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Characterization of cells derived from inflamed intra-bony periodontal defects

Introduction: Regeneration of intra-bony periodontal defects should be supported by formation of new blood vessels and nerve fibres to ensure nutrition and innervation of the newly formed tissues. Aim of the present study was to evaluate the neurovascular properties of human stem cells derived from inflamed periodontal ligaments (ihPDLSCs).

Methods: Cultures of ihPDLSCs were established from granulation tissue of intra-bony periodontal defects (n = 4). Expression of epitopes characteristic for mesenchymal (CD73, CD90, CD105, CD146, STRO-1), embryonic (SSEA-4) and hematopoietic (CD34, CD45) stem cells were analysed by flow cytometry. Neuronal, endothelial and osteoblastic differentiation was induced by respective media. Changes in cell morphology were observed microscopically. Matrix mineralization was visualized and quantified using Alizarin Red S staining. Gene expression of neurogenic (NEFL, NCAM1, ENO2, TUBB3), angiogenic (VEGFR1, VEGFR2, PECAM1, ANGPT2) and osteogenic (RUNX2, SP7, APL, BMP2, BGLAP, SPP1, IBSP) markers was assessed by qRT-PCR.

Results: Cultures of ihPDLSCs showed high expression of CD73 and CD90, medium to high expression of CD105 and CD146, low to medium expression of SSEA-4 and low expression of STRO-1, CD34 and CD45. Trilineage differentiation potential was documented by histomorphologic changes, pronounced matrix mineralization and significant upregulation of stage-specific markers characteristic for neuronal (NEFL, NCAM1, ENO2), endothelial (VEGFR1, VEGFR2, PECAM1) and osteoblastic (ALP, BMP2) differentiation.

Conclusions: Our data provide evidence that cells isolated from granulation tissue of intra-bony periodontal defects have properties characteristic for mesenchymal stem cells. As these cells have the potential to undergo neuronal, endothelial and osteoblastic differentiation, the preservation of granulation tissue during regenerative periodontal surgery may be valuable to promote the healing of intra-bony periodontal defects.

Keywords: mesenchymal stem cells; regenerative periodontal surgery; inflamed intra-bony defects; granulation tissue; trilineage differentiation potential; in vitro

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Background

Intra-bony periodontal defects caused by periodontitis often show a rapid progression and represent a serious risk for tooth loss. During the destructive process, an inflammatory soft tissue – the granulation tissue – progressively replaces the healthy periodontium. The granulation tissue, considered as tissue of minor value, is generally resected during regenerative periodontal surgery [12, 13, 51, 62, 63]. The resulting tissue deficiency is usually associated with the development of gingival recessions, in particular in patients with advanced intra-bony periodontal defects [57]. Bone substitutes and occlusive membranes acting as defect fillers and barriers are routinely used to avoid a soft tissue collapse into the defect [17]. However, these exogenous materials can lead to unwanted side effects, like incomplete resorption of the bone substitutes or membrane exposure, and adversely affect the regenerative healing processes [43, 64].

Therefore, our group has introduced the granulation tissue preservation technique (GTPT, Fig. 1), which attempts to maintain as much granulation tissue as possible during regenerative periodontal surgery. We have shown in a case series that the preservation of granulation tissue as 'autologous' material has a positive influence on the clinical and radiographic treatment outcome [28]. Thus, we observed only a negligible development of gingival recessions and a significant bone fill in most cases. The maintenance of the vascular network, the increased wound stability and the preservation of mesenchymal stem cells (MSCs) are possible explanations for that. It has been suggested that the recruitment of progenitor cells, which are able to differentiate into specialized cells, plays a critical role within periodontal wound healing processes [8]. As all periodontal tissues originate from the dental mesenchyme, which is derived from the cranial neural crest [35], MSCs are considered as the main progenitor cells for periodontal regeneration.

Various sources of MSCs have been identified in the oral cavity. MSCs have been shown to reside in the pulp (dental pulp stem cells – DPSCs) [27] and the apical papilla (stem cells from the apical papilla – SCAP) [67] of permanent teeth, in the pulp of deciduous teeth (stem cells from human exfoliated deciduous teeth – SHED) [48], in the alveolar bone (bone marrow stem cells – BMSCs) [66], in the gingiva (gingival mesenchymal stem cells – GMSCs) [78] and in the periodontal ligament (healthy human periodontal ligament stem cells – hhPDLSCs) [65]. It has been also reported that MSCs may survive under inflammatory conditions. Therefore, stem cell properties have been found in cells isolated from the inflamed dental pulp [56], the inflamed gingiva [25] and the inflamed periodontal granulation tissue (inflamed human periodontal ligament stem cells – ihPDLSCs) [54].

Regenerative periodontal surgery aims at renewing all tooth supporting tissues including the periodontal ligament (PDL), the root cementum and the alveolar bone [36]. Angiogenesis and neurogenesis play a decisive role in periodontal regeneration, because formation of blood vessels and nerve fibres is required to ensure nutrition and innervation of the newly formed tissues [3, 42, 71]. Objective of the present study was to examine, if ihPDLSCs have the potential to promote the healing processes following regenerative periodontal surgery. The hypothesis of the present study was that ihPDLSC cultures derived from intra-bony periodontal defects contain a MSC population that is able to

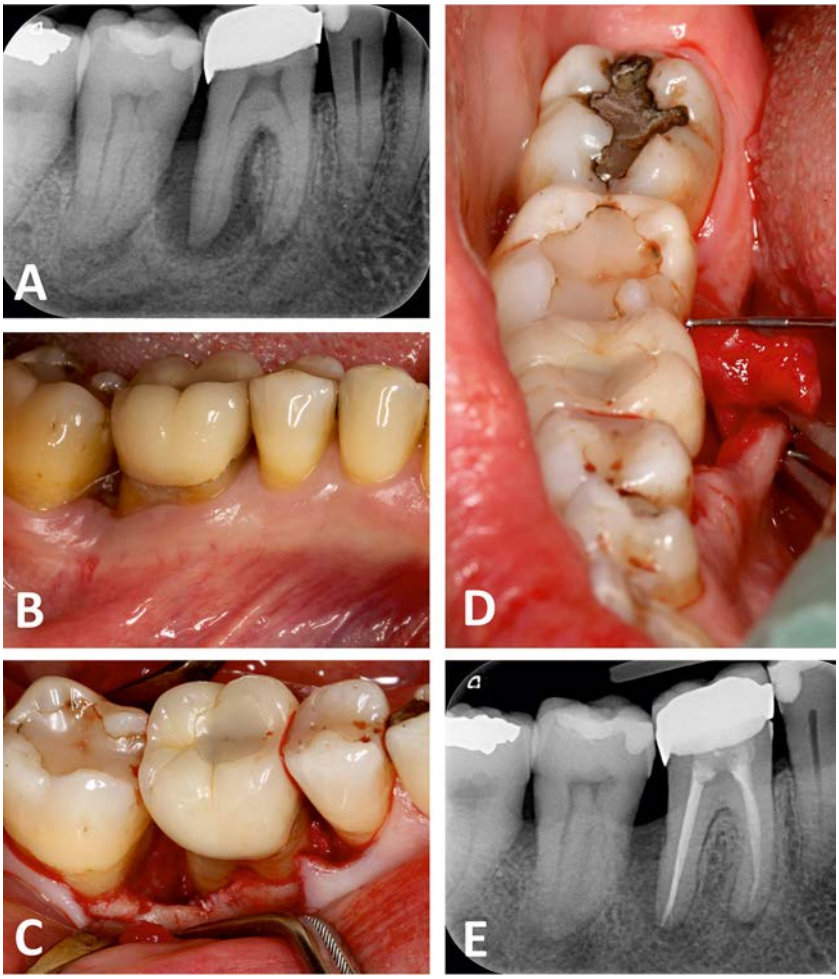


Figure 1A–E Representative treatment of an intra-bony periodontal defect (IPD) using the granulation tissue preservation technique (GTPT): The preoperative radiograph showed a pronounced bone loss at the distal root of the lower right first molar (**A**). The mucoperiosteal flap including the intra-lesional granulation tissue (GT) was thoroughly detached from the IPD and mobilized as far as it was necessary for an effective scaling and root planning (**B–D**). The radiograph 2 years after surgery and endodontic treatment revealed a significant bone fill in the former defect (**E**).

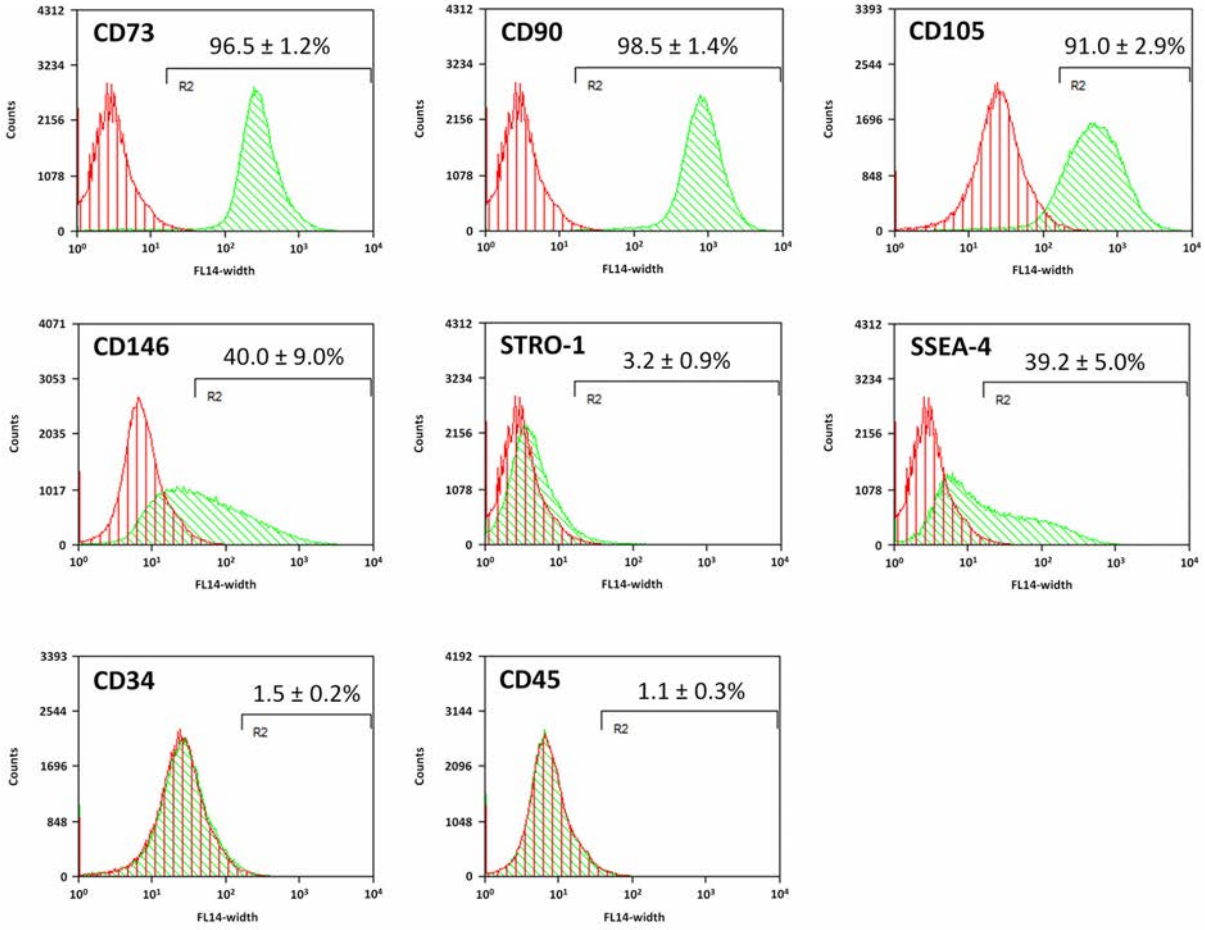


Figure 2 Representative data of the immunophenotypic characterization with flow cytometry: Single-parameter histograms show expression of mesenchymal (CD73, CD90, CD105, CD146, STRO-1), hematopoietic (CD34, CD45) and embryonic (SSEA-4) stem cell markers (red/vertical lines: unstained control; green/diagonal lines: cells expressing marker of interest).

undergo neurogenic, angiogenic and osteogenic differentiation.

Methods

Isolation and culture of ihPDLSCs

Four systemically healthy patients (aged 46.75 ± 2.63 years, 2 women, 2 men) diagnosed for severe chronic periodontitis were selected as donors for the biopsies. All patients received a non-surgical periodontal therapy to reduce local signs of inflammation. The surgical intervention was performed at teeth with a residual periodontal defect exhibiting a pocket probing depth > 6 mm, bleeding on probing and a radiographically evident intra-bony component of ≥ 3 mm. Since all surgical interventions were conducted for the purpose of periodontal regeneration, the GTPT was applied [28]. To get access to the bacterially contaminated root surface(s), a circumferential marginal incision was conducted at the defect-related teeth and the buccal and oral gums including the intra-lesional granulation tissue were sharply dissected from the underlying bone using microsurgical instruments. The mucoperiosteal flaps with the adherent granulation tissue were elevated as far as needed for an effective mechanical debridement (Fig. 1). The residual inflammatory granulation tissue was collected from the bottom of the intra-bony periodontal defect using curettes and scalers and used for the cell culture establishment of the present study. The defect-related root surface(s) were thoroughly debrided with hand instruments and a sonic device. Afterwards, the regenerative procedure was conducted. This included the application of a 24 % ethylene-diamine-tetra-acetic acid (EDTA) gel (PrefGel, Straumann, Freiburg, Germany), irrigation with sterile physiologic saline solution and application of enamel matrix proteins (Emdogain, Straumann). At the end of the surgical procedure, the intra-lesional granulation tissue and mucoperiosteal flaps were repositioned and fixed with interrupted sutures.

Immediately after collection, the inflammatory granulation tissue was minced into the smallest pieces possible and digested in -minimal essential medium (-MEM; Gibco, Grand Island, NY, USA) containing 3 mg/ml collagenase type I (Gibco) and 4 mg/ml dispase II (Sigma-Aldrich, Steinheim, Germany) for 1 h at 37°C. Cell-containing medium was passed through a strainer with a pore size of 70 µm (EASYstrainer, Greiner bio-one, Frickenhausen, Germany). The resulting ihPDLSCs were seeded into

	QuantiTect Primer Assay	Protein/enzyme (abbreviation)	Catalogue number	Detected transcript(s)
Neurogenic	Hs_ENO2_1_SG	enolase 2 (ENO2)	QT00084889	NM_001975 (2423 bp)
	Hs_NCAM1_1_SG	neural cell adhesion molecule (NCAM1)	QT00071211	NM_000615 (5977 bp) NM_001076682 (4944 bp) NM_001242608 (4831 bp)
	Hs_NEFL_1_SG	neurofilament, light polypeptide (NEFL)	QT00096369	NM_006158 (3854 bp)
	Hs_TUBB3_1_SG	tubulin, beta 3 class III (TUBB3)	QT00083713	NM_006086 (1794 bp)
Angiogenic	Hs_ANGPT2_1_SG	angiopoietin 2 (ANGPT2)	QT00100947	NM_001118887 (5267 bp) NM_001118888 (5114 bp) NM_001147 (5270 bp)
	Hs_PECAM1_1_SG	platelet and endothelial cell adhesion molecule 1 (PECAM1)	QT00081172	NM_000442 (6831 bp) XM_005276880 (4006 bp) XM_005276881 (3972 bp) XM_005276882 (3966 bp) XM_005276883 (3943 bp) XM_006721944 (2438 bp) XM_006721945 (2452 bp)
	Hs_FLT1_1_SG	fms-related tyrosine kinase 1 (FLT1) or vascular endothelial growth factor receptor 1 (VEGFR1)	QT00073640	NM_002019 (7123 bp)
	Hs_KDR_1_SG	kinase insert domain receptor (KDR) or vascular endothelial growth factor receptor 2 (VEGFR2)	QT00069818	NM_002253 (6055 bp)
Osteogenic	Hs_ALPL_1_SG	alkaline phosphatase (ALPL)	QT00012957	NM_000478 (2606 bp) NM_001127501 (2441 bp) NM_001177520 (2325 bp) XM_005245818 (2573 bp) XM_005245820 (1379 bp) XM_006710546 (2558 bp)
	Hs_BGLAP_1_SG	bone gamma carboxyglutamic acid-containing protein (BGLAP)	QT00232771	NM_199173 (562 bp)
	Hs_BMP2_1_SG	bone morphogenic protein 2 (BMP2)	QT00012544	NM_001200 (3150 bp)
	Hs_IBSP_1_SG	integrin-binding sialoprotein (IBSP)	QT00093709	NM_004967 (1595 bp)
	Hs_RUNX2_1_SG	runt-related transcription factor 2 (RUNX2)	QT00020517	NM_001015051 (5487 bp) NM_001024630 (5553 bp) NM_004348 (5720 bp) NM_001278478 (5235 bp) XM_006715231 (5304 bp) XM_006715233 (2944 bp) XM_006715234 (875 bp)
	Hs_SP7_1_SG	sp7 transcription factor (SP7)	QT00213514	NM_001173467 (3173 bp) NM_152860 (2995 bp) NM_001300837 (3211 bp)
	Hs_SPP1_1_SG	secreted phosphoprotein 1 (SPP1)	QT01008798	NM_000582 (1616 bp)

Table 1 QuantiTect Primer Assays (Qiagen) used for the qRT-PCR analyses of genes related to neuronal, endothelial and osteoblastic differentiation

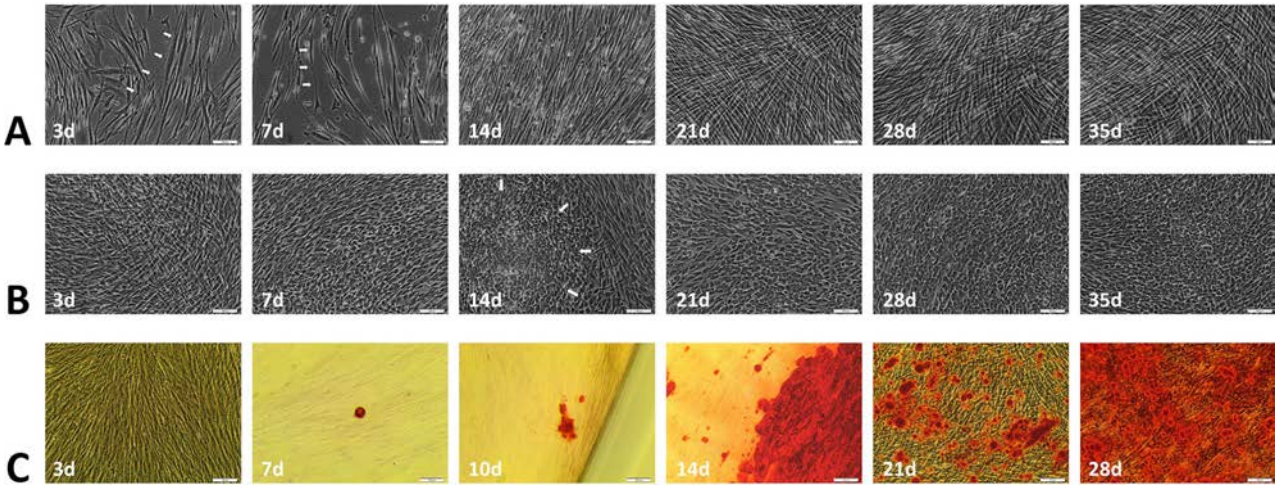


Figure 3A–C Microscopic images documenting the histomorphologic changes occurring during neuronal (**A**), endothelial (**B**) and osteoblastic (**C**) differentiation: Already 3 days after exposure to neurogenic medium, the formation of a neuron-like phenotype with a 'drawn-out' cell body (open arrow) and dendrite-like processes (white arrows) was observed (**A**). During angiogenic differentiation, the cell morphology changed from spindle-shaped (characteristic for fibroblasts) to polygonal (characteristic for endothelial cells). At the beginning of the endothelial differentiation, both morphologies were simultaneously present, as illustrated by the microscopic image at day 14 (white arrows indicating endothelial-like cells). Afterwards, a continuously increasing amount of the polygonal phenotype was observed (**B**). During osteoblastic differentiation, an increasing amount of AR-S positive mineralized deposits was detectable. At the end of the osteogenic differentiation experiment, the entire surface of the well was covered by AR-S positive mineralized tissue (**C**).

cell culture flasks containing complete culture medium (CCM) consisting of -MEM supplemented with 15 % foetal bovine serum (FBS, Biochrom, Berlin, Germany), 100 U/ml penicillin, 100 µg/ml streptomycin (both from Biochrom), 2.5 µg/ml Amphotericin B (Capricorn Scientific, Ebsdorfergrund, Germany) and 100 µM L-ascorbic acid phosphate (Sigma-Aldrich). The cultures were incubated in a humidified atmosphere at 37 °C and 5 % CO_2. Cells from passages 2 to 4 were used for the experiments.

Characterization of ihPDLSC cultures with flow cytometry

The antigen profiles of ihPDLSC cultures at passages 2 and 3 were analysed by flow cytometry, as previously described [2]. Cells were seeded in 75 cm^2 culture flasks and expanded in CCM until confluency. Cells were trypsinized, washed with phosphate-buffered saline (PBS) and re-suspended in FACS buffer consisting of PBS supplemented with 1 % bovine serum albumin (BSA) and 0.1 % sodium azide (NaN_3). For each sample, 1x 10^6 cells/100 µl FACS buffer were Fc-blocked with 1 µg of human IgG (Sigma-Aldrich) for 15 min on ice. Afterwards, cells were stained by incubation with the following fluorochrome-conjugated mouse anti-human antibodies for 25 min in the dark on ice: CD73-FITC (fluorescein isothiocyanate), CD90-FITC, CD105-APC (allophycocyanin), CD146-PE (phycoerythrin), STRO-1-FITC, CD34-APC, CD45-PE and SSEA-4-FITC (all from BioLegend, Fell, Germany). For flow cytometry analysis, a BD LSR II Flow Cytometer (BD Biosciences, Heidelberg, Germany) was used. A total of 100,000 events were acquired for each sample. Data were analysed using the Summit 5.1 software (Beckman Coulter, Fullerton, USA). Flow cytometry experiments were repeated 4 times for each donor.

Induction of neurogenic differentiation

For neurogenic differentiation, ihPDLSCs were seeded into six-well plates coated with 0.1 % gelatine (Sigma-Aldrich) at 1x 10^5 cells/well and expanded in neurobasal A medium (Gibco) supplemented with B27 supplement (Gibco), 2 mM L-glutamine (Gibco), 20 ng/ml epidermal growth factor (EGF, Biochrom), 40 ng/ml recombinant human basic fibroblast growth factor (rh-bFGF, Biochrom), 100 U/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml amphotericin B. Cells were cultured for 5 weeks and the neurogenic medium was changed every 2 to 3 d. Neurogenic differentiation was assessed with an inverted microscope for the detection of morphological changes towards a neuron-like phenotype and with quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) for the expression of the neuronal markers neurofilament light polypeptide (NEFL), neural cell adhesion molecule 1 (NCAM1), tubulin beta 3 class III (TUBB3), and enolase 2 (ENO2).

Induction of angiogenic differentiation

For induction of angiogenic differentiation, cells were seeded into six-well plates coated with collagen I (Santa Cruz Biotechnology, Heidelberg, Germany) at 1x 10^5 cells/well. Cultures were expanded in CCM until they reached confluency. Afterwards, cells were exposed to angiogenic medium consisting of M199 medium (Gibco) supplemented with 5 % FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml amphotericin B, 50 µg/ml heparin (Sigma-Aldrich), 1 µg/ml hydrocortisone (Sigma-Aldrich), 60 µg/ml en-

dothelial cell growth supplement (ECGS, PromoCell, Heidelberg, Germany), 10 ng/ml EGF (Biochrom), 25 ng/ml rh-bFGF (Biochrom) and 50 ng/ml vascular endothelial growth factor (VEGF, Gibco). Cells were cultured for 5 weeks; the angiogenic medium was changed every 2 to 3 d. Angiogenic differentiation was assessed by evaluation of phenotypic changes and qRT-PCR for the expression of the angiogenic markers angiopoietin 2 (ANGPT2), vascular endothelial growth factor receptor 1 (VEGFR1), vascular endothelial growth factor receptor 2 (VEGFR2) and platelet endothelial cell adhesion molecule 1 (PECAM1).

Induction of osteogenic differentiation

For osteogenic differentiation, cells were seeded into six-well plates at 1x 10^5 cells/well and expanded in CCM until they reached confluency. Subsequently, cells were exposed to osteogenic medium consisting of CCM supplemented with 5 mM -glycerophosphate (Sigma-Aldrich), 1.8 mM monopotassium phosphate (KH_2PO_4, Sigma-Aldrich) and 10 nM dexamethasone (Sigma-Aldrich). Cells were cultured for 4 weeks; the osteogenic medium was changed every 2 to 3 d. Osteogenic differentiation was analysed by the Alizarin Red S (AR-S) mineralization assay and by qRT-PCR for the expression of the osteogenic markers bone morphogenic protein 2 (BMP2), secreted phosphoprotein 1 (SPP1), runt related transcription factor 2 (RUNX2), Sp7 transcription factor (SP7), bone gamma-carboxyglutamate protein (BGLAP), integrin binding sialoprotein (IBSP) and alkaline phosphatase (ALP). For the in vitro mineralization assay, cultures were washed twice with PBS without Ca^{2+} and Mg^{2+} (Biochrom) and fixed with 10 % neutral buffered formalin solution (Sigma-Aldrich) for 1 h at room temperature. Afterwards, cells were washed twice with distilled water and stained with 1 % AR-S (pH 4.0, Sigma-Aldrich) for 20 min at room temperature. To reduce non-specific staining, cells were washed 4 times with 2 ml distilled water and mineralized deposits were visualized and photographed with an inverted microscope (Olympus Optical Co., Ltd., Japan). For quantification of calcified tissues, AR-S was extracted by adding 1.5 ml cetylpyridinium chloride (CPC) buffer (10 %, w/v) in 10 mM disodium monohydrogen phosphate (Na_2HPO_4, pH 7.0) for 2 h at 37°C. Aliquots of 200 µl were transferred to a 96-well plate and the optical density of the solution was measured at 550 nm using a microplate spectrophotometer (Spectra Max 250, MWG Biotech, Sunnyvale, CA, USA).

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

To analyse changes in gene expression during neurogenic, angiogenic and osteogenic differentiation, a two-step qRT-PCR was applied. Total mRNA was isolated using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). An additional on-column DNA digestion was conducted to eliminate genomic DNA (RNase-free DNase Set, Qiagen). Afterwards, the RNA concentration was measured using a microplate reader (Synergy H1, BioTek, Bad Friedrichshall, Germany). The cDNA was synthetized using 1 µg of the isolated RNA and the QuantiTect Reverse Transcription Kit (Qiagen). For amplification and real-time quantification of cDNA targets, the QuantiTect SYBR Green PCR Kit, QuantiTect Primer Assays (Table 1) and the Rotor-Gene Q cycler (all from Qiagen) were used. All PCR reactions consisted of an initial incubation step of 5 min at 95°C to activate the HotStarTaq DNA polymerase and 40 cycles of denaturation (at 95°C for 5 sec), annealing and extension (at 60°C for 10 sec). A standard melting curve was used to validate the specificity of the reaction products. PCR raw data were processed using LinRegPCR to perform baseline correction, to determine the window-of-linearity and to determine the PCR efficiency per sample and per amplicon group [59]. Actin beta (ACTB), beta-2-microglobulin (B2M), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 18S ribosomal RNA (RRN18S), succinate dehydrogenase flavoprotein subunit (SDHA2) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (YWHAZ) were used as housekeeping genes. The 2 most stable housekeeping genes selected by geNorm were used to normalise the adjusted PCR data [73].

Statistics

The gene expression of independent biological replicates was standardized by logarithmic transformation, mean centring and auto-scaling, as described by Willems et al. [76]. Statistical analysis was performed by one-way ANOVA with Tukey's multiple comparison test using GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, CA 92037, USA). A value of $p \leq 0.05$ was considered statistically significant.

Results

Immunophenotypic characterization

Specific cell surface markers were selected to show that the established and in vitro expanded ihPDLSC cultures contain cells with MSC-like properties. Despite certain intra- and inter-individual differences, all ihPDLSC cultures exhibited a similar expression pattern of cell surface molecules (Table 2, Fig. 2). A high expression was detected for the MSC markers CD73 and CD90 (> 95 % of the population), a medium to high expression was observed for CD146 (76.2 ± 24.3) and CD105 (82.6 ± 14.6) and a low expression was documented for STRO-1 (5.2 ± 3.8). The embryonic marker SSEA-4 exhibited low to medium expression levels (30.3 ± 12.5). Furthermore, the hematopoietic progenitor cell antigen CD34 and the leukocyte common antigen CD45 showed a negligible expression level (< 2 %) in almost all cases. Considerable intra- and inter-individual differences were found for the markers CD105, CD146, SSEA-4 and CD34.

Multilineage differentiation potential

Multipotency belongs to the key properties of MSCs. Therefore, the neurogenic, angiogenic and osteo-

CD73	CD90	CD105	CD146	STRO-1	SSEA-4	CD34	CD45
97.6 ± 1.8	98.9 ± 0.9	82.6 ± 14.6	76.2 ± 24.3	5.2 ± 3.8	30.3 ± 12.5	3.6 ± 4.0	1.3 ± 0.5

Table 2 Immunophenotypic characterization with flow cytometry (expression of mesenchymal [CD73, CD90, CD105, CD146, STRO-1], embryonic [SSEA-4] and hematopoietic [CD34, CD45] stem cell markers of all donors [n = 4] expressed as mean value [%] ± standard deviation)

genic differentiation potential was evaluated. Initial morphological changes characteristic for neurogenic differentiation were microscopically detected 3 d after induction. During the differentiation process, the fibroblast specific, spindle-shaped morphology changed into a neuron-like phenotype with a 'drawn-out' cell body and dendrite-like extensions. In addition, the longer the differentiation process lasted, the more the orientation of cells changed from a random to a parallel pattern (Fig. 3A). To analyse the neurogenic differentiation potential of ihPDLSCs at mRNA level, qRT-PCR was conducted for neurogenic markers. A continuously increasing expression of NEFL, NCAM1 and ENO2 could be observed (Fig. 4A). Expression of NEFL was significantly increased at day 14, 21, 28 and 35 ($p < 0.001$), expression of NCAM1 was significantly increased at day 21 ($p < 0.05$), 28 ($p < 0.01$) and 35 ($p < 0.001$), and expression of ENO2 was significantly increased at day 3, 7, 14, 21, 28 and 35 ($p < 0.001$) when compared to day 0 (reference). Expression of TUBB3 did not significantly change during the entire observation period.

The spindle-shaped morphology of fibroblasts changed toward a polygonal endothelial cell-like phenotype during cultivation in angiogenic medium. In addition, the shoal-like arrangement of fibroblasts converted into a cobblestone-like pattern (Fig. 3B). At mRNA level, a continuously increasing expression of VEGFR2, VEGFR1 and PECAM1 was detected (Fig. 4B). Expression of VEGFR2 and PECAM1 was significantly increased at day 3, 7, 14, 21, 28 and 35 ($p < 0.001$) and expression of VEGFR1 was significantly increased at day 7, 14, 21, 28 and 35 ($p < 0.001$) when compared to day 0. As ANGPT2 was not detectable in 3 out of our 4 donors, statistics were not performed for this marker.

During the 4 weeks of induction, ihPDLSCs exhibited osteogenic potential as determined by the presence of AR-S positive mineralized deposits and by increased expression of osteogenic markers. First AR-S positive nodules were observed 3 to 7 d after induction. These were primarily located in the periphery of the well. Afterwards, mineralization rapidly increased and after 14 to 21 d of induction 70–80 % of the adherent monolayer was covered by AR-S positive calcium accumulations (Fig. 3C). These microscopic observations were confirmed by spectrophotometric AR-S quantification using the CPC extraction method (Fig. 5). AR-S concentration per well was significantly increased at day 14, 21 and 28 ($p < 0.001$) when compared to day 3, 7 and 10. Significant changes in gene expression were observed for ALP and BMP2 (Fig. 4C). Expression of ALP significantly increased at day 3, 7, 10 and 14 ($p < 0.001$) and subsequently decreased to non-significant values. Expression of BMP2 was significantly increased at day 3, 7, 10, 14, 21 and 28 ($p < 0.001$) when compared to day 0 and showed the highest values at day 21. Expression of RUNX2 and BGLAP did not significantly change throughout the entire observation period. As IBSP, SP7 and SPP1 were not reliably detectable, data were not further analysed for these markers.

Discussion

Re-establishment of a biocompatible root surface, exclusion of the gingival epithelium, sufficient wound stability and presence of progenitor cells are prerequisites to regenerate destroyed periodontal structures. MSCs as the ideal progenitor cells for periodontal regeneration have been shown to reside in the intact periodontal ligament, in the adjacent alveolar bone and in the blood stream [33]. During wound healing, MSCs are assumed to migrate into the periodontal defect and subsequently differentiate into the cell types required for periodontal regeneration [34]. Due to the bacterial contamination and the inflammatory properties, granulation tissue of intra-bony periodontal defects has not been considered as appropriate source for MSCs that could be useful in regenerating destroyed tissues. Therefore, it is routinely removed during regenerative periodontal surgery [12, 13, 51, 62, 63]. However, the clinical and radiographic outcomes of the GTPT, which has been developed and documented by our group, suggest that the preservation of granulation tissue may positively influence the healing processes following regenerative periodontal surgery.

Hypothesis of the present study was that ihPDLSCs show characteristics of MSCs and maintain their multilineage differentiation potential, which is needed for periodontal regeneration. Microscopic observations revealed that ihPDLSCs comprise a heterogeneous mixture of cells that predominantly show a spindle-shaped, fibroblast-specific morphology. Flow cytometry analysis was used for immune-phenotypic characterization. Although several articles have been published regarding MSC surface antigens, there is no general consensus, which combination of CD markers is appropriate to identify MSCs with sufficient accuracy. Dominici et al. [15] have published minimal criteria for defining MSCs. These criteria include that more than 95 % of the MSC population must express CD73, CD90 and CD105. Since expression of CD73, CD90, CD105 and CD44 is not only observed in MSCs but also

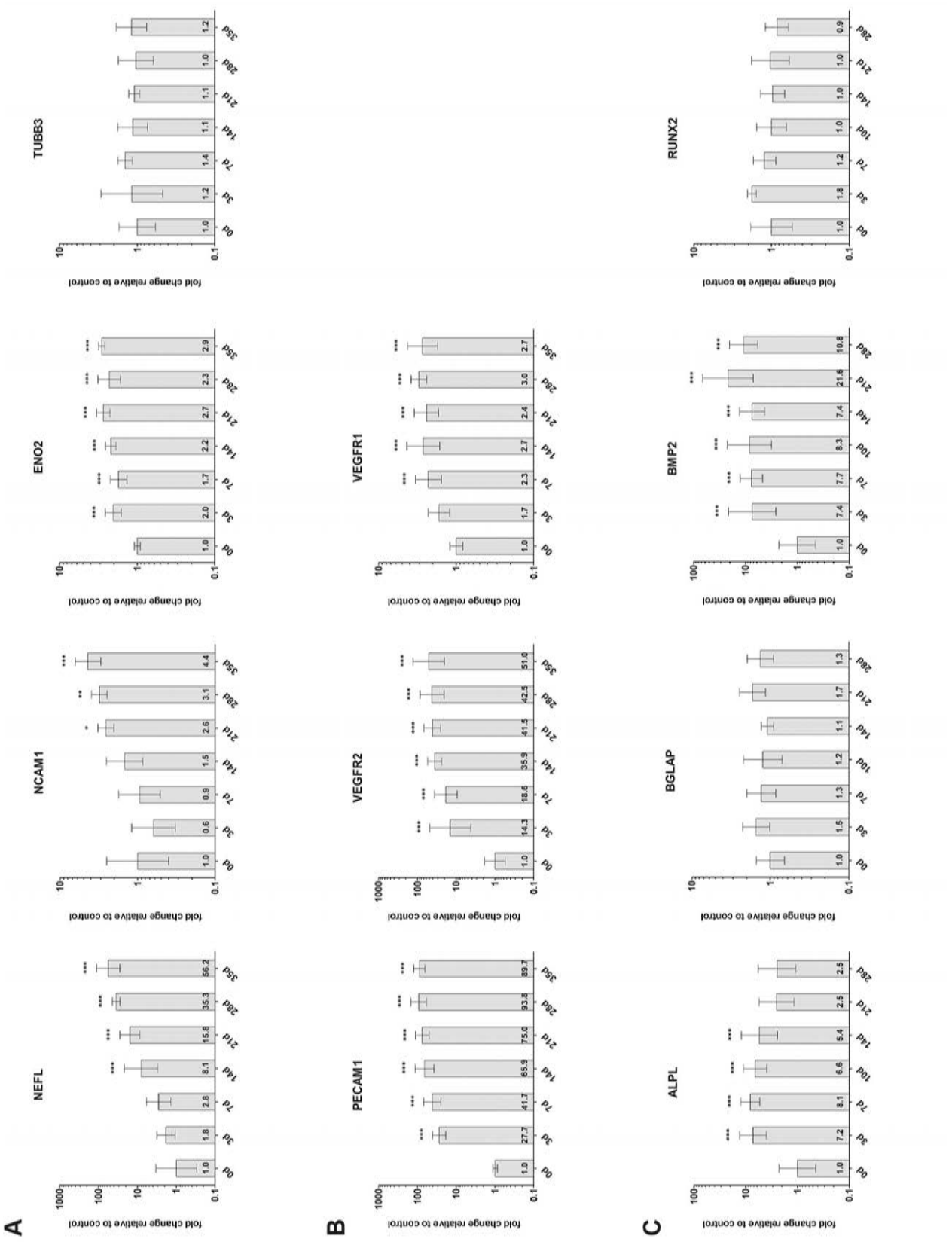
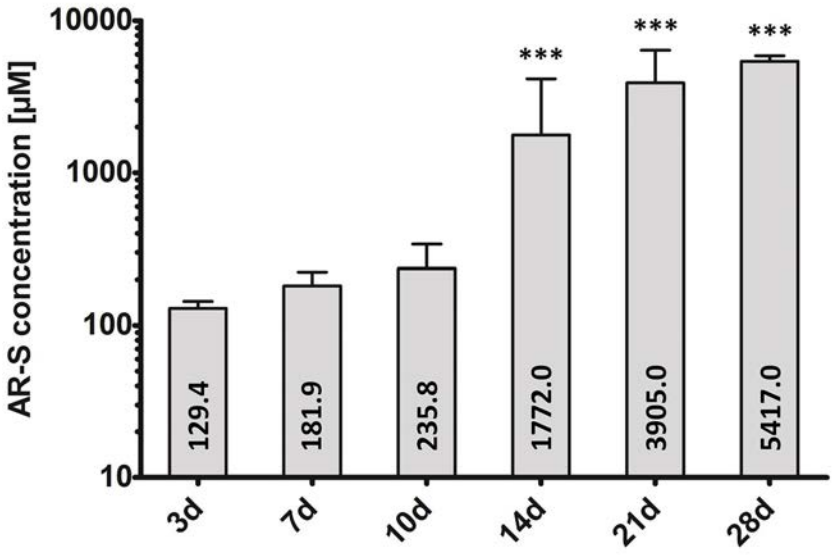


Figure 4A–C Gene expression of markers characteristic for neuronal (**A**), endothelial (**B**) and osteoblastic (**C**) differentiation: The qRT-PCR data of all donors (n = 4) were standardized using logarithmic transformation, mean centring and auto-scaling, as described by Willems et al. [76]. Data are expressed as recalculated average with upper and lower confidence interval. One-way ANOVA with Tukey's multiple comparison test was used to detect significant differences to day 0 (*P < 0.05; **P < 0.01; ***P < 0.001). The neuronal markers included neurofilament light polypeptide (NEFL), neural cell adhesion molecule 1 (NCAM1), tubulin beta 3 class III (TUBB3) and enolase 2 (ENO2). The endothelial markers included vascular endothelial growth factor receptor 1 (VEGFR1), vascular endothelial growth factor receptor 2 (VEGFR2) and platelet and endothelial cell adhesion molecule 1 (PECAM1). The osteoblastic markers included alkaline phosphatase (ALP), bone gamma-carboxyglutamate protein (BGLAP), bone morphogenic protein 2 (BMP2) and runt related transcription factor 2 (RUNX2).



(Fig. 1–5, Tab. 1 and 2: Knut Adam)

Figure 5 Quantification of matrix mineralization during the osteoblastic differentiation experiments using the AR-S extraction method. Data from all donors (n = 4) are expressed as mean AR-S concentration [µM] ± standard deviation. AR-S concentrations were significantly increased at day 14, 21 and 28 when compared to day 3, 7 and 10 (***P < 0.001; one-way ANOVA with Tukey's multiple comparison test).

in fibroblasts and stromal cells [49], further markers are required. Thus, expression of CD146, Stro-1 and SSEA-4 was additionally investigated in the present study. Our data revealed that ihPDLSCs exhibit a high expression of CD73, CD90, CD105 and CD146. Considerable inter-individual differences in the expression of CD105 and CD146 were detected. This heterogeneity is reflected by the results of other studies about ihPDLSCs, where expression of CD105 ranged from 51.9 to 96.0 % and expression of CD146 from 9.2 to 81.1 % [32, 41, 44, 54, 72]. Expression of Stro-1 was quite low and obviously lower than in other studies about ihPDLSCs [32, 41, 44, 54]. In this context, Lv et al. [45] reported that Stro-1 is not universally expressed in all types of MSCs and that its expression gradually may become lost in culture. A similar phenomenon can be observed for CD34, a marker for hematopoietic stem cells (HSCs) and endothelial progenitors, which is commonly used to distinguish MSCs from HSCs. Mitchell et al. [47] reported that CD34 expression dramatically decreases with each passage in culture. This could explain why CD34 expression showed significant inter-individual differences and was higher than 2 % in 2 out of our 4 donors. Stage-specific embryonic antigen 4 (SSEA-4) is known as an embryonic stem cell-associated marker. However, SSEA-4 is not only expressed in human embryonic stem cells but also in adult MSC populations [24]. Liu et al. [44] reported that ihPDLSCs express SSEA-4 by 13.56 ± 4.21 %, which is remarkably lower than in our study (30.27 ± 12.50). Although the exact percentage remains unclear, the flow cytometry data of our study indicate that a relevant fraction of the heterogeneous ihPDLSC population expresses surface antigens, which allow to identify them as MSCs.

The ability to differentiate into 3 different lineages under inductive culture conditions represents another characteristic of MSCs. Generally, the potential to generate adipocytes, osteoblasts and chondrocytes is regarded sufficient to prove multipotency. However, differentiation potential of MSCs is not only restricted to tissues of mesodermal origin. Thus, trans-differentiation into ectodermal (e.g. neurons) and endodermal (e.g. hepatocytes) lineages was successfully conducted in vitro [18, 39]. As neurogenesis, angiogenesis and osteogenesis belong to the biological key processes in periodontal regeneration, we attempted to generate neurons of ectodermal origin as well as endothelial cells and osteoblasts of mesodermal origin in vitro. Our data provide a detailed view on the expression changes chronologically occurring during neural, endothelial and osteoblastic differentiation.

As all periodontal tissues originate from the neural crest, it is not surprising that hhPDLSCs spontaneously express neuronal markers, as shown by Heng et al. [30]. However, we could show that a baseline expression of neuronal markers is also present in ihPDLSCs. After induction of neurogenic differentiation, ihPDLSCs showed a continuously increasing expression of the neuronal markers NEFL, NCAM1 and ENO2. Neurofilaments, like NEFL, are neuronal intermediate filaments representing the most abundant structural cytoskeletal proteins of myelinated axons [70]. During development of the nervous system, the expression of neurofilaments follows a stereotypic program, which is phylogenetically conserved and repeated during axonal regeneration [70]. NCAM1 belongs to the immunoglobulin superfamily and regulates neurite outgrowth in the developing nervous system [58]. In addition, regeneration of central and peripheral neuronal fibres was reported to be associated with an upregulation of NCAM1 expression [53]. ENO2 is a glycolytic enzyme predominantly localized in the cytoplasm of neurons. Expression of ENO2 is upregulated when axons are damaged suggesting that ENO2 is involved in neuronal regeneration [10]. The significant increase of these neuronal markers strongly indicates that ihPDLSCs have the potential to generate neuron-like cells and to play an important role in the re-innervation of tissues regenerated following periodontal surgery.

However, expression of TUBB3 did not significantly change. TUBB3 is a component of neuronal microtubules and involved in many cellular functions such as intracellular organization, coordinated vesicle transport and cell division [37]. TUBB3 is one of the earliest neuronal cytoskeletal proteins in the development of

the central nervous system and seems to participate in early neuritogenesis [38]. Foudah et al. [23] reported that a very high percentage of undifferentiated hhPDLSCs and other types of MSCs are positive for TUBB3 and that this expression does not change up to passage 16. This phenomenon implies that TUBB3 is constitutively expressed in MSCs, not influenced by spontaneously occurring differentiation or senescence processes and, therefore, not solely neuron-specific. Widera et al. [75] investigated the neurogenic differentiation potential of ihPDLSCs. They were able to detect high levels of neuron-specific markers, such as MAP2, NEFL, NEFM and NEFH after neural induction with retinoic acid. Their conclusion that ihPDLSCs possess the ability to generate neural precursors was confirmed by the results of our study.

Angiogenesis, defined as new blood vessel sprouting from pre-existing ones, is an essential process involved in development, wound healing and regeneration [16, 19, 22]. After induction of angiogenic differentiation, a significant increase in the expression of the endothelial marker proteins VEGFR2, VEGFR1 and PECAM1 was observed. VEGF is regarded as the most important factor stimulating angiogenesis in healthy and diseased tissues [20, 50]. Most angiogenic functions of VEGF, including proliferation, survival, migration and permeability of endothelial cells, are triggered by VEGFR2 [14]. Although its precise function remains unclear, VEGFR1 seems to be a decoy receptor for VEGF [6, 19]. Thus, VEGFR1 moderates the angiogenic effects of VEGF by preventing VEGF binding to VEGFR2. PECAM1 is an efficient signalling molecule, expressed on all cells within the vascular compartment and involved in angiogenesis [55, 77]. The expression profiles of VEGFR2, VEGFR1 and PECAM1 in the present study strongly suggest that endothelial trans-differentiation occurred. However, ANGPT2 was generally not expressed during the entire angiogenic differentiation experiments. Endothelial cells in adults persist in a quiescent state and proliferate only following activation. ANGPT1-mediated Tie2 activation is required to maintain this vascular quiescence [21]. ANGPT2, as functional antagonist of ANGPT1, destabilizes quiescent endothelia and, thus, promotes angiogenesis in particular in combination with VEGF [21, 61]. The lack of ANGPT2 expression in our experiments can be explained by the 'artificial' in vitro conditions, which are not unconfined comparable to the in vivo situation of a quiescent-resting endothelium. In this context, Korff et al. [40] investigated the synergistic effects of VEGF and ANGPT2 in a 3-dimensional spheroidal co-culture model. They reported that endothelial cells exhibit different responsiveness to VEGF stimulation depending on the culture conditions. Thus, endothelial cells grown in monoculture were able to form capillary-like sprouts after stimulation with VEGF, while endothelial cells grown in co-culture with smooth muscle cells were only able to generate sprouts, when VEGF was combined with ANGPT2. This suggests that ANGPT2 is obligatory for endothelial differentiation when cells are grown in co-culture, but dispensable when cells are grown in monoculture like in our study. Amin et al. [1] investigated the angiogenic and vasculogenic differentiation potential of hhPDLSCs. They detected an upregulation of the endothelial markers VEGFR2, Tie1, Tie2, VE-cadherin and vWF. Further studies consistently revealed that MSCs derived from healthy oral tissues are able to differentiate into endothelial-like cells [4, 60]. Our study discovered for the first time, that ihPDLSCs as a MSC population derived from inflamed tissue retain their angiogenic differentiation potential required for neovascularization.

As regeneration of intra-bony periodontal defects involves formation of new alveolar bone, differentiation of MSCs to osteoblasts is a prerequisite. Osteoblast differentiation is dynamically controlled by stage-specific signal transduction and transcription factors, such as RUNX2 and SP7 [52]. In vivo, RUNX2 is strongly expressed in pre-osteoblasts and immature osteoblasts, but finally down-regulated in mature osteoblasts [46]. This indicates that RUNX2 is crucial for the initial steps of osteoblast differentiation. In the present study, RUNX2 was strongly expressed in ihPDLSCs throughout the entire differentiation experiment. A definite increase in the expression of RUNX2 was detectable 3 days after induction. Wang et al. [74] investigated the osteogenic differentiation potential of SCAP using osteogenic differentiation media containing dexamethasone, -glycerophosphate and KH_2PO_4, as used in our study. Similar to our results, they reported that expression of RUNX2 was upregulated 3 days after induction and reduced afterwards. SP7 represents another transcription factor essential for bone development and osteoblastogenesis [52]. Despite some obvious inter-individual differences, a reproducible expression pattern was detected. Thus, expression of SP7 was initially upregulated (peaking at day 3, 7, or 10), afterwards considerably downregulated and frequently not detectable (data not shown). Therefore, expression of RUNX2 and SP7 seems to be associated with the early stages of osteoblast differentiation, but not with matrix mineralization in vitro. Bone morphogenetic proteins (BMPs) are multi-functional growth factors that belong to the transforming growth factor (TGF-) superfamily [7]. It has been reported that BMP2 strongly promotes the differentiation of MSCs into osteoblasts [29, 52] and also enhances bone matrix production by osteoblastic cells [69]. Expression of BMP2 was significantly upregulated during the entire osteogenic differentiation experiment. It showed highest values at day 21 when matrix mineralization had largely covered the well surface. Conversely, ALP expression was significantly upregulated from day 3 to 14 and downregulated at day 21. These results are in agreement with data reported by Hoemann et al. [31] who investigated in vitro osteogenesis assays in BMSCs. They documented that confluent osteogenic cultures follow a two-stage developmental process consisting of a 1– to 2-week initiation phase with increased ALP activity and a subsequent maturation phase, in which matrix

mineralization occurs. During increased ALP activity, the extracellular matrix undergoes modifications in composition and organization, which prepares it effectively for the following mineralization process [68]. While the upregulation of ALP expression is stimulated by dexamethasone, the matrix mineralization is induced by -glycerophosphate, which is rapidly converted to glycerol and inorganic phosphate in vitro [31]. BGLAP, SPP1 and IBSP belong to the non-collagenous proteins in the bone matrix. They are produced at the late stages of osteoblastic maturation and participate in matrix mineralization in vivo [68]. BGLAP was strongly expressed and did not significantly change in our study. However, expression of SPP1 and IBSP was significantly decreased and frequently suppressed to undetectable levels (data not shown), although an increased expression of both proteins is considered essential for matrix mineralization in vivo [68]. Similar to the results of our study, Cheng et al. [11] reported that exposure of BMSCs to dexamethasone resulted not only in substantial matrix mineralization, but also in drastically suppressed levels of SPP1 and IBSP in vitro. Since high concentrations of SPP1 and IBSP inhibit hydroxyapatite formation in vitro [5, 26], it seems likely, that already minor concentrations of SPP1 and IBSP are sufficient to initiate and perpetuate the mineralization process.

Other investigations have already shown the osteogenic differentiation potential of ihPDLSCs [9, 32, 54]. However, the data of the present study provide for the first time a detailed view on both, the process of matrix mineralization and the expression of osteogenic markers, chronologically occurring after induction with dexamethasone. These data strongly indicate that ihPDLSCs are able to generate osteoblast-like cells and that ihPDLSCs have many features in common with BMSCs during osteoblastic differentiation.

The cell cultures used in the present study were unexceptionally isolated from granulation tissues harvested from the bottom of intra-bony periodontal defects. Attachment loss is continuously proceeding in inflammatory periodontal diseases. Thus, granulation tissues derived from the bottom of intra-bony periodontal defects have been exposed to the inflammatory conditions for a shorter period of time than granulation tissues harvested from a more coronal part. It is conceivable that the regenerative capacity of the granulation tissue may increase gradually to the bottom of the periodontal defect and that the cell populations differ in the different parts of the defect. Further investigations are required to clarify this uncertainty.

Conclusions

Granulation tissue derived from intra-bony periodontal defects represents an inflamed tissue containing cell populations with properties characteristic for mesenchymal stem cells. The present in-vitro-study highlights the expression changes chronologically occurring during neurogenic, angiogenic and osteogenic differentiation of inflamed human periodontal ligament stem cells. Our data strongly suggest that these cells are able to undergo neuronal, endothelial and osteoblastic differentiation. As formation of nerves, blood vessels and alveolar bone are key processes in the regeneration of periodontal tissues, we strongly believe that the preservation of granulation tissue, to date considered as tissue of minor value and routinely removed, could promote the healing processes in intra-bony periodontal defects. This knowledge may lead to a paradigm shift in regenerative periodontal surgery, where the preservation of granulation tissue as 'autologous' tissue could replace exogenous materials like bone substitutes or occlusive membranes. In addition, the granulation tissue of intra-bony periodontal defects may be considered as an easily accessible source for MSC in regenerative medicine.

Declarations

Ethics approval and consent to participate

The present study was reviewed and approved by the Ethical Committee of Hannover Medical School (No. 1096). A written informed consent was obtained from all subjects included in the study.

Consent for publication

The patient who contributed to the realization of the manuscript through clinical and radiographic pictures gave her written consent for publication.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Conflicts of interest:

The authors declare that there is no conflict of interest within the meaning of the guidelines of the International Committee of Medical Journal Editors.

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