pericyte-like characteristics. We showed that SHEDs expressed pericyte markers such as NG2, α-SMA, PDGFRβ, and CD146. SHEDs expressed different expression level of pericyte markers, which implied the existence of subpopulations. Moreover, in the results of FACS analysis, the expression of NG2 was divided into two populations and CD146 was broadly expressed. In this study, we were unable to determine the relationship between subpopulation of SHEDs and the efficacy of *in vivo* angiogenesis. Sorting subpopulations and culturing according to the expression levels of pericyte markers, could lead to a better understanding of the biology of SHEDs in the future.

Pericytes are located within blood vessels and interact with endothelial cells to regulate homeostasis of blood vessels (Armulik et al., 2005; Gaengel et al., 2009). Moreover, in some reports, the relationship between pericyte and diseases has been suggested (Melero-Martin et al., 2007; Ren and Duffield, 2013). *In vivo* Matrigel plus assay was used to confirm functional pericyte-like characteristics of SHEDs. In our result, transplantation of SHEDs alone or HUVECs alone could not form vessel-like structure *in vivo*. This result is in agreement with a previous report (Melero-Martin et al., 2008). However, when SHEDs and HUVECs were transplanted together, many vessel-like structures were observed with host erythrocytes in the lumen. Co-localization of SHEDs and HUVECs in perivascular region suggested their reciprocal relationship. These data suggested that SHEDs might have pericyte-like characteristics and have a functional role as pericytes in formation of *in vivo* vessel-like structures. Moreover, the reciprocal interactions between SHEDs and HUVECs might be involved in this process.

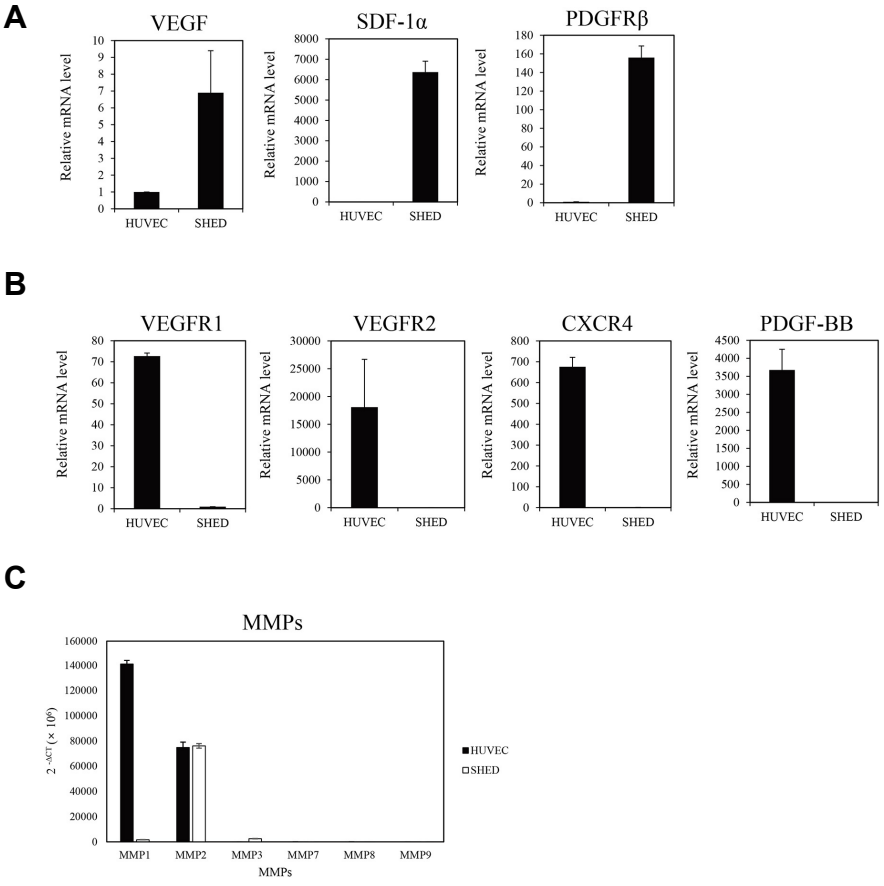


Fig. 4. The expression of angiogenic factors/receptors and MMPs. The expression of angiogenic factors/receptors and MMPs was compared between SHEDs and HUVECs by qPCR. (A) The expression of VEGF, SDF-1α, and PDGFRβ was higher in SHEDs than HUVECs. (B) On the contrary, the expression of VEGFR1, VEGFR2, CXCR4, and PDGF-BB was higher in HUVECs than SHEDs. (C) SHEDs had higher expression of MMP-2 than other MMPs; whereas, HUVECs showed higher expression of MMP-1 and -2 than other MMPs.

VEGF is a potent mitogen and chemoattractant for endothelial cells and induces the release of MMP-2, MMP-9, and MT1-MMP by endothelial cells (Beck and D'Amore, 1997). Recently, in slice chamber model, the significance of VEGF signaling was reported in dental pulp (Bento et al., 2013). PDGFB-PDGFRβ is involved in mural cells including smooth muscle cells and pericytes, which can contribute to the recruitment of pericytes (Andrae et al., 2008; Gaengel et al., 2009). SDF-1α-CXCR4 axis is well defined in angiogenesis and neovascularization (Petit et al., 2007). In the result of qPCR, VEGF, SDF-1α, and PDGFRβ were expressed highly in SHEDs. On the contrary, the expression of VEGFR1, VEGFR2, CXCR4, and PDGF-BB was higher in HUVECs than SHEDs. These selective expression patterns of angiogenic factors and their receptors suggested the reciprocal interactions between SHEDs and HUVECs during the formation of *in vivo* vessel-like structures.

The MMPs are a family of zinc-containing endopeptidases that degrade various components of the ECM. MMPs are involved in angiogenesis and metastasis of cancer (Rundhaug, 2003). MMP-2, MMP-9, and MT1-MMP have all been implicated in angiogenesis in mouse knock-out models (Fang et al., 2000; Itoh et al., 1998; Vu et al., 1998; Zhou et al., 2000). MMPs derived from SHEDs and HUVECs could contribute to *in vivo* angiogenesis. Among MMPs, MMP-2, which is important to neovascularization (Barnett et al., 2007; Cheng et al., 2007), was highly expressed in SHEDs and HUVECs. The expression of MMPs from SHEDs and HUVECs could contribute to the remodeling of extracellular matrix and their migration.

In vivo angiogenesis remains one of the obstacles that need to be overcome, especially in the field of tissue engineering, because adequate supply of nutrients and oxygen is necessary for successful regenerative therapeutics. Our study suggested that SHEDs could be used as a perivascular source to form functional vessel-like structures *in vivo*. As underlying mechanisms of *in vivo* angiogenesis, the expression of angiogenic chemokines and cytokines from SHEDs and HUVECs could be helpful for the recruitment of host blood cells to mediate proper vessel-like organization. Our work would contribute to the understanding of *in vivo* angiogenesis in tissue engineering and potential clinical applications of SHEDs.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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