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Pro-angiogenic impact of dental stem cells *in vitro* and *in vivo*

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Abstract Within the field of dental tissue engineering, the establishment of adequate tissue vascularization is one of the most important burdens to overcome. As vascular access within the tooth is restricted by the apical foramen, it is of major importance to implement effective vascularization strategies in order to recreate viable components of teeth and periodontal tissues. However, while the current regenerative approaches focus on the use of dental stem cells (DSCs), little is known about these cells and their ability to promote angiogenesis. Therefore, the present study aimed to elucidate the paracrine angiogenic properties of postnatal DSCs, in particular dental pulp stem cells (DPSCs), stem cells from the apical papilla (SCAPs) and dental follicle precursor cells (FSCs). An antibody array, together with RT-PCR and ELISA, pointed out the differential expression of pro-angiogenic as well as anti-angiogenic factors by cultured DSCs and human gingival fibroblasts (HGF-1). Despite the secretion of proliferation-promoting factors, DSCs caused no notable increase in the proliferation of human microvascular endothelial cells (HMEC-1). With regard to other aspects of the angiogenic cascade, DPSCs, SCAPs and HGF-1 significantly promoted endothelial migration in a transwell migration assay. DPSCs also had a pronounced effect on endothelial tubulogenesis, as was shown by an *in vitro* Matrigel™ assay. In the last part of this study, a chorioallantoic membrane assay demonstrated a sustained pro-angiogenic impact of DPSCs and SCAPs in an *in vivo* setting. Collectively, these data indicate a predominant pro-angiogenic influence of DPSCs and SCAPS *in vitro* and *in vivo* in comparison to FSCs, suggesting that both stem cell populations could potentially promote the vascularization of regenerated dental tissues.
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Introduction

Dental pulp tissue is a highly innervated and vascularized soft connective tissue which, while encased in dentin, appears to be very vulnerable to external insults such as trauma, chemical irritation or microbial infection (Huang, 2009). Despite the relatively high success rate of traditional endodontic treatment, which involves the replacement of the diseased or necrotic pulp by gutta percha, treatment of immature teeth in particular, remains to be a challenge as any factor that interferes with normal pulp physiology may conflict with the completion of root development (Hargreaves et al., 2013; Huang, 2008). Arrested root development following pulp necrosis can lead to weakening

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of the root structure, making the tooth susceptible to fractures and thereby reducing its survival rate (Andreasen et al., 2002; Cvek, 1992; Nosrat et al., 2014). Recently, more regenerative endodontic procedures have been introduced, involving root canal disinfection followed by the induction of a blood clot in order to promote the release of growth factors and to attract residing stromal cells (Huang, 2008; Nosrat et al., 2014). In addition, the use of appropriate scaffolds in combination with angiogenesis-promoting growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) has also been proposed and tested in animal models, although the nature of the regenerated tissues in the latter approach is often unclear due to its reliance on cell homing (Huang et al., 2013; Kim et al., 2010; Mullane et al., 2008; Suzuki et al., 2011). Furthermore, an often recurring concern in revitalization/cell homing approaches to regenerate pulp tissue is the size of the apex needed to allow proper revascularization, as vascular supply within the tooth is limited by the apical foramen (Huang et al., 2013). Multiple studies suggest a higher rate of pulp revascularization and healing with apical sizes between 1.1 and 1.5 mm (Andreasen et al., 1995; Kling et al., 1986). However, a recent study of Iohara et al. indicated the regeneration of pulp tissue in canals with only 0.7 mm apices when using a stromal cell-based method (Iohara et al., 2011). Stromal cells with similar surface characteristics as BMSCs can be found in numerous tissues within the human body, such as adipose tissue, umbilical cord, tendons and also teeth (Arana et al., 2013; Bi et al., 2007; Huang et al., 2009; Kim et al., 2013). Over a decade ago, Gronthos et al. were the first to report the presence of a stem cell population within the dental pulp (Gronthos et al., 2000). Besides dental pulp stem cells (DPSCs), dental tissues also harbor other stromal cells such as stem cells from the apical papilla (SCAPs) and dental follicle precursor cells (FSCs) (Morsczeck et al., 2005; Sonoyama et al., 2008). These cells can be relatively easy isolated from human third molars, a procedure which involves little discomfort for the patient compared to bone marrow aspiration (Hilkens et al., 2013; Huang et al., 2009; Vanhelleputte et al., 2003). Numerous studies already suggested the potential role of DSCs in the repair of diseased and damaged dental tissues, as they are capable of forming dentin–pulp complexes and periodontal tissues *in vivo* (Huang et al., 2009; Huang et al., 2008; Iohara et al., 2013; Rosa et al., 2013; Sonoyama et al., 2008). Apart from their well-known role in generating calcified tissues, little is known about the angiogenic properties of DSCs. With regard to DPSCs, a number of studies mentioned the expression of paracrine angiogenic factors such as VEGF, bFGF and platelet-derived growth factor (PDGF) under basal conditions and after injury or hypoxia (Aranha et al., 2010; Nakashima et al., 2009; Tran-Hung et al., 2008; Tran-Hung et al., 2006). Furthermore, a couple of studies have indicated the *in vitro* differentiation capacity of DPSCs into endothelial or pericyte-like cells, which could indicate the potential incorporation of these cells into newly formed blood vessels although proof of true functionality is limited at this stage (d'Aquino et al., 2007; Karbanova et al., 2011; Marchionni et al., 2009). Although SCAPs and FSCs are considered to be more immature dental stem cell populations residing in strongly vascularized tissues, literature merely indicates their expression of endostatin, bFGF and angiogenesis-regulating transcription factors such as hypoxia-inducible factor 1 alpha (HIF1A) (Guo et al., 2013; Huang et al., 2009). Despite their expression of angiogenic factors, the effects of both dental stem cell populations on endothelial cell behavior and blood vessel formation have not yet been described. Within the oral environment, an additional cell population with potential angiogenic properties can be found, namely gingival fibroblasts. In certain inflammatory and pathological conditions human gingival fibroblasts (HGF) have been known to secrete angiogenic factors such as urokinase (uPA), monocyte chemotactic protein-1 (MCP-1), interleukin 8 (IL-8) and VEGF (Ogura et al., 2001; Okada et al., 2009; Suthin et al., 2003). Furthermore, these cells are capable of promoting the proliferation of human umbilical cord vein endothelial cells (HUVECs) (Okada et al., 2009). In order to potentiate any therapeutic application of DSCs in dental tissue engineering, a more elaborate angiogenic profiling is required. In the present study, the paracrine angiogenic properties of DSCs and HGF are further elucidated by determining the angiogenic expression profile at mRNA and protein level, after which the impact of these cells on endothelial proliferation, migration and tube formation is assessed *in vitro*. Finally, a chorioallantoic membrane (CAM) assay is performed to evaluate the potential of DSCs and HGF to induce blood vessel formation *in vivo*.

Materials and methods

Cell culture

Dental tissues were obtained with informed consent from patients (15–20 years of age) undergoing extraction of third molars for orthodontic or therapeutic reasons at Ziekenhuis Maas en Kempen, Bree, Belgium. Written informed consent of patients below the age of 18 was obtained *via* their guardians. This study was approved by the medical ethical committee from Hasselt University. Immediately after extraction, dental follicles and apical papillae were gently removed from the teeth with forceps. The pulp tissue was obtained by means of forceps after mechanically fracturing the teeth with surgical chisels. Dental tissues were rinsed with Minimal essential medium, alpha modification (αMEM, Sigma-Aldrich, St. Louis) supplemented with 100 U/ml Penicillin and 100 μg/ml Streptomycin (Sigma), 2 mM L-glutamine (Sigma) (further referred to as standard DSC culture medium) containing 10% FBS (Biochrom AG, Berlin, Germany), after which dental stem cells were isolated according to the explant method as described previously by our group (Hilkens et al., 2013). Briefly, dental tissues were mechanically minced into fragments of 1–2 mm^3, which were cultured in 6-well plates in standard DSC culture medium, to allow cellular outgrowth. All DSCs were routinely screened for the expression of the following markers: CD31 (negative), CD34 (negative), CD44, CD45 (negative), CD90, CD105 and Stro-1 (data not shown). A human microvascular endothelial cell line (HMEC-1) was purchased from the Center of Disease Control and Prevention (Atlanta, GA). The cells were cultured in MCDB 131 medium (Invitrogen, Carlsbad, CA) supplemented with 100 U/ml Penicillin and 100 μg/ml Streptomycin, 10 mM L-glutamine, 10% FBS, 10 ng/ml human epidermal growth factor (hEGF, Gibco, Paisley, UK) and 1 μg/ml hydrocortisone (Sigma) (further referred to as standard endothelial culture medium). Human gingival

fibroblasts (HGF-1, CRL-2014, ATCC, Molsheim Cedex, France) were cultured in αMEM supplemented with 100 U/ml Penicillin and 100 μg/ml Streptomycin, 2 mM L-glutamine and 20% FBS (further referred to as standard HGF-1 culture medium). All cell cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. The culture medium was changed every 2–3 days and all cultures were regularly monitored with an inverted phase-contrast microscope (Nikon, Eclipse TS100, Nikon Co., Shinjuku, Tokyo, Japan). When reaching 80–90% confluence, cells were harvested using 0.05% Trypsin/EDTA (Sigma) and sub-cultured for further experiments.

Dental stem cell conditioned medium

DSCs and HGF-1 (passages 2–4) were seeded at 20,000 cells/cm^2 in standard culture medium. After 24 h of culturing, the cells were rinsed twice with phosphate buffered saline (PBS) and the medium was replaced with standard DSC culture medium containing 0.1% FBS. Following 48 h of incubation, the conditioned medium was harvested and stored at −80 °C.

Antibody array

In order to identify which angiogenesis-related proteins are secreted by DSCs and HGF-1, a general screening was performed by means of a human angiogenesis antibody array (Proteome profiler™, R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Briefly, while array membranes were blocking, conditioned medium of DSCs and HGF-1 was incubated with a detection antibody cocktail. Standard DSC culture medium supplemented with 0.1% FBS was incorporated as a negative control. After 1 h of incubation, the sample/antibody mixture was added to the array membranes for overnight incubation. After rinsing the membranes, Streptavidin–HRP was added for 30 min at room temperature. In order to visualize the results, chemiluminescent reagents (ECL plus, GE Healthcare, Little Chalfont, UK) were added to the array membranes as described by the manufacturer. Afterwards, the membranes were exposed to X-ray film during 2 min (optimal exposure time). The visualized dots were quantified using Image J Software complemented with a dot blot analyzer plug-in. This assay was performed three times on matched samples of three different donors. The results were validated by reverse transcriptase polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA).

Reverse transcriptase polymerase chain reaction

DSCs and HGF-1 (passages 2–3) were seeded at 4000 cells/cm^2 and 2000/cm^2, respectively, in standard culture medium. When reaching 80–90% confluence, the cells were recovered using 0.05% Trypsin/EDTA and cell pellets were made. Total RNA was extracted from the cell pellets as described by the RNeasy Plus Mini Kit (Qiagen). After determining the concentration and the purity of the isolated RNA by means of a spectrophotometer (NanoDrop 2000, Thermo Scientific, Pittsburgh, PA), 700 ng of RNA was reverse transcribed into cDNA using the Reverse Transcription System (Promega, Leiden, The Netherlands). cDNA was amplified through RT-PCR according to the following protocol: initialization (5 min, 94 °C), 35 cycles of denaturation (1 min, 95 °C), annealing (1 min, primer-specific Tm) and elongation (45 s, 72 °C), and additional elongation (10 min, 72 °C), after which the samples were cooled to 4 °C. Reaction mixtures (25 μl) consisted of: 1 μM of each primer, 1× PCR buffer, 200 μM dNTPs and 0.75 U of Taq polymerase (Roche Diagnostics, Basel, Switzerland). All primers were provided by Eurogentec S.A. and are listed in Table 1. Samples were separated on a 1.2% agarose gel and visualized by means of ethidium bromide. This reaction was performed on 6 different patient samples of each cell population, of which 3 representative samples are shown.

Enzyme-linked immunosorbent assay

In order to obtain a clear view of the concentration range of secreted angiogenic factors, the following ELISAs were performed on conditioned medium of DSCs and HGF-1 (passages 2–4) according to the manufacturer's instructions: angiopoietin-1 (ANGPT1) (Raybiotech, Norcross, GA), insulin-like growth factor binding protein 3 (IGFBP3) (Raybiotech) and vascular endothelial growth factor (VEGF) (Raybiotech). All ELISAs were conducted on at least 6 different patient samples of each cell population.

Immunofluorescent stainings

Immunofluorescent stainings were performed on dental tissues (pulp, apical papilla and follicle) as well as DSCs. In order to maximize the amount of available tissue, different donors were used for whole tissue and cell isolation purposes. Dental tissues were isolated as explained earlier and fixed in 4% paraformaldehyde (PFA) at room temperature. After dehydration in graded ethanol, the tissues were embedded in paraffin and serial sections of 7 μm were made. Following deparaffinization and rehydration, antigen retrieval was performed by boiling the tissue sections in 1× citrate buffer (Dako, Glostryp, Denmark). After cooling down, the tissue sections were blocked for 30 min at room temperature using 10% normal donkey serum in PBS (Millipore, Billerica, MA). With regard to the cellular stainings, DSCs were seeded at 2500 cells/cm^2 in standard DSC culture medium on glass cover slips (12 mm, Menzel GmbH, Braunschweig, Germany). When reaching 70–80% confluency, the cells were fixed in 4% PFA at room temperature. Before blocking the cells for 20 min with 10% normal donkey serum, the cells were pretreated with 0.05% Triton X-100 (Boehringer, Mannheim, Germany) in PBS for 30 min at 4 °C. Primary antibodies, namely mouse monoclonal anti-human VEGF (1:50; R&D Systems) and rabbit polyclonal anti-human CD146 (1:50; Abcam, Cambridge, UK) were added to incubate overnight at 4 °C. Matched isotype controls were used as a negative control condition. Afterwards, the sections were washed and incubated with the appropriate secondary antibodies, namely Alexa Fluor 555-labeled donkey anti-mouse antibody (1:500, Invitrogen) and Alexa Fluor 488-labeled donkey anti-rabbit antibody (1:500, Invitrogen), for 30 min at room temperature. The specificity of the secondary antibody was verified by omitting the primary antibody (data not shown). Following

Table 1
Primer designation for angiogenic factors and housekeeping genes.

Gene	GenBank accession number	Primer	Sequence	Tm (°C)	Product size (bp)
Angiogenic factors					
Angiogenin	NM_001145.4	Forward	CCT-GGG-CGT-TTT-GTT-GTT-GG	62.2	352
		Reverse	TGT-GGC-TCG-GTA-CTG-GCA-TG		
Dipeptidyl peptidase IV	NM_001935.3	Forward	GGC-ACC-TGG-GAA-GTC-ATC-GGG-A	65.6	237
		Reverse	GGG-CAG-ACC-AGG-ACC-GGA-AC		
Endothelin 1	NM_001955.4	Forward	TTG-CCA-AGG-AGC-TCC-AGA-AAC-AGC	64	206
		Reverse	ACG-GAA-CAA-CGT-GCT-CGG-GA		
Insulin-like growth factor binding protein 3	NM_001013398.1	Forward	TTG-CAC-AAA-AGA-CTG-CCA-AG	62	275
		Reverse	CAA-CAT-GTG-GTG-AGC-ATT-CC		
Pentraxin 3	NM_002852.3	Forward	TCC-CCA-TTC-AGG-CTT-TCC-TCA-GCA	65.2	277
		Reverse	ACG-GCG-TGG-GGT-CCT-CAG-TG		
Pigment epithelial-derived factor	NM_002615.5	Forward	ATC-CAC-AGG-CCC-CAG-GAT-GCA-G	65.4	235
		Reverse	GCT-CGT-GCT-GGA-TCG-CAC-CC		
Plasminogen activator inhibitor 1	NM_003256.3	Forward	ATA-CTG-AGT-TCA-CCA-CGC-CC	62.1	320
		Reverse	GTG-GAG-AGG-CTC-TTG-GTC-TG		
Tissue inhibitor of matrix metalloproteinase 1	NM_003254.2	Forward	GCT-TCT-GGC-ATC-CTG-TTG-TT TTT-GCA-	60	462
		Reverse	GGG-GAT-GG A-T A A-AC		
Tissue inhibitor of matrix metalloproteinase 4	NM_003256.3	Forward	CAA-GAG-GTC-AGG-TGG-TAA	54	446
		Reverse	ACA-GCC-AGA-AGC-AGT-ATC		
Thrombospondin 1	NM_003246.2	Forward	CAG-GGC-TCC-TGT-CGC-TCT-CCA	56.6	793
		Reverse	ACA-TTC-TGC-AGC-ACC-CCC-TGG-AA		
Urokinase	NM_002658.3	Forward	GCC-ATC-CCG-GAC-TAT-ACA-GA	59.7	417
		Reverse	AGG-CCA-TTC-TCT-TCC-TGG-GT		
Housekeeping genes					
p2-Microglobulin	NM_004048.2	Forward	CTC-ACG-TCA-TCC-AGC-AGA-GA	60	213
		Reverse	CGG-CAG-GCA-TAC-TCA-TCT-TT		
p-Actin	NM_001101.3	Forward	AAA-TCT-GGC-ACC-ACA-CCT-TC	60	185
		Reverse	AGA-GGC-GTA-CAG-GGA-TAG-CA		

counterstaining cellular nuclei with 4′,6-diamidino-2-phenylindole (DAPI, Invitrogen), all samples were incubated with 0.1% Sudan Black in 70% ethanol in order to minimize autofluorescence. Afterwards, DSCs and sections were mounted with 'fluorescent mounting medium' (Dako). Pictures were taken with a Nikon Eclipse 80i fluorescent microscope equipped with a Nikon DS-2MBWc digital camera. All stainings were performed three independent times on different donor samples and representative pictures are shown. Hematoxylin–eosin (H&E) stained tissue sections were also included in order to clearly indicate the perivascular region within the dental tissues.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

In order to determine the impact of DSCs and HGF-1 on the proliferation of endothelial cells, a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed. HMEC-1 were seeded at 15,625 cells/cm^2 in a flat bottom 96-well plate in standard endothelial culture medium. After 24 h of culturing, the cells were rinsed with PBS and incubated with either conditioned medium of DSCs and HGF-1, standard DSC culture medium containing 10% FBS (positive control) or standard DSC culture medium containing 0.1% FBS (negative control). All conditions were performed in triplicate. Following 72 h of culturing, the different conditions were replaced with 500 μg/ml MTT (Sigma) in standard DSC culture medium containing 0.1% FBS. After 4 h of incubation, the MTT solution was removed and a mixture of 0.01 M glycine (Sigma) in DMSO (Sigma) was added to dissolve the formed formazan. The absorbance was measured at a wavelength of 540–550 nm with a Benchmark microplate reader (Bio-Rad Laboratories, Hercules, CA). This assay was repeated 4 independent times on a total of at least 9 patient samples of each stem cell population.

Transwell migration assay

DSCs (passages 2–5) and HGF-1 were seeded at 50,000 cells/cm^2 in a 24-well plate in standard culture medium. After 24 h of culturing, the cells were rinsed with PBS and incubated with standard DSC culture medium containing 0.1% FBS to allow the secretion of angiogenic factors. The next day, tissue

culture inserts (ThinCert™, 8 μm pore size, Greiner Bio-One, Frickenhausen, Germany) were seeded with HMEC-1 at 150,000 cells/cm^2 in standard DSC culture medium containing 0.1% FBS and placed above the wells. Standard DSC culture medium containing 10% FBS and 0.1% FBS was used as a positive and negative control in the wells beneath. Following 24 h of incubation, transmigrated HMEC-1 were fixed with 4% paraformaldehyde (PFA) at room temperature and stained with 0.1% crystal violet in 70% ethanol. Per insert, two representative pictures were taken with an inverted phase-contrast microscope (Nikon Eclipse TS100) equipped with a ProgRes® C3 digital microscope camera (Jenoptik AG, Jena, Germany). The amount of migration (expressed as mean area percentage) was quantified using AxioVision software 4.6.3 (Carl Zeiss Vision, Aalen, Germany). This assay was independently performed on at least 12 different patient samples of each cell population.

Tube formation assay

In order to evaluate the effect of DSCs and HGF-1 on tubulogenesis, a tube formation assay was performed. The inner wells of an Angiogenesis μ-slide (Ibidi GmbH, Planegg/Martinsried, Germany) were coated with growth factor-reduced BD Matrigel™ Basement Membrane Matrix (BD Biosciences, Franklin Lakes, NJ). After the matrix had set, the outer wells of the μ-slide were seeded with a cell suspension containing 5000 HMEC-1 and conditioned medium of DSCs or HGF-1. Standard DSC culture medium containing 10% FBS and 0.1% FBS was used as a positive and negative control, respectively. The cultures were maintained for 24 h at 37 °C in a humidified atmosphere containing 5% CO_2. The next day, 2 representative pictures per well were taken with an inverted phase-contrast microscope (Nikon Eclipse TS100) equipped with a ProgRes® C3 digital microscope camera (Jenoptik AG). Per picture, 5 blood vessels were measured starting from the middle of each branching point using Image J Software. This assay was performed 5 independent times on samples of 5 different donors.

Chorioallantoic membrane assay

A chorioallantoic membrane (CAM) assay was performed in order to examine the angiogenic behavior of DSCs and HGF-1 in an *in vivo* setting. Fertilized white leghorn chicken eggs (*Gallus gallus*) were incubated for 3 days at 37 °C in a humidified atmosphere. After 3 days (E3), 3–4 ml albumen was removed from the eggs in order to detach the developing CAM from the egg shell. A small opening was made in the shell, which was covered afterwards with cellophane tape before the eggs were returned to the incubator. Six days later (E9), the CAM was incubated with Matrigel™ droplets containing 50,000 DSCs (passages 3–4) or HGF-1. Pure Matrigel™ droplets were applied as a negative control. Following 3 days of incubation (E12), the eggs were opened and the CAM was carefully dissected out of the eggs to assess angiogenesis. Pictures were taken with a stereomicroscope (Wild M3Z Stereomicroscope, Heerbrugg, Switzerland) equipped with a Nikon digital net camera DN100. In order to quantify angiogenesis, two concentric circles (radii 1.5 mm and 2 mm) were drawn and intersecting blood vessels were counted independently 2 times in a double-blind fashion. This assay was performed 4 independent times on samples of 4 different donors, leading to a total of at least 26 eggs per experimental condition.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 Software (GraphPad Software, La Jolla, CA). Data distribution was assessed by means of a D'Agostino & Pearson normality test. In the case of a Gaussian distribution, experimental groups were compared using a one way ANOVA with a Tukey–Kramer multiple comparison post-hoc test or a repeated measures ANOVA with a Newman–Keuls post-hoc test. Non-parametric data were compared with a Kruskall–Wallis test combined with a Dunns post-hoc test. Differences were considered to be statistically significant at p-values ≤ 0.05. All data were expressed as mean ± standard deviation (s.d.).

Results

Angiogenic expression profile of DSCs

The first part of this study focused on the identification of the angiogenic factors expressed by DSCs and HGF-1. In a first stage, a general screening of conditioned medium was performed using an antibody array. Dot blot analysis showed the relative expression of a wide variety of angiogenic proteins by DSCs as well as HGF-1 (Fig. 1A, arrays). The array legend can be found in supplemental Fig. 1. The following pro-angiogenic (green) and anti-angiogenic factors (red) showed no real trends towards differential expression between the different cell populations or displayed a relatively low expression pattern and were validated by RT-PCR: uPA, endothelin-1 (EDN1), dipeptidyl peptidase IV (DPPIV), angiogenin (ANG), plasminogen activator inhibitor 1 (PAI-1), thrombospondin-1 (THBS1), tissue inhibitor of matrix metalloproteinase 1 and 4 (TIMP1/4), pentraxin-3 (PTX3) and pigment epithelium-derived factor (PEGF) (Fig. 1A, graph). The entire graph can be found in supplemental Fig. 2. Visualization by means of gel electrophoresis indicated a variable expression of these factors between the different cell populations as well as between the different patient samples. Angiogenic factors of which the antibody array indicated a trend towards differential expression (Fig. 1A, graph), namely ANGPT1, IGFBP3 and VEGF, were validated through ELISA in order to obtain a clear view of the range of the secreted concentrations (Table 2). Analysis revealed no significant differences between the different cell populations in terms of ANGPT1 expression levels. With regard to the expression of IGFBP3, FSCs secreted a significantly higher concentration compared to DPSCs and SCAPs. DPSCs, on the other hand, demonstrated notably higher levels of VEGF secretion in comparison to the other cell populations (Fig. 1C). Since the expression of angiogenic factors such as VEGF can be induced by culture conditions (Potapova et al., 2007), an immunofluorescent staining was carried out in order to evaluate the *in situ* expression of this protein in dental tissues. Dental pulp, as well as apical papilla and dental follicle showed positive expression of VEGF throughout the whole tissue. Co-expression with CD146 was also detected, in particular in perivascular regions (Fig. 2A, white arrows). Cultured dental

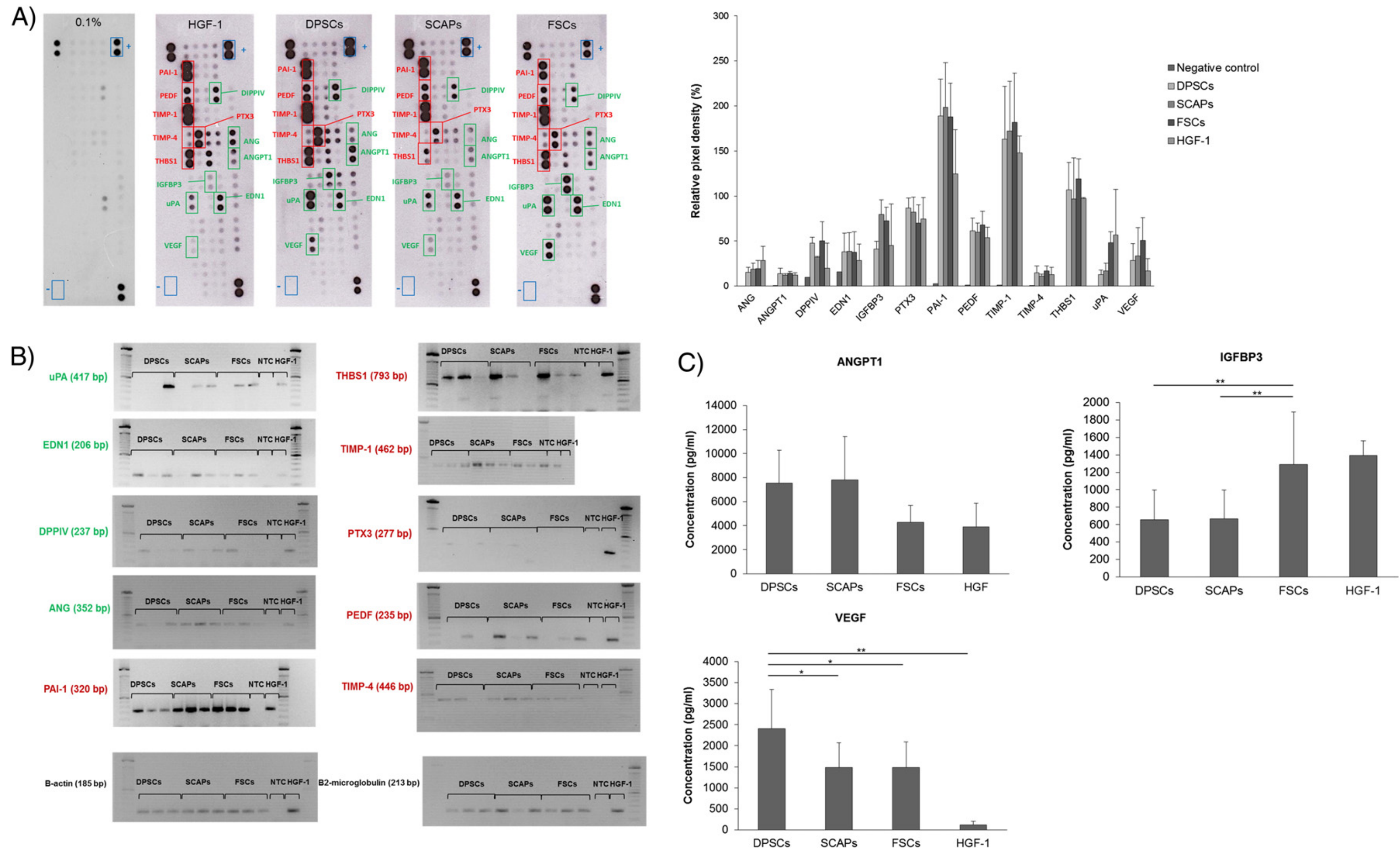

Figure 1 Angiogenic expression profile of dental stem cells. A. Detection of protein spots on the array membrane. Pro-angiogenic factors (green) as well as anti-angiogenic factors (red) were secreted in the conditioned medium of dental pulp stem cells (DPSCs), stem cells from the apical papilla (SCAPS), dental follicle precursor cells (FSCs) and human gingival fibroblasts (HGF-1). Culture medium containing 0.1% fetal bovine serum (FBS) was used as a negative control condition. The graph shows relative levels of protein secretion by the different cell populations. This assay was performed three times on matched samples of three different donors. B. Reverse transcriptase polymerase chain reaction. This assay was performed on 6 different patient samples. Three representative samples are shown. C. Enzyme-linked immunosorbent assay. ELISAs were performed on conditioned medium of at least 6 different patient samples. * = p-value < 0.05; ** = p-value < 0.01.

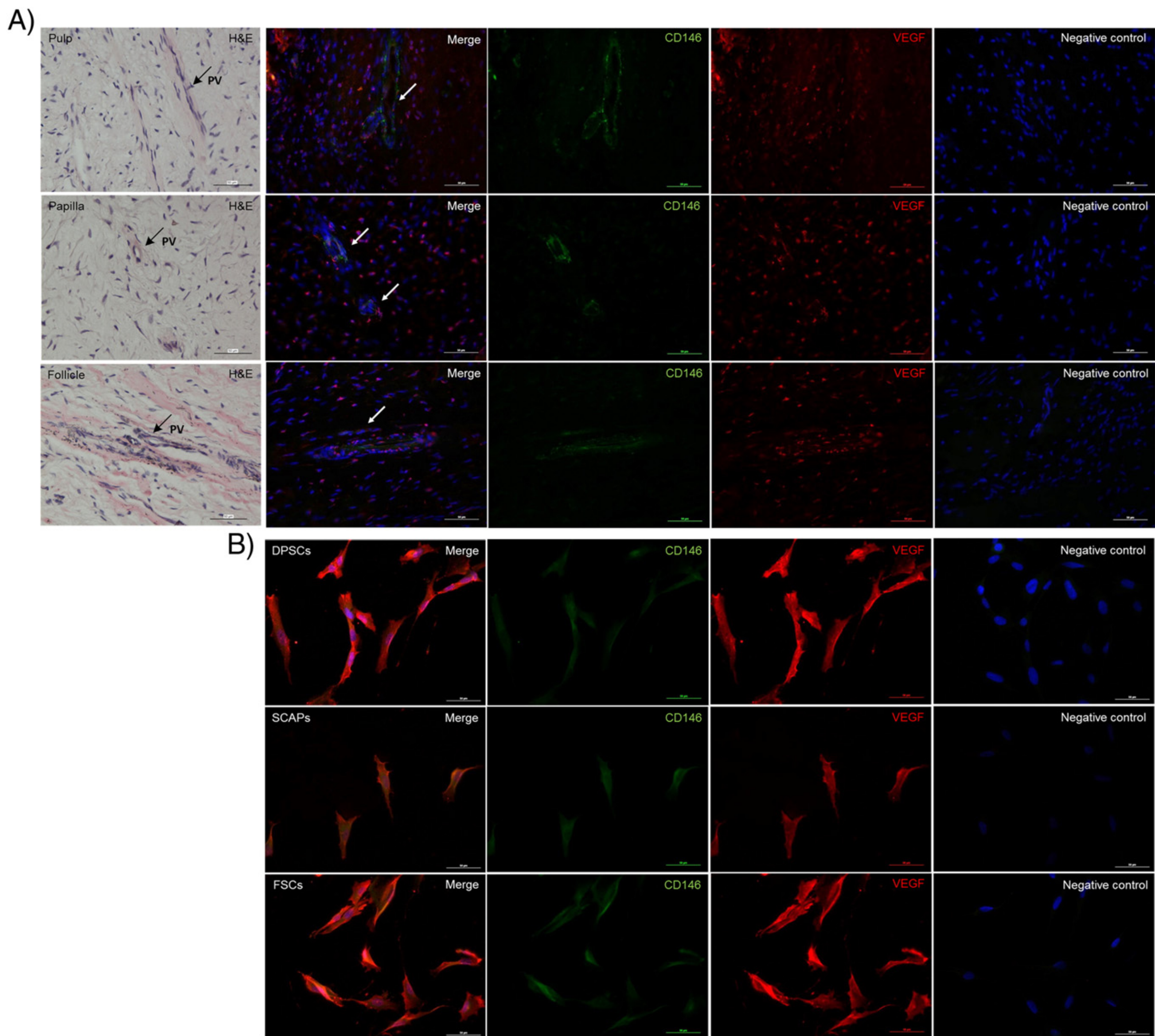

Figure 2 Expression of VEGF in dental tissues and cultured dental stem cells. A. *In situ* expression of VEGF in all dental tissues (dental pulp, apical papilla and dental follicle). Co-expression of VEGF and CD146 was mainly observed around the perivascular region (white arrows). H&E stained sections were added to indicate the perivascular region (black arrow, PV). B. Co-expression of VEGF and CD146 in dental pulp stem cells (DPSCs), stem cells of the apical papilla (SCAPs) and dental follicle precursor cells (FSCs). Negative control: matched isotype control. This staining was performed three independent times of samples of three different donors. Representative pictures are shown. Scale bars = 50 μm.

stem cells were also positive for VEGF expression as well as CD146 (Fig. 2B).

Functional analysis of the angiogenic potential of DSCs *in vitro*

As angiogenesis is a well-orchestrated biological reaction, involving a multitude of regulating factors, it is not only important to determine which of these factors are secreted by DSCs but also to assess the potential influence of DSCs on the behavior of endothelial cells. Therefore, multiple *in vitro* assays were performed, mimicking the different steps of the angiogenic process. One of the first events within the angiogenic cascade comprises the proliferation of endothelial cells. Hence, in a first stage the proliferation-stimulating capacity of DSCs was tested by means of a MTT assay. After incubating HMEC-1 with conditioned medium for 72 h, no apparent difference in proliferation was detected compared to the control conditions (Fig. 3).

During angiogenesis, endothelial cells also respond to chemotactic stimuli in order to migrate towards the site which requires vascular supply (Carmeliet, 2000). Accordingly, the chemotactic potential of DSCs was evaluated using

Table 2
Range of secreted concentrations of angiogenic factors. * = p-value < 0.05; ** = p-value < 0.01.

Concentration (pg/ml)				
Angiogenic factor	DPSCs	SCAPs	FSCs	HGF-1
Angiopoietin-1	7547 ± 2744	7S03 ± 3629	4296 ± 1380	3905 ± 1977
Insulin-like growth factor binding protein 3	656.7 ± 338.6	666.3 ± 330	1290 ± 601.4 (**)	1394 ± 166.2
Vascular endothelial growth factor	2403 ± 935 (*)	1482 ± 591.4	1482 ± 611.1	120.5 ± 82.73

a transwell migration system, which involved 24 h incubation of HMEC-1 (seeded in a culture insert) with DSCs and HGF-1 (seeded in the wells beneath) in order to induce transmigration (Fig. 4A). In particular DPSCs, SCAPs and HGF-1 caused a significant increase in endothelial cell migration (Fig. 4A, graph).

With regard to tubulogenesis, a Matrigel™ assay was applied to examine the effect of DSCs on endothelial tube formation. Following 24 h of incubation, the conditioned medium of DPSCs in particular induced a pronounced increase in tubulogenesis compared to the negative control situation. Furthermore, analysis indicated a significantly higher impact of DPSC conditioned medium compared to SCAPs and HGF-1, respectively (Fig. 4B).

Collectively, these data suggest a paracrine pro-angiogenic effect of DSCs, in particular DPSCs and SCAPs, *in vitro*.

Angiogenesis *in vivo*

To confirm whether DSCs could sustain their paracrine effects in an *in vivo* setting, a CAM assay was performed. After 3 days of incubation, a characteristic spoke wheel pattern was observed in all test samples, as the capillaries grew radially towards the different cell populations (Fig. 5A, arrows). Representative pictures were taken and two concentric circles were drawn to allow quantification of intersecting blood vessels in a double-blind fashion. Compared to the control condition, DPSCs and SCAPs caused a significant increase in blood vessel count, while this was not the case for FSCs and HGF-1 (Fig. 5B).

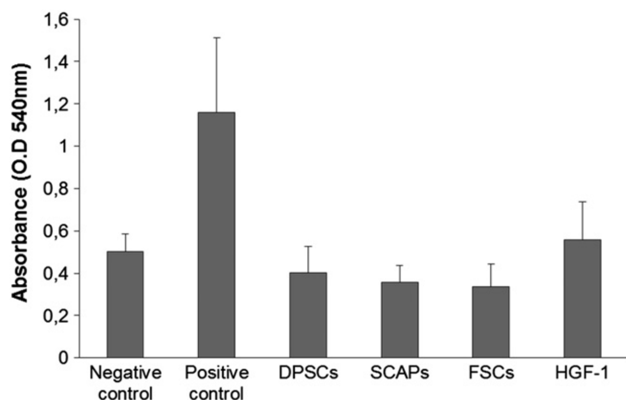

Figure 3 Cell proliferation assay. Endothelial proliferation following 72 h of incubation with conditioned medium from dental pulp stem cells (DPSCs), stem cells of the apical papilla (SCAPs), dental follicle precursor cells (FSCs) and human gingival fibroblasts (HGF-1). Negative control: culture medium containing 0.1% fetal bovine serum (FBS). Positive control: culture medium containing 10% FBS. The conditioned medium of dental stem cells as well as human gingival fibroblasts was not able to increase endothelial proliferation, as compared to the positive control medium. This assay was repeated 4 independent times on at least 9 different patient samples.

Discussion

Within the human body, three mechanisms of blood vessel formation can be distinguished, namely vasculogenesis, arteriogenesis and angiogenesis. In particular angiogenesis, *i.e.* the sprouting of new capillaries from pre-existing blood vessels, is the most predominant mechanism in the adult body (Carmeliet, 2000). Capillary sprouting does not only play an important role in physiological processes, but it also is a determining factor for the survival of transplanted cells and tissues. Especially within the field of dental tissue engineering, the establishment of adequate tissue vascularization is an important burden to overcome, as dental blood supply is restricted to the apical foramen (Huang, 2009; Huang et al., 2013). Multiple studies already indicated apical size as a key factor in pulpal healing and vascularization (Andreasen et al., 1995; Kling et al., 1986). Next to the size of the root apex, the nature of the regenerated tissue is also of major importance, an aspect which is rather ambiguous in current revitalization/cell homing-based regenerative procedures (Huang et al., 2013; Kim et al., 2010; Mullane et al., 2008; Suzuki et al., 2011). However, recent studies indicated the regeneration of organized and vascularized dental tissues after dental stem cell transplantation, even when the size of the apical foramen was limited to 0.7 mm (Iohara et al., 2013; Iohara et al., 2008; Rosa et al., 2013). Therefore, the main focus of this study was to compare the paracrine angiogenic potential of postnatal DSCs, in particular DPSCs, SCAPs and FSCs, together with a HGF-1 cell line as an additional population from the oral environment with potential angiogenic properties.

In a first part of this study, the presence of angiogenic proteins in the conditioned medium of DSCs and HGF-1 was determined by means of an antibody array. This general screening demonstrated the differential secretion of multiple angiogenesis-related factors by the studied cell populations. In-depth expression analysis at mRNA level indicated the expression of angiogenesis-stimulating (uPA, EDN1, DPPIV and ANG) as well as angiogenesis-inhibiting (PAI-1, THBS1, TIMP1/4, PTX3 and PEGF) factors by DSCs and HGF-1. A subset of these factors, namely uPA, EDN1 and THBS1, were previously reported in primary rat and human cultures

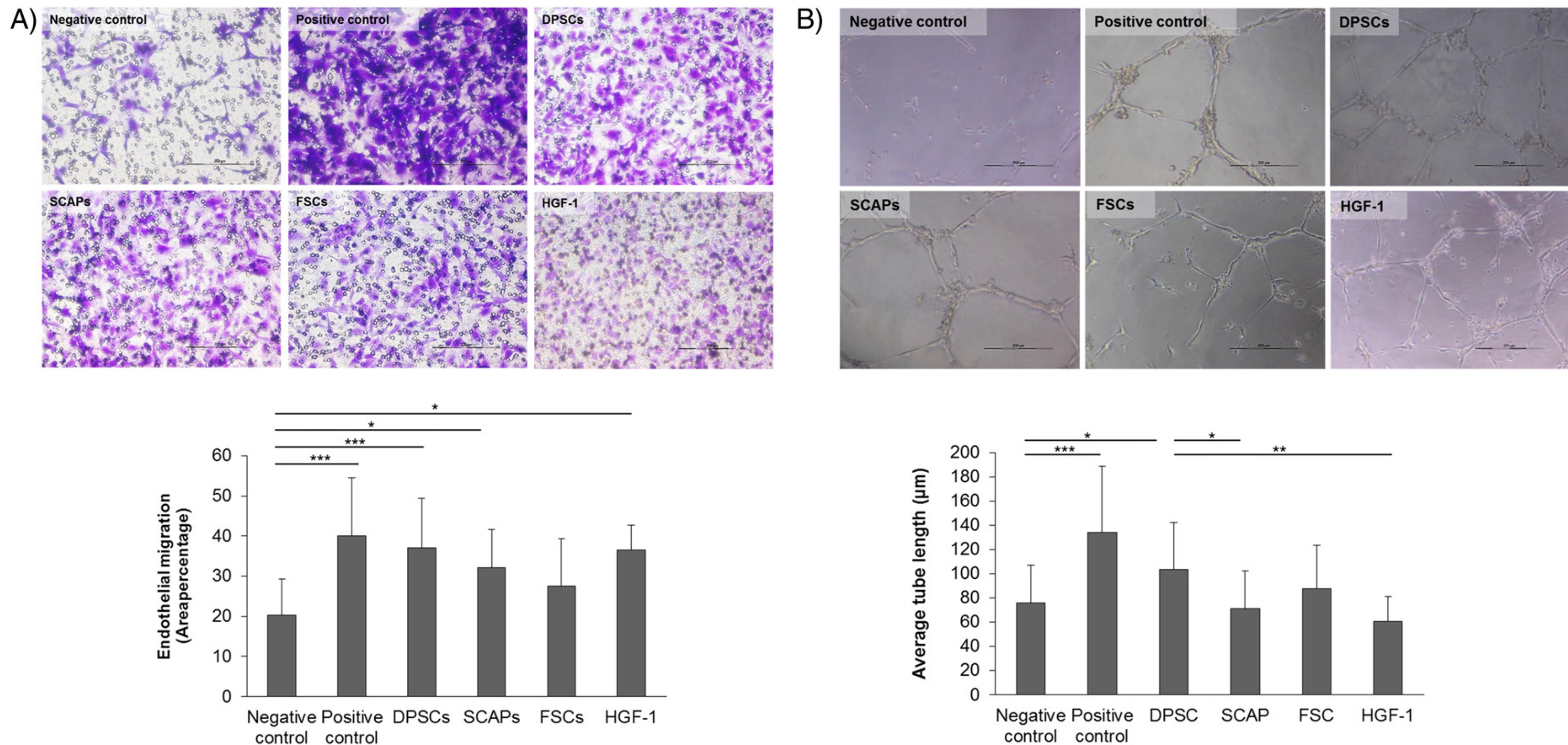

Figure 4 Impact of dental stem cells on endothelial migration and tube formation. A. Representative endothelial transmigration following 24 h of incubation with dental pulp stem cells (DPSCs), stem cells of the apical papilla (SCAPs), dental follicle precursor cells (FSCs) and human gingival fibroblasts (HGF-1). Negative control: culture medium containing 0.1% fetal bovine serum (FBS). Positive control: culture medium containing 10% FBS. Scale bars = 200 μm. The graph shows the mean area percentage of endothelial migration. DPSCs, SCAPs and HGF-1 caused a significant increase of endothelial migration as compared to the negative control medium. This assay was independently repeated on at least 12 different patient samples. B. Representative endothelial tube formation following 24 h of incubation with conditioned medium of DPSCs, SCAPs, FSCs and HGF-1. Negative control: culture medium containing 0.1% FBS. Positive control: culture medium containing 10% FBS. Scale bars = 200 μm. The graph shows the average endothelial tube length (μm). DPSC conditioned medium significantly increased endothelial tube formation. DPSCs significantly differed from SCAPs and HGF-1 regarding their impact on tubulogenesis. This assay was performed 5 independent times on matched samples of 5 different donors. * = p-value < 0.05; ** = p-value < 0.01; *** = p-value < 0.0001.

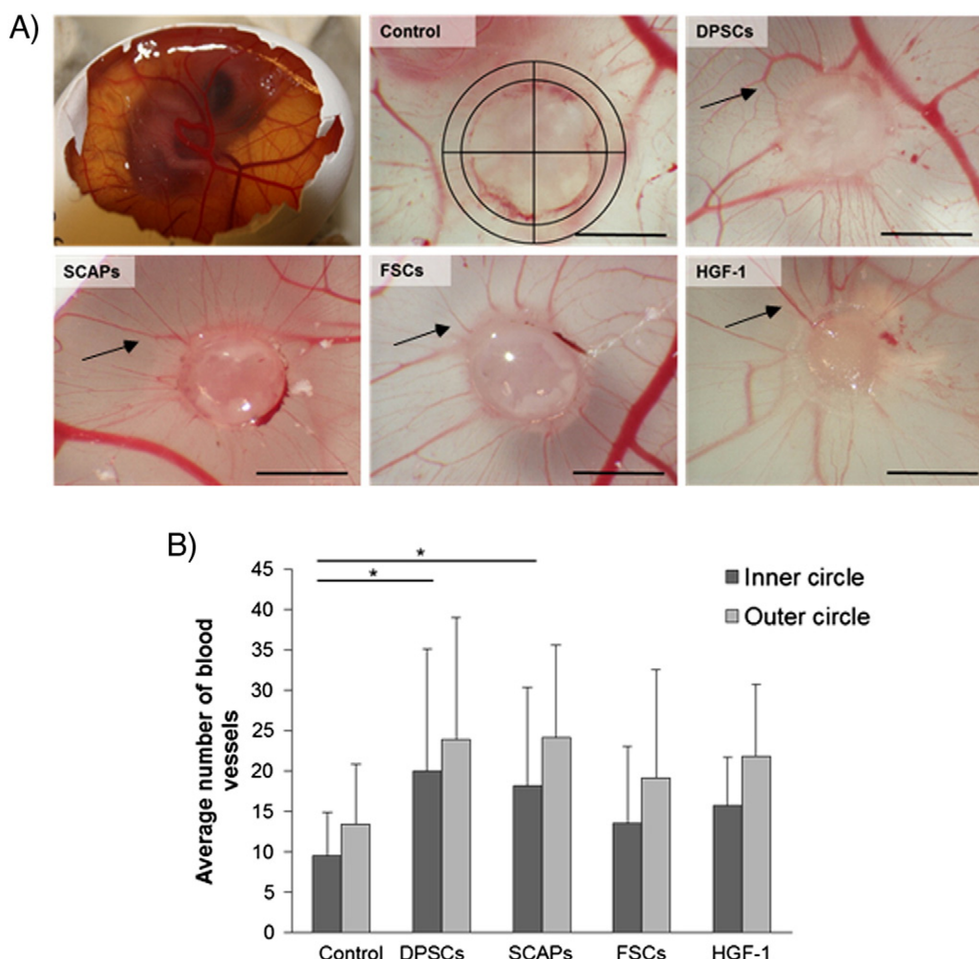

Figure 5 Chorioallantoic membrane assay. A. Representative vascularization of the chorioallantoic membrane (CAM) following 3 days of incubation with growth factor-reduced Matrigel™ containing dental pulp stem cells (DPSCs), stem cells of the apical papilla (SCAPs), dental follicle precursor cells (FSCs) and human gingival fibroblasts (HGF-1). Matrigel™ alone was used as a negative control condition. A characteristic spoke wheel pattern was observed in all test conditions (arrows). Scale bars = 2 mm. B. Average number of blood vessels. DPSCs and SCAPs significantly increased the number of capillaries intersecting both circles. This assay was performed 4 independent times on matched samples of 4 different donors, leading to a total of at least 26 eggs per experimental condition. * = p-value < 0.05.

of gingival fibroblasts (Chin et al., 2009; Koh et al., 2004; Ogura et al., 2001). A number of studies also indicated the (inflammation-induced) expression of other angiogenic factors by primary HGF-1, such as MCP-1, bFGF, ANGPT2, CXCL12 and CXCR4, some of which were also minimally detected in our antibody array (Hosokawa et al., 2005; Koh et al., 2004; Okada et al., 2009). At the protein level, ELISA showed the secretion of ANGPT1, IGFB3 and VEGF by DSCs and HGF-1. ANGPT1 primarily plays a role in tubulogenesis and vessel stabilization, while IGFB3 is thought to have a dual function; *i.e.* stimulation of angiogenesis on the one hand by promoting endothelial motility and inhibition of angiogenesis on the other hand by downregulating pro-angiogenic factors such as VEGF and bFGF (Benini et al., 2006; Granata et al., 2007; Liu et al., 2007). VEGF is a well-known protein which serves many functions within the angiogenic cascade, such as the regulation of vessel permeability, the stimulation of endothelial proliferation and migration, and the downregulation of endothelial apoptosis (Distler et al., 2003). No significant differences were demonstrated between the different cell populations in terms of ANGPT1 expression. However, VEGF was shown to be secreted at substantially high levels by DPSCs, as previously reported by others (Tran-Hung et al., 2008; Tran-Hung et al., 2006). Earlier studies also mentioned the inflammation-induced expression of VEGF by primary cultures of HGF-1 (Hosokawa et al., 2005; Suthin et al., 2003). In accordance with a previous report of Götz et al. which highlighted the expression of the IGF system in human permanent teeth, IGBP3 was secreted by all DSC populations, though at significantly higher concentrations by FSCs (Gotz et al., 2006).

With regard to the functional analysis of DSCs and HGF-1, several *in vitro* tests were performed with endothelial cells (HMEC-1) to assess their influence on the different steps of the angiogenic process. Despite the expression of multiple mitogens such as VEGF, EDN1, DPPIV and ANG, DSCs were not able to increase the proliferation of HMEC-1. A potential explanation for this discrepancy could be the concentration of the secreted proliferation-stimulating factors, which was presumably too

low to cause a sustainable effect. Another explanation may lie in the expression of proliferation-inhibiting factors, such as PTX3 and THBS1; the actions of these proteins could be predominant in comparison to the angiogenesis-promoting factors and tip the angiogenic balance towards an inhibitory state. In contrast, Iohara et al., demonstrated a significant increase of human umbilical vein endothelial cell (HUVEC) proliferation following incubation with conditioned medium of porcine pulp-derived CD31$^-$ CD146$^-$ stem cells (Iohara et al., 2008). However, besides considering the potential species-related differences between swine and human, it also has to be kept in mind that the aforementioned subset of DPSCs could potentially display more pronounced angiogenic properties than DPSCs in general (Nakashima et al., 2009). In comparison, studies regarding the proliferation-stimulating capacity of BMSCs yielded similar conflicting results. While Potapova et al. and others mentioned a significant increase in HUVEC proliferation caused by BMSCs, Gruber et al., demonstrated no proliferation-promoting effect of BMSC conditioned medium (Chen et al., 2008; Gruber et al., 2005; Kinnaird et al., 2004; Potapova et al., 2007). However, the constitution of the applied conditioned medium appears to play an important role, as the addition of fetal bovine serum can increase the expression of proliferation-stimulating factors such as VEGF and in that way bias the outcome of the experiment (Potapova et al., 2007). For that reason, the conditioned medium in this study only contained 0.1% FBS in order to avoid artificial upregulation of VEGF or other mitogens.

Another important aspect within the angiogenic cascade is endothelial migration. Since the expression analysis demonstrated that DSCs and HGF-1 express multiple factors which are known to affect migration, such as ANGPT1, EDN1, IGFBP3, uPA and VEGF, a transwell migration assay was carried out to assess their chemotactic potential. Following 24 h of incubation, DPSCs, SCAPs and HGF-1 significantly increased endothelial transmigration, while FSCs had no substantial impact. Given the high secretion of IGFBP3 by FSCs, the lack of a pronounced migration-stimulating effect was rather unexpected. However, the aforementioned dual role of IGFBP3 taken together with the lower secretion of VEGF and ANGPT1 probably established suboptimal conditions for endothelial migration. HGF-1 on the other hand, display a secretion profile similar to FSCs in terms of VEGF, IGFBP3 and ANGPT1 and do significantly enhance endothelial migration. This discrepancy can be explained by the potential contribution of other (yet to be identified) angiogenic factors which influence endothelial migration. With regard to the chemotactic properties of BMSCs, similar observations were made by Potapova et al. and others, who mentioned a significant increase in HUVEC transmigration caused by stromal cell-conditioned medium (Gruber et al., 2005; Potapova et al., 2007).

In terms of tubulogenesis, functional assays showed a pronounced effect of DPSCs on endothelial tube formation, an outcome which also differed significantly from SCAPs and HGF-1. Earlier studies of Tran-Hung et al. and others, reported a similar increase and stabilization of endothelial tubular structures following direct co-culture of HUVECs and DPSCs, indicating a more pericyte-like behavior of DPSCs (Dissanayaka et al., 2012; Janebodin et al., 2013; Tran-Hung et al., 2006). Human and murine BMSCs on the other hand, are also capable of promoting endothelial tube formation, as was shown by a number of studies (Estrada et al., 2009; Lin et al., 2012; Sorrell et al., 2009; Wu et al., 2007). Since ANGPT1 and VEGF play an important role in the induction of tubulogenesis, the aforementioned increase can probably be explained by the angiogenic secretion profile of the different cell populations as DPSCs displayed a notably higher VEGF secretion compared to SCAPs, FSCs and HGF-1.

In the last part of this study, a CAM assay was conducted in order to determine the angiogenic properties of DSCs in an *in vivo* setting. According to earlier reports of Laschke et al., the CAM assay is an ideal model to study vascular development due to its capability to support the ingrowth of blood vessels and its lack of a complete immune system which allows for the assessment of xenografts without rejection (Laschke et al., 2006). Following incubation with DSCs as well as HGF-1, a characteristic spoke wheel pattern could be distinguished caused by the radial ingrowth of blood vessels (Baiguera et al., 2012). In particular DPSCs and SCAPs significantly enhanced neoangiogenesis. In comparison, BMSCs were also found to promote blood vessel ingrowth in a CAM assay, notwithstanding the altered model the authors applied (Gruber et al., 2005; Oskowitz et al., 2011).

Despite the expression of several angiogenesis-inhibiting factors such as TIMP, PAI-1, THBS1 and PTX3, these data collectively suggest a predominant pro-angiogenic impact of DSCs, and in particular DPSCs and SCAPs, *in vitro* and *in vivo*.

Conclusion

This study was the first to describe the angiogenic properties of SCAPs and FSCs in an *in vitro* and *in vivo* setting. Furthermore, it compares three different dental stem cell populations, namely DPSCs, SCAPs and FSCs, together with a HGF-1 cell line with regard to their angiogenic expression profile and impact on endothelial cell behavior *in vitro* and *in vivo*. DSCs seemed to have a predominant pro-angiogenic impact on endothelial migration and tube formation, *in vitro* as well as in an *in vivo* set-up. Our results suggest a stronger angiogenic profile and function of DPSCs and SCAPs in comparison to FSCs and HGF-1, encouraging further investigation of both of these stem cell populations as potential therapeutic tools. The dental field can significantly benefit from the angiogenic properties of DSCs, in particular in pulp regeneration and whole tooth engineering, as vascular supply is an important burden to overcome in these applications.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2014.03.008.

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