Journal of
PERIODONTAL RESEARCH

J Periodont Res 2013; 48: 276–285

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/jre.12005

Characterization and analysis of migration patterns of dentospheres derived from periodontal tissue and the palate

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Keeve PL, Dittmar T, Gassmann G, Grimm W-D, Niggemann B, Friedmann A. Characterization and analysis of migration patterns of dentospheres derived from periodontal tissue and the palate. J Periodont Res 2013; 48: 276–285. © 2012 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd

Background and Objective: Stem cells derived from periodontal and palatal tissues may be useful for regenerative therapies of periodontal tissues. In addition to the use of single periodontium-derived stem cells (pdSCs) and palatal-derived stem cells (paldSCs), the application of pdSC and paldSC dentospheres, providing a pool of vital stem cells, may be a useful approach. As cell migration is a prerequisite for stem cells to regenerate a three-dimensional tissue environment, we characterized pdSCs and paldSCs and investigated the migratory activity of dentospheres within a three-dimensional environment. We also investigated the capacity of the dentospheres to grow on zirconium dioxide surfaces.

Material and Methods: The capacity of pdSCs and paldSCs to differentiate into the neuronal and osteogenic lineages was proved by RT-PCR and immunohistochemistry through the detection of specific lineage markers, such as alkaline phosphatase, glutamate decarboxylase 1 (also known as GAD67, the 67-kDa isoform of glutamate decarboxylase), neurofilament-M and β-III-tubulin. The expression profile of surface molecules on pdSCs and paldSCs was analyzed by flow cytometry. Adhesion and growth of pdSC/paldSC dentospheres on zirconium dioxide surfaces were determined using confocal laser-scanning microscopy. The migratory behavior of the cells was analyzed using a three-dimensional collagen matrix migration assay.

Results: Both pdSCs and paldSCs were positive for epidermal growth factor receptor, CC chemokine receptor 2 and CXC chemokine receptor 4 expression and were able to grow on zirconium dioxide surfaces. Cell-migration experiments revealed that both stem-cell populations responded similarly to epidermal growth factor (EGF), monocyte chemotactic protein 1 (MCP-1) and stromal cell-derived factor 1alpha (SDF-1α). Stimulation with EGF resulted in an increased migratory activity of both stem-cell types, whereas the locomotory behavior of the cells was impaired by both MCP-1 and SDF-1α.

Conclusion: Dentospheres represent a pool of vital pdSCs/paldSCs. As a result of the migratory activity demonstrated, along with the capacity to grow on zirconium dioxide surfaces, dentospheres may be useful for regenerative purposes in periodontal tissues.

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Key words: cell migration; dentospheres; regeneration; stem cells

Accepted for publication August 8, 2012

Stem-cell research has become an important field for tissue regeneration and implementation of regenerative medicine. In addition to the use of stem cells of various origins (adult stem cells, embryonic stem cells and induced pluripotent stem cells) for curing disease [e.g. liver cirrhosis, heart attack or neurodegenerative diseases; for a review see (1)], stem cells have also been suggested to be useful for the regeneration of dental and periodontal tissues (2–4). However, irrespective of the pluripotency of embryonic stem cells and induced pluripotent stem cells, stem cells derived from both dental and periodontal tissues are still chiefly used for regenerative purposes of dental/periodontal tissues (5–8). Dental pulp stem cells display multidifferentation potential, with the capacity to give rise to at least three distinct cell lineages: osteogenic/odontogenic cells, adipogenic cells and neurogenic cells (5,9,10). Likewise, the periodontal ligament represents a cell-renewal system in steady state. (pdSCs), exhibiting features of somatic stem cells, have been identified in a perivascular location (11–13). In addition, stem cells derived from palatal tissues (14) might be another source of stem cells suitable for the regeneration of dental/periodontal tissues. In a recent study it was demonstrated that (paldSCs) possess an increased osteogenic-differentiation capacity compared with pdSCs (4).

In addition to the differentiation capacity, the stem-cell migration activity is another important parameter to be considered in regeneration. Stem cells are locally applied to colonize a destined area and later differentiate and regenerate to a three-dimensional tissue environment; this requires the cells to have the ability to migrate – a process that is triggered and guided by growth factors, cytokines and chemokines (15–18). However, when used in regenerative periodontal therapy, pdSCs have to be applied in a three-dimensional tissue environment as spheroid clusters – so-called dentospheres – which poses the question of whether stem cells within dentospheres still have migratory activity. Therefore, in the present study we analyzed the migratory activity of pdSCs, grown as dentospheres, in response to epidermal growth factor (EGF), monocyte chemotactic protein-1 (MCP-1) and stromal cell-derived factor 1alpha (SDF-1α). In addition to pdSCs, we investigated the migratory activity of paldSCs, which have also been suggested to be suitable for use in the regeneration of dental/periodontal tissues.

Material and methods

The study protocol was approved by the Ethics Commission of the Witten/Herdecke University (Ethical Board Protocol 54/2009).

Culture of pdSCs and paldSCs

The stem-cell isolation procedure has previously been described in detail by Widera *et al.* (19). In brief, both pdSCs and paldSCs were cultured in serum-free medium [Dulbecco's modified Eagle's minimum essential medium (DMEM)/F12; Gibco, Eggenstein, Germany] containing fibroblast growth factor 2 (20 ng/mL; Chemicon, Hofheim, Germany), EGF (20 ng/mL; R&D Systems, Wiesbaden, Germany) and B27 supplement (60 μL/mL; Gibco), as described previously (19). After primary periodontium-derived dentospheres appeared at days 8–10, they were dissociated using Accutase (PAA, Pasching, Austria) to derive secondary dentospheres. The subculture protocol consisted of dentosphere passage every 3–4 d, which involved exchanging the whole culture medium and adding new growth factors.

Differentiation of pdSCs and paldSCs

Cells were differentiated into the neuronal and the osteogenic lineages, as described previously (4,19). In brief, osteogenic differentiation of pdSCs and paldSCs was achieved by seeding dissociated cells (1×10^5–2×10^5 cells) onto coverslips and subsequent culture for up to 3 wk in osteogenic differentiation medium [DMEM (PAA) supplemented with 10% fetal calf serum (PAA), 1% penicillin/streptomycin (PAA), 10 mM β-glycerophosphate, 50 μm L-ascorbic acid 2-phosphate and 100 nM dexamethasone 21-phosphate (all chemicals were purchased from Sigma Aldrich, Deisenhofen, Germany)]. Expression of alkaline phosphatase in paraformaldehyde-fixed cells was determined using FastRed (Sigma-Aldrich) staining, according to the manufacturers' instructions. FastRed staining was visualized by confocal laser scanning microscopy (Leica TCS SP5; Leica Bensheim, Bensheim, Germany). For neuronal differentiation, dissociated cells (1×10^5 cells) were cultured for 4 d in neuronal induction medium consisting of normal serum-free culture medium (DMEM/F12, B27 supplement, EGF and basic fibroblast growth factor 2) supplemented with 5 μM retinoic acid (Sigma-Aldrich). Subsequently, re-aggregated dentospheres were dissociated again and then cultured on poly-D-lysine/laminin-coated culture slides (BD Biocoat, Heidelberg, Germany) for an additional 3 d in serum-free DMEM/F12 medium containing B27 supplement. Cells were fixed with 4% paraformaldehyde (Sigma-Aldrich), permeabilized with 1% Triton X-100 (volume by volume in phosphate-buffered saline) and stained with the following antibodies: anti-GAD67 (clone 1G10.2, mouse monoclonal; Merck Millipore, Billerica, MA, USA), anti-neurofilament M (rabbit polyclonal; Merck Millipore) and β-III-tubulin (clone TU-20, mouse monoclonal; Merck Millipore). After thorough washing, cells were stained with either goat-anti-mouse or goat-anti-rabbit secondary Cy3-conjugated antibodies (both from Dianova, Hamburg, Germany). Before visualization by confocal laser scanning microscopy (Leica TCS SP5; Leica Bensheim), the DNA of fixed cells was stained with Sytox Green (Invitrogen, Karlsruhe, Germany). Undifferentiated dissociated pdSCs and paldSCs were used as controls.

RT-PCR

RNA was isolated from 1×10^6 cells (both undifferentiated control cells

and differentiated neuronal lineage cells) using the NucleoSpin® RNA II Kit from Macherey-Nagel (Macherey-Nagel GmbH, Düren, Germany), in accordance with the manufacturers' instructions. Reverse transcription of RNA into complementary DNA was performed using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, St Leon-Rot, Germany), as described in the instruction manual. PCR was performed in a 25-μL reaction mixture containing 1.25 U of *Taq* polymerase, 10× reaction buffer, 2 mM $MgCl_2$, 200 μM of each deoxyribonucleotide triphosphate (all reagents were purchased from Fermentas) and 100 pM primers (Invitrogen). The cycling conditions were as follows: an initial denaturation of 5 min at 94°C; 40 cycles of 1 min at 94°C, 1 min at the appropriate annealing temperature and 1 min at 72°C; and a final elongation for 10 min at 72°C. The primer pairs, their specific annealing temperature and product length are summarized in Table 1.

Table 1. Summary of primer pairs for PCR

Name	Annealing temperature (°C)	Mean product size(bp)	Primer	Sequence (5′ to 3′)
CD133	61	497	Forward	CACCGCTCTAGATACTGCTGTTGA
			Reverse	TGATGGACCATGGACTATAACGTG
KLF4	61	253	Forward	GCTGTGGATGGAAATTCGCC
			Reverse	TGTAGTGCTTTCTGGCTGGG
SOX2	61	278	Forward	TGCAGTACAACTCCATGACCA
			Reverse	GTGCTGGGACATGTGAAGTCT
OCT4	61	391	Forward	GTGGAGGAAGCTGCAAACA ATGAAA
			Reverse	GACCGAGGAGTTACAGTG CAGTGAAG
GAD67	61	414	Forward	CTTCCAGCCAGACAAGCAGTATGA
			Reverse	TGGGTTGGAGATGACCATCCGGAA
NF-M	62	599	Forward	TTGGACTCGCTGGGCAACCC
			Reverse	CGGATGGCCGCCTCAGTGTC
β-III-TUB	61	160	Forward	CTCAGGGGCCTTTGGACATC
			Reverse	CAGGCAGTCGCAGTTTTCAC
β-ACTIN	60	99	Forward	CAGGCTGTGCTATCCCTGTA
			Reverse	CATACCCCTCGTAGATGGGC

β-III-TUB, β-III-tubulin; GAD67, glutamic acid decarboxylase 1; KLF4, Kruppel-like factor 4; NF-M, neurofilament M; OCT4, octamer-binding transcription factor 4; SOX2, SRY (sex determining region Y)-box 2.

Aggregation of pdSCs and paldSCs on zirconium dioxide surfaces

Zirconium dioxide surfaces served as test substrates for culture of the target cells and represent one category of material commonly used in dental implantology. The aggregation behavior of target cells on the test substrates was studied using confocal laser scanning microscopy (Leica TCS SP5; Leica Bensheim) combined with a commercial staining test of cell vitality (vital dye CellTracker™ Green; Invitrogen). Therefore, after 4 d of culture, convex-shaped 7 × 7-mm^2 zirconium dioxide surfaces were placed in the culture medium and culture was continued for three more days. Then, the samples were analyzed using confocal laser scanning microscopy (Leica TCS SP5; Leica Bensheim).

Flow cytometry analysis

The periodontium-derived dentospheres were dissociated using Accutase (PAA) to obtain a single-cell suspension. Before analysis using flow cytometry, Fc receptors were blocked with FcR Blocking Reagent (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), in accordance with the manufacturers' instructions. Cells (1×10^6 cells) were stained either with unconjugated primary antibodies [which were then detected with phycoerythrin (PE)-conjugated secondary antibodies] or with PE-conjugated primary antibodies, according to the appropriate datasheets provided. The unconjugated primary antibodies (19) used were: CD133 (clone AC141; Miltenyi Biotec GmbH), CD34 (clone AC136; Pharmingen, BD Bioscience, Heidelberg, Germany), erbB2 (clone 9G6; Merck Biosciences, Darmstadt, Germany), EGFR (clone 528; Merck Biosciences), CXCR1 (clone 8F1; Santa Cruz Biotechnology, Heidelberg, Germany) and CXCR2 (clone 5E8; Santa Cruz Biotechnology). The conjugated antibodies used were: PE-CD117 (clone Ab81; Santa Cruz Biotechnology), PE-CXCR4 (Clone XY R&D Systems) and PE-CCR2 (clone 48607; R&D Systems). Isotype-matched antibodies, either unconjugated or fluorescein isothiocyanate/PE-conjugated, served as controls. Isotype controls were purchased from Pharmingen (BD Bioscience). Fluorescence-activated cell sorter analysis was performed using a FACS Calibur flow cytometer (Becton Dickinson, Heidelberg, Germany). Data analysis was performed using WinMDI software http://www.methods.info/software/flow/winmdi.html.

Cell-migration assay

Cell-migration analysis was performed in self-constructed glass chambers by applying the three-dimensional (3D) collagen matrix migration assay [for a detailed overview see (17,20,21)], with slight modifications. In brief, collagen lattices were generated by mixing 50 μL of dentosphere suspension with 100 mL of buffered collagen solution (pH 7.4), containing 1.67 mg/mL of collagen type I, in minimal essential Eagle's medium (Flow Laboratories, McLean, VA, USA). Depending on the experimental set-up, 1 μg/mL of SDF-1α (R&D Systems GmbH), 100 ng/mL of EGF or 1 μg/mL of MCP-1 (Merck Biosciences) was added to the mixture. The suspension was used to fill self-constructed glass chambers and then allowed to polymerize for 30 min at 37°C in a 5% CO_2-humidified atmosphere. The

migratory behavior of the cells in the 3D collagen matrix was recorded for 15 h at 37°C. After recording, 30 cells were selected randomly by hand, and their paths were digitized at 15-min intervals by computer-assisted cell tracking. In addition to the mean locomotory activity of the cells, the following migration parameters were analyzed: the percentage of moving cells, the period of active movement (time active), the speed (distance over time) and the distance the cells migrated (distance migrated).

Statistical analysis

One-way analysis of variance (ANOVA) was used to evaluate differences between test and control groups for each stem-cell line separately (pdSCs and paldSCs). The Shapiro–Wilk test and Levene's test were used first, to ensure the correct application of one-way analysis of variance (ANOVA). Descriptive statistics were expressed as mean ± standard deviation. Statistical analysis was performed using statistical application software (PASW Statistics (SPSS), version 18.0.0.; IBM Corp., Somers, NY, USA).

Results

Characterization of pdSCs and paldSCs

To verify the stem-cell phenotype of pdSCs and paldSCs, both cell types were differentiated into osteogenic and neuronal lineages, in accordance with previously published data (4,14,19), and were subsequently analyzed by RT-PCR and immunohistochemistry. Figure 1 summarizes the RT-PCR data. In brief, both pdSCs and paldSCs were negative for CD133 and octamer-binding transcription factor 4 (OCT4), but were positive for SRY (sex determining region Y)-box 2 (SOX2) and Kruppel-like factor 4 (KLF4) (Fig. 1), which, for CD133, OCT4, SOX2 and pdSCs, is in accordance with previous data (19). Analysis of the neuronal differentiation markers glutamic acid decarboxylase 1 (also known as GAD67, the 67-kDa isoform of glutamate decarboxylase), neurofilament M (NF-M) and β-III-tubulin revealed that both GAD67 and β-III-tubulin were already expressed in undifferentiated pdSCs and paldSCs (Fig. 1). By contrast, NF-M was found to be expressed only in pdSCs and paldSCs that were differentiated into the neuronal lineage.

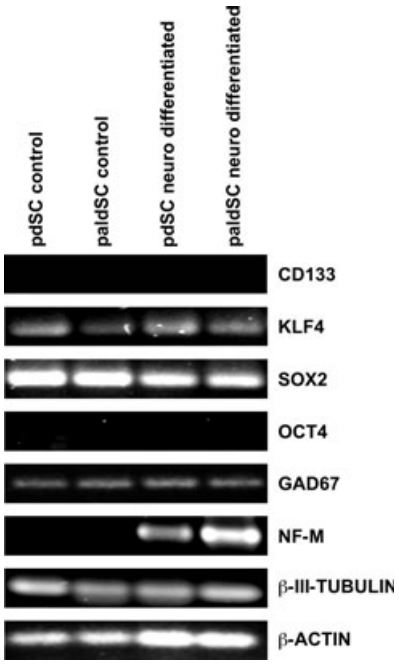

Fig. 1. RT-PCR analysis of periodontium-derived stem cells (pdSCs) and palatal-derived stem cells (paldSCs). Expression of stem-cell and neuronal differentiation markers in undifferentiated pdSCs and paldSCs and in pdSCs and paldSCs that were differentiated into the neuronal lineage was determined by RT-PCR. Both pdSCs and paldSCs showed an identical pattern of expression of the markers analyzed. Interestingly, both glutamic acid decarboxylase (GAD67) and β-III-tubulin were clearly detectable in undifferentiated pdSCs and paldSCs. KLF4, Kruppel-like factor 4; NF-M, neurofilament M; OCT4, octamer-binding transcription factor 4; SOX2, SRY (sex determining region Y)-box 2.

Immunohistochemistry, with FastRed staining, clearly showed the osteogenic-differentiation capacity of pdSCs and paldSCs (Fig. 2A,B). Interestingly, in contrast to RT-PCR data, GAD67 and β-III-tubulin were detectable only in neuronal differentiated pdSCs and paldSCs (Fig. 2C,D, G,H). Whether this discrepancy might be attributed to post-transcriptional gene repression mediated by microRNAs (miRNAs) (22) is not yet clear. NF-M expression was clearly visible in neuronal differentiated pdSCs and paldSCs (Fig. 2E,F), which is in accordance with RT-PCR data.

Flow cytometry analysis of pdSCs and paldSCs

Staining of both pdSCs and paldSCs for epidermal growth factor receptor and c-erbB-2 (both are receptors for EGF), CC chemokine receptor 2 (the receptor for MCP-1), CXC chemokine receptor (CXCR)1 and CXCR2 (receptors for interleukin-8), and CXCR4 (the receptor for SDF-1α), as well as stem cell-related marker molecules, yielded similar results (Fig. 3). Cells were positive for epidermal growth factor receptor, CC chemokine receptor 2 and CXCR4, but were negative for CD34, CD133, Stro-1, c-erbB-2, CXCR1 and CXCR2 (Fig. 3).

Aggregation of pdSCs and paldSCs on zirconium dioxide surfaces

Both cell types demonstrated a similar pattern of aggregation when cultured on zirconium dioxide surfaces. The aggregates mainly appeared as dentospheres, containing exclusively vital cells, on the zirconium dioxide surface (Fig. 4), indicating that entire dentospheres were likely to adhere to the surface. There were no signs of nonvital cells within single dentospheres. All aggregates detected by confocal laser scanning microscopy (Leica TCS SP5; Leica Bensheim) appeared to be well attached to the substrate surface. Among these aggregates, however, small cell clusters and single cells were detectable. Staining with CellTracker™ Green revealed that the attached cells were all vital (Fig. 4).

Cell-migration assay

An example of the capability of the cells to spread out of the primary dentosphere and to start to migrate

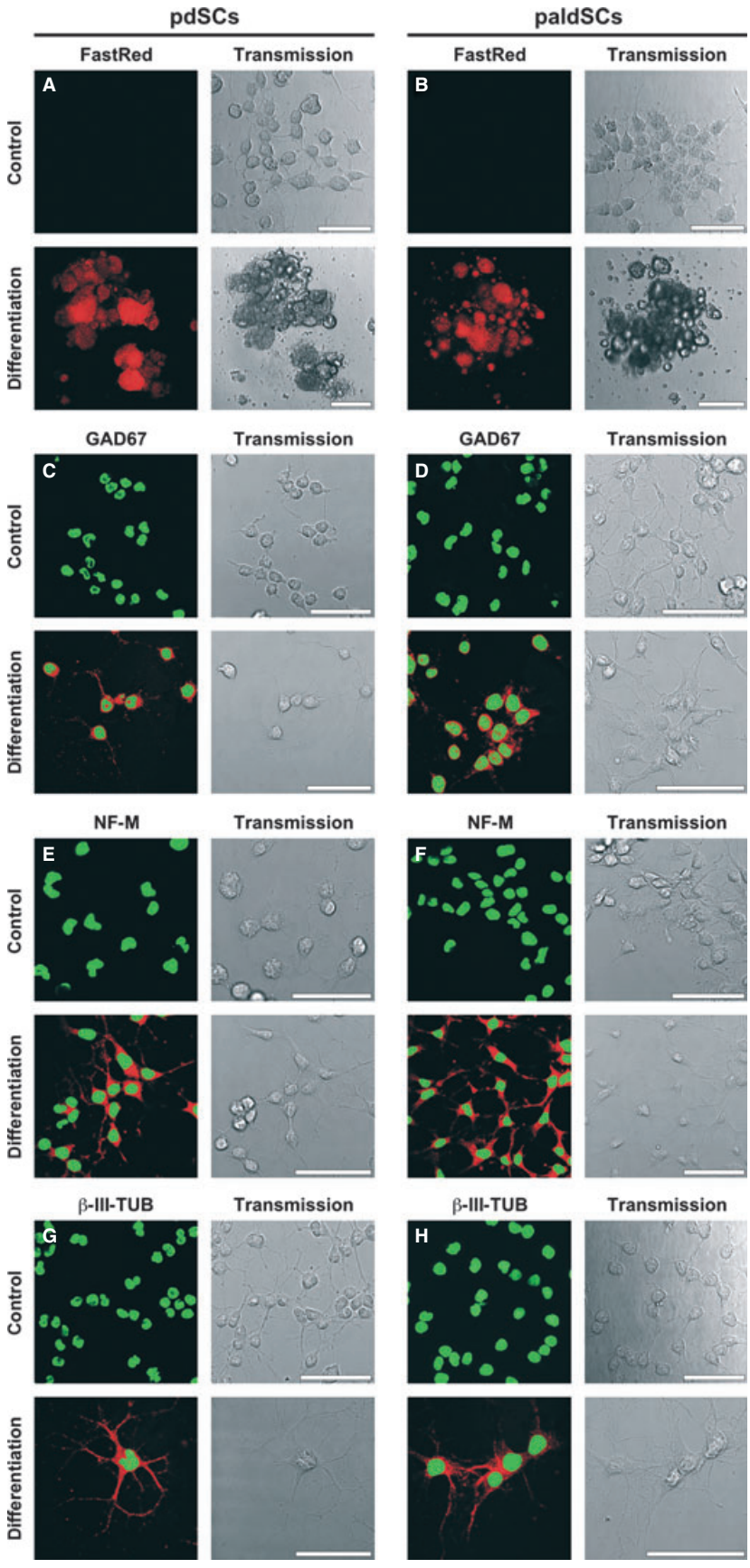

Fig. 2. Immunohistochemistry analysis of periodontium-derived stem cells (pdSCs) and palatal-derived stem cells (paldSCs). Representative images are shown of pdSCs and paldSCs that were differentiated into the osteogenic and neuronal lineages. FastRed staining of alkaline phosphatase (A, B), glutamic acid decarboxylase 1 (GAD67) (C, D), neurofilament M (NF-M) (E, F) and β-III-tubulin (β-III-TUB) (G, H). The control was undifferentiated cells. Bar = 50 μm.

into the periphery is shown in Figure 5. Both stem-cell types exhibited a similar, spontaneous migration rate of about 40% (Fig. 6A). Addition of EGF, MCP-1 and SDF-1α to both pdSCs and paldSCs yielded similar results. Both stem-cell types responded well to EGF with an increased migratory activity, but this effect was more prominent for pdSCs than for paldSCs (Fig. 6A). The migratory activity of pdSCs increased from 36.23 ± 3.10% to 48.02 ± 4.73% ($p < 0.001$; Fig. 6A) upon stimulation with EGF. By contrast, the EGF-dependent migratory activity of paldSCs was moderately (but significantly) increased to 40.66 ± 2.92% ($p < 0.05$; Fig. 6A). Nonetheless, the EGF-mediated increased locomotory activity of both stem-cell populations was attributed to both an increased number of moving cells and time of active movement (Fig. 6B,C).

In contrast to EGF, both SDF-1α and MCP-1 impaired the migratory activity of pdSCs and paldSCs: the inhibitory effect was stronger for SDF-1α than for MCP-1 and in general was more prominent for paldSCs than for pdSCs (Fig. 6A). Interestingly, the inhibitory effect of 1 μg/mL of SDF-1α on pdSCs was not caused by a decreased number of moving cells – in fact, more cells migrated in the presence of SDF-1α (control: 78.12 ± 16.77% vs. 1 μg/mL of SDF-1α: 89.19 ± 5.03%) (not significant; Fig. 6B) – but rather to a decreased time of active movement (control: 37.63 ± 2.51% vs. 1 μg/mL of SDF-1α: 30.28 ± 3.38%) (not significant;

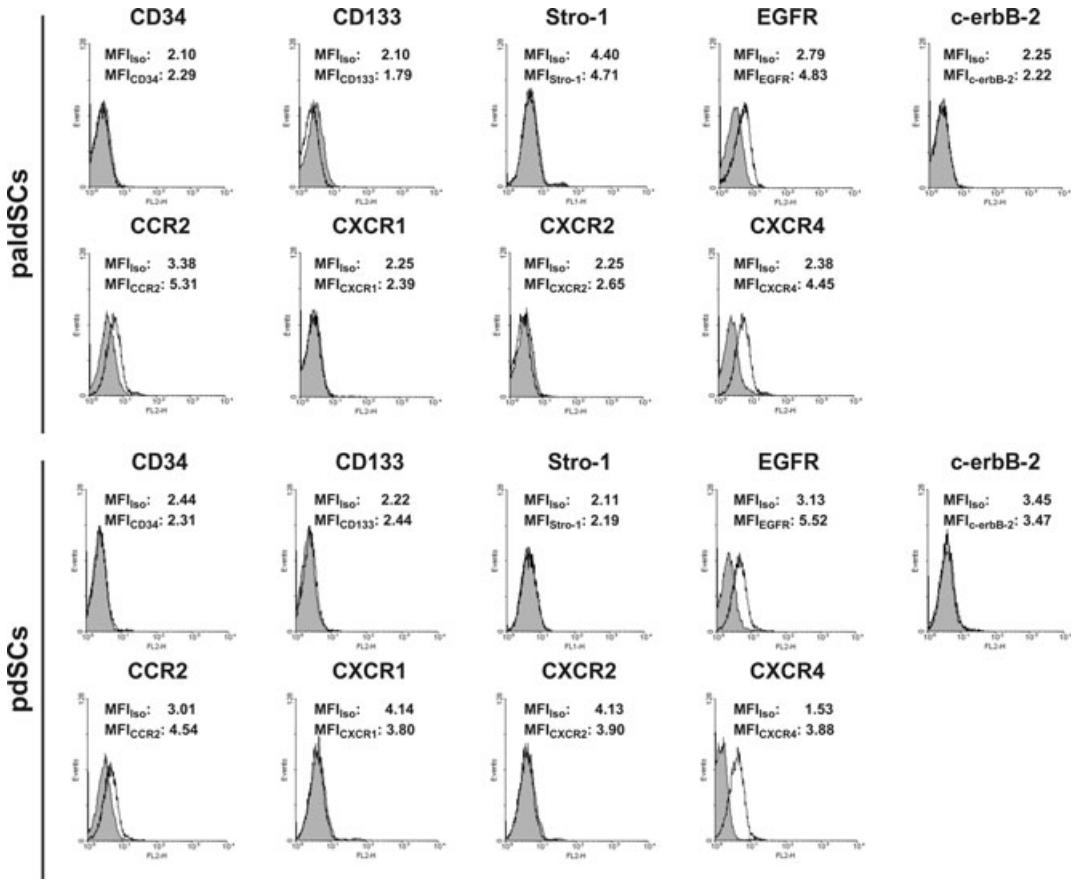

Fig. 3. Flow cytometry analysis of periodontium-derived stem cells (pdSCs) and palatal-derived stem cells (paldSCs). Representative data of three independent measurements are shown. The gray histogram represents the isotype control and the white histogram indicates expression of the appropriate target molecule. CCR, CC chemokine receptor; CXCR, CXC chemokine receptor; EGFR, epidermal growth factor receptor; MFI, mean fluorescence intensity.

Fig. 6C). By contrast, the SDF-1α-mediated migration of paldSCs was attributed to both a reduced number of moving cells and time of active movement (Fig. 6B,C).

Compared with SDF-1α, the inhibitory effect of MCP-1 on pdSCs was weaker, but still significant (control: 36.23 ± 3.10% vs. 1 μg/mL of MCP-1: 32.97 ± 4.09%) ($p < 0.05$; Fig. 6A). However, MCP-1 had a stronger inhibitory effect on paldSCs than on pdSCs. Here, the migratory activity of the paldSCs was decreased from 36.85 ± 2.70% (control) to 25.53 ± 3.10% (1 μg/mL of MCP-1) ($p < 0.001$; Fig. 6A). In contrast to SDF-1α, MCP-1 rather impaired the migration of paldSCs by decreasing the time of active movement (control: 35.35 ± 5.78% vs. 1 μg/mL of MCP-1: 24.71 ± 3.71%) ($p < 0.001$; Fig. 6C) and not by reducing the number of moving cells (control: 81.18 ± 15.99% vs. 1 μg/mL of MCP-1: 74.44 ± 9.76%) (not significant; Fig. 6B).

Discussion

The aim of the present study was to evaluate the response of the pdSCs and paldSCs, grown as dentospheres, to growth factors and chemokines. A topic of interest in the context of using these cells in a periodontally involved environment could be an application option for regenerative purposes. Such an application requires colonization of a 3D defect by progenitor cells before their immobilization in a self-autonomous manner. However, in order to retain sufficient tissue function, the locally applied stem cells have to migrate into the periphery – a process that is triggered by growth factors, chemokines and other soluble factors (15,16) – and to differentiate functionally into different tissue types.

Both stem-cell populations analyzed in this study are capable of differentiating into the osteogenic and neuronal lineages *in vitro*, which is in accordance with previously published data (4,19). Interestingly, both *GAD67* and β-III-tubulin mRNAs had already been detected in undifferentiated pdSCs and paldSCs, but not in neuronal differentiated cells. However, intron-spanning primer pairs were used for amplification of *GAD67* and β-III-tubulin, and the data indicate that undifferentiated pdSCs and paldSCs do express these neuronal markers at the mRNA level. But how do these findings relate to the immunohistochemistry data showing that neither *GAD67* nor β-III-tubulin are expressed in undifferentiated pdSCs and paldSCs? A suitable

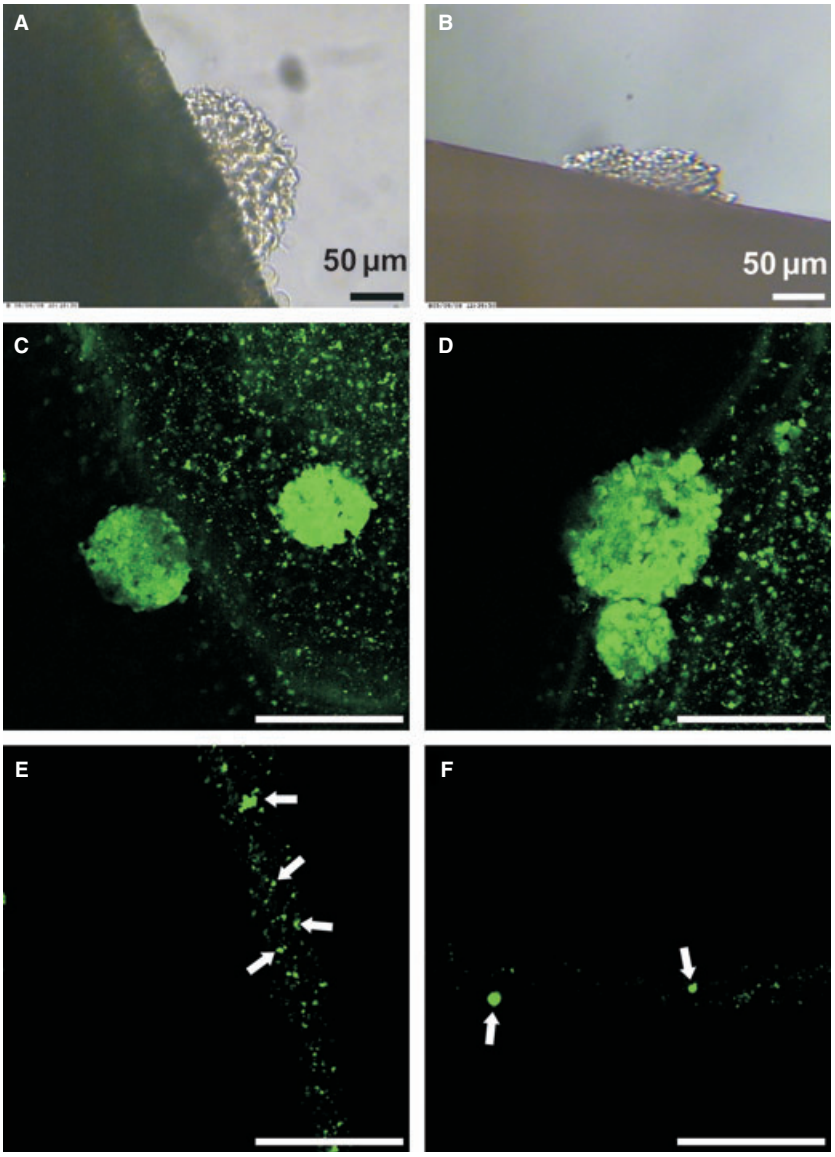

Fig. 4. Confocal laser scanning microscopy data of dentospheres grown on zirconium dioxide surfaces. Zirconium dioxide surfaces were co-cultured with dentospheres. After 3 d the viability of cells was analyzed by CellTracker™ Green staining. (A, B) Transmission light images showing representative dentospheres growing on the zirconium dioxide surface. (C, D) CellTracker™ Green-stained dentospheres growing on the zirconium dioxide surface. Owing to the cross-reactivity of CellTracker™ Green with the surface, the latter is also slightly stained. However, dentospheres can be clearly distinguished from the surface. (E, F) Single cells and small cell clusters located on the zirconium dioxide surface (marked by an arrow). Representative images of periodontium-derived stem cell (pdSC) dentospheres and single cells are shown. Identical data were obtained for palatal-derived stem cells (paldSCs) (data not shown). Bar = 50µm.

explanation for this observation might be that the translation of *GAD67* and β-III-tubulin mRNAs is repressed by miRNAs in undifferentiated pdSCs and paldSCs (22). In fact, many studies demonstrated that *in vitro* transient overexpression or inhibition of brain-specific miRNAs in stem cells significantly directed differentiation along neuronal cell lineages (23), revealing the impact of miRNAs on stem-cell differentiation.

We have recently demonstrated the efficacy of pdSCs to differentiate into periodontal ligament-like tissues, elements of bone and osteocyte-lacunae in the bone tissue of an athymic rat model after 6 wk (4). However, in this previous study, single-cell suspensions of pdSCs, rather than dentospheres, were used for coating collagen sponges. Because dissociation of dentospheres might be associated with an unwanted loss of stem cells and consequently a putative decrease in regenerative efficacy, we investigated whether dentospheres themselves could be used for regeneration.

The potential use of dentospheres for regenerative purposes has the prerequisites that the stem cells within the cell cluster are migratory active and start to spread out of the dentosphere after application. In this study, the effects of EGF, MCP-1 and SDF-1α were investigated because both stem-cell populations were positive for the corresponding receptors. Both MCP-1 and SDF-1α are up-regulated in response to tissue degeneration and inflammation. MCP-1 triggers the recruitment of monocytes and their transendothelial migration (24,25), whereas SDF-1α has been associated with the stress-induced recruitment of human $CD34^+$ haematopoietic stem and progenitor cells (HSPCs) in the liver (26) and in the heart tissues after myocardial infarction (27). Likewise, EGF is an important growth factor that mediates the migration, infiltration, proliferation and differentiation of several cell types during wound-healing processes (28).

Our data showed that pdSCs and paldSCs spread out of the dentospheres in a radial manner that is dependent on the supplied factors (EGF, MCP-1 and SDF-1alpha), indicating that the dentospheres consist of viable cells. Interestingly and unexpectedly, both chemokines (SDF-1α or MCP-1) impaired the locomotory activity, whereas EGF induced the migratory activity, of both cell types in this experiment. Our findings seem to be contradictory to data published by Widera *et al*. (19). In their work, the migration of previously dissociated pdSC dentospheres was successfully induced by 20 ng/mL of SDF-1α and 20 ng/mL of MCP-1

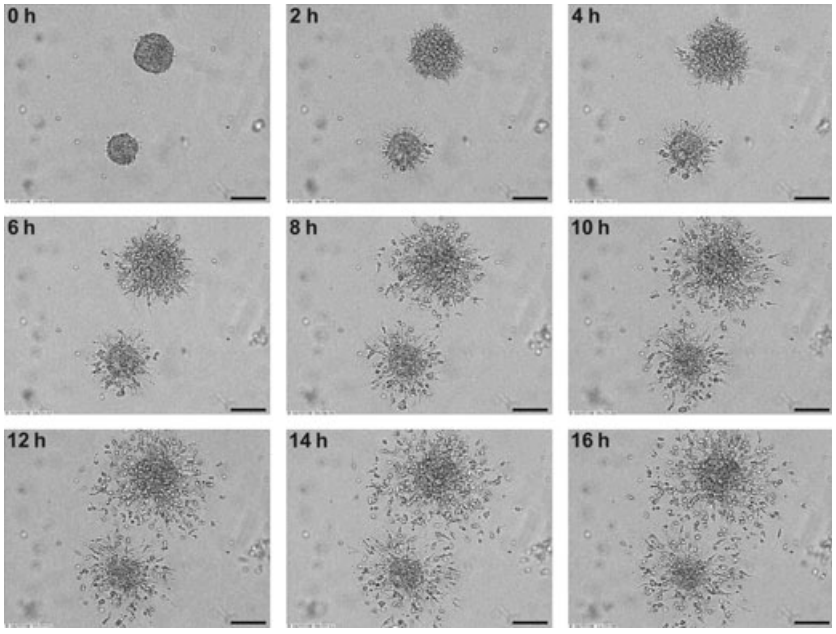

Fig. 5. Screen shot images of a time-lapse film of two dentospheres embedded within a three-dimensional collagen matrix. Screen shots were taken every 2 h (in real time) from the time-lapse film. After 2 h single cells have already started to migrate out of the dentosphere into the periphery. Representative data for both periodontium-derived stem cells (pdSCs) and palatal-derived stem cells (paldSCs) are shown. Bar = 100μm.

(19). However, it has to be taken into account that a direct comparison of our study with that of Widera *et al.* cannot really be performed because different cell-migration assays were used that differ in fundamental properties. Widera and colleagues used a modified Boyden chamber/Transwell assay for their work. Even though this assay is well established and widely used, it has some limitations, probably because of its simplicity. For instance, only those cells that have moved into the lower compartment are considered for analysis, whereas migratory active cells that remain in the upper compartment are neglected. Likewise, because of the experimental set-up, the modified Boyden chamber/Transwell assay is not suitable for studying cell migration in real time at a single-cell level. This was why we chose the 3D collagen matrix migration assay. As a result of the continuous monitoring, the migration of single cells (and even of cell clusters) could be resolved in real time, which is essential in the context of studying the migration of single pdSCs and paldSCs out of a dentosphere.

Nonetheless, the diametral results of the migratory activity of pdSCs in the study of Widera *et al.* (19) and in this work remain ambiguous. One possible explanation may be the culture conditions. In previous studies we demonstrated that the migratory behavior of cells is strongly dependent on the cell-culture conditions and the cytokines used (21,29). However, for the culture of pdSCs and paldSCs, a standard procedure, in accordance with Widera *et al.*, was used (19). This method is routinely used for the culture of neurospheres and, with slight modifications, for mammospheres (30,31). As the culture conditions used have been optimized for the culture of neurospheres, mammospheres and dentospheres with the aim of maintaining their stem-cell state, it is questionable whether further modifications of the culture conditions could be carried out without initiating cell-differentiation processes. In a previous study, Lang and colleagues (32) investigated the effect of different concentrations of interleukin-8 on the migratory activity of neutrophil granulocytes. The migratory activity of the cells decreased as the concentration of interleukin-8 was increased (32), indicating that chemokines not only direct the migration of cells along a gradient, but are also capable of modulating the cells' migratory activity in a concentration-dependent manner. Under a physiological point of view this mechanism ensures that chemokine-recruited cells slow down their locomotion once they have reached the area of highest chemokine concentration. As 50-fold higher levels of MCP-1 and SDF-1α were used in in the study by Lang *et al.* (32) compared to the present study we conclude that the decreased migration of both pdSCs and paldSCs in response to SDF-1α and MCP-1 could be attributed to an effect similar to that described by Lang and colleagues (32).

The primary aim of the present study was to explore whether pdSCs and paldSCs could be applied as dentospheres and whether single pdSCs and paldSCs can migrate out of these spheres into the surrounding tissue. The 3D collagen matrix migration assay was developed as an *in-vitro* model to mimic the *in-vivo* situation as closely as possible. As comparison of data obtained from our assay with intravital microscopy cell-migration data yielded similar results, we conclude that the migratory activity of pdSCs and paldSCs, demonstrated in our experimental set-up, can be adopted in a 3D *in-vivo* situation. The advantage of dentospheres might be that a reservoir of viable stem cells can easily be delivered into the tissues, which may then initiate a regenerative process. We thus conclude that the use of dentospheres in regenerative purposes may be a suitable approach, but further investigations are required.

Acknowledgements

The authors are grateful to the Molthera AG Company (Sprockhövel, Germany) for providing the researchers with the cells of interest and for

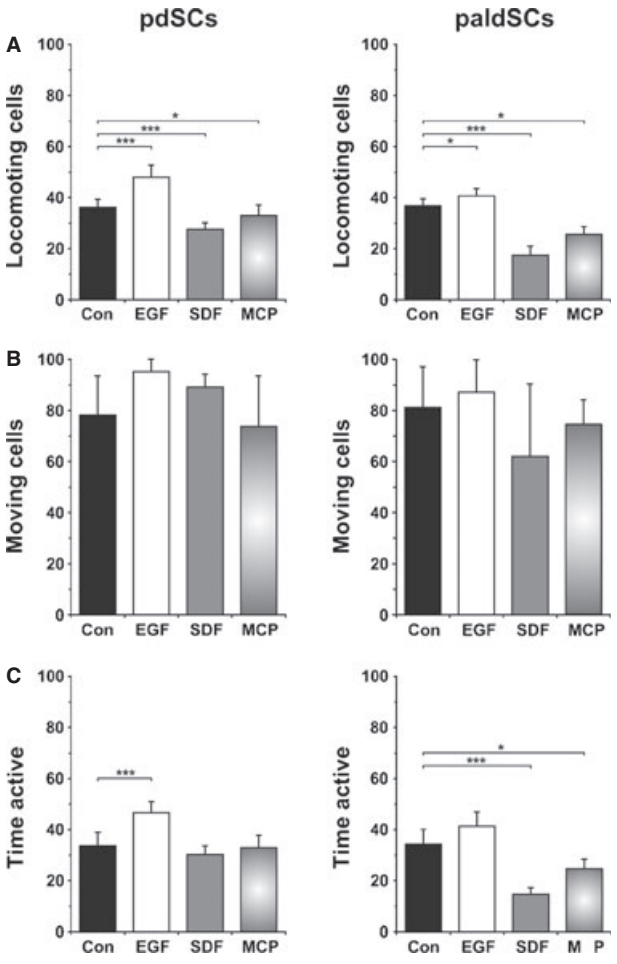

Fig. 6. Cell-migration analysis of periodontium-derived stem cell (pdSC) and palatal-derived stem cell (paldSC) dentospheres stimulated with epidermal growth factor (EGF), stromal cell-derived factor 1alpha (SDF) and monocyte chemotactic protein 1 (MCP). (A) Locomoting cells. (B) Moving cells. (C) Time active. Data shown are the mean of at least four independent experiments. Con, unstimulated control cells. Statistical significance: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

financial support. Our gratitude goes also to Silvia Keil for her technical assistance in the laboratory.

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