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Cells from granulation tissue of intra-bony periodontal defects reveal neurogenic and angiogenic differentiation potential and express the embryonic transcription factors NANOG, OCT4 and SOX2

Introduction: Aim of the present study was to assess if potent mesenchymal stem cells reside in the granulation tissue of intra-bony periodontal defects (IPD). The regeneration of IPD requires formation of new blood vessels and nerve fibers to ensure the neurovascular supply of the regenerated periodontal tissues (alveolar bone, periodontal ligament, root cementum). The main focus was to investigate the expression of osteogenic markers (BMP2, BMP4) and embryonic pluripotency factors (NANOG, OCT4, SOX2) during neurogenic and angiogenic differentiation.

Methods: Cells from granulation tissues of 5 systemically healthy subjects (mean age: 44.04 ± 5.73 years; range: 35 to 49 years) were used for the present investigation. The expression of mesenchymal (CD73, CD90, CD105, CD146, STRO1), hematopoietic (CD34, CD45) and embryonic stem cell markers (SSEA4, NANOG, OCT4 and SOX2) was analysed using flow cytometry. Neurogenic and angiogenic differentiation was induced with respective differentiation media for 5 weeks. Cultures grown in the maintenance medium without differentiation substances were used as negative controls. Changes in cell morphology were documented with an inverted microscope. The mRNA-expression of proteins characteristic for neurons (NEFL, NCAM1, ENO2), endothelial cells (ANGPT1, VEGFR1, VEGFR2, PECAM1), osteoblasts (BMP2, BMP4), and embryonic stem cells (NANOG, OCT4, SOX2) was analysed using qRT-PCR.

Results: The data of flow cytometry revealed a very high expression of CD73 (97.66 ± 1.92 %) and CD90 (98.87 ± 0.93 %), a high expression of CD105 (78.02 ± 12.81 %) and CD146 (80.64 ± 23.87 %) and a low expression of STRO1 (5.29 ± 3.62 %). The pluripotency factors demonstrated a very high expression of OCT4 (94.60 ± 1.95 %) and SOX2 (98.27 ± 0.36 %) and a lower but significant expression of NANOG (52.09 ± 6.98 %) and SSEA4 (29.76 ± 12.38 %). The neurogenic and angiogenic differentiation was documented

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through morphologic changes towards a neuron- and endothelial cell-like phenotype and through the continuously increasing mRNA-expression of neuronal (NEFL, NCAM1, ENO2) and endothelial markers (VEGFR1, VEGFR2, PECAM1). The expression of BMP2 and NANOG was significantly up-regulated and the expression of BMP4 and SOX2 was significantly down-regulated in cells grown in both differentiation media. OCT4 did not significantly change, but there was a clear down-regulation in 3 out of our 5 donors.

Conclusions: The granulation tissue of IPD contains cells with properties of mesenchymal stem cells. Therefore, it is a possible source for regenerative therapeutic applications. The embryonic pluripotency factors NANOG and SOX2 were influenced by differentiation media and seem to be involved in the regulation of multipotency and differentiation in mesenchymal stem cells from inflamed periodontal granulation tissue.

Keywords: inflamed periodontal granulation tissue; mesenchymal stem cells; neurogenic differentiation; angiogenic differentiation; bone morphogenic proteins; embryonic pluripotency factors

Introduction

The ultimate goal of regenerative periodontal surgery is the structural and functional regeneration of tissues that have been lost due to periodontal diseases. Histological examinations have shown that the sole removal of the microbial biofilm results in the formation of a long junctional epithelium along the mechanically debrided root surfaces. This is known as reparative healing [8]. A regenerative healing can only be achieved through the application of specific surgical techniques, like the guided tissue regeneration or the bio-modification of the root surfaces with enamel matrix derivatives [18, 38, 47]. There is consensus in the scientific literature that periodontal regeneration also requires the presence of progenitor cells. These cells differentiate under the influence of growth and differentiation factors into osteoblasts for the regeneration of the alveolar bone, into fibroblasts for the regeneration of the periodontal ligament, and into cementoblasts for the regeneration of the root cementum [22]. All these periodontal tissues originate from the ectomesenchyme of the neural crest [10]. Therefore, mesenchymal stem cells can be regarded as ideal progenitor cells for periodontal regeneration.

Intra-bony periodontal defects resulting from inflammatory periodontal diseases represent the most frequent indication for regenerative periodontal surgery. These defects are characterized by the formation of intra-bony pockets, in which the healthy periodontal tissues are replaced through an inflammatory granulation tissue. This tissue has been assumed to negatively influence the treatment outcome and is generally resected during regenerative periodontal surgery [11]. The granulation tissue preservation technique (GTPT, Fig. 1) was introduced 2013 by our group and has led to promising results from a clinical and radiographic perspective [20]. Possible explanations are

1. the preservation of the blood vessel network favoring the initial wound healing,
2. the preservation of an endogenous matrix as soft tissue support, and
3. the preservation of multipotent mesenchymal stem cells, which are required for the regeneration of the periodontal tissues.

Mesenchymal stem cells can be found in many healthy tissues of the oral cavity, but they are also present in inflamed tissues. Thus, cells isolated from the pulp of teeth with irreversible pulpitis as well as from granulation tissue of intra-bony periodontal defects were shown to have properties of mesenchymal stem cells [30, 31, 33]. Granulation tissue of intra-bony periodontal defects is easily accessible through a simple minimally invasive surgical intervention. Therefore, it is a useful source for the extraction of mesenchymal stem cells. The isolation of mesenchymal stem cells from inflammatory granulation tissue, their *ex vivo* expansion and finally their replantation in an increased number, is far away from being routine in regenerative periodontal surgery. However, it seems likely that the increased number of undifferentiated progenitor cells may have a positive influence on the regeneration of periodontal defects.

During periodontal regeneration, the formation of nerve fibers and blood vessels is required to ensure the neurovascular supply of the regenerated periodontal tissues. Therefore, aim of the present study was to assess the neurogenic and angiogenic differentiation potential of inflamed human periodontal ligament stem cells (ihPDLSCs). Special attention was given to the expression of bone morphogenic proteins and embryonic transcription factors. Bone morphogenic proteins (BMPs) belong to the transforming growth factor β superfamily. In addition to postnatal bone formation, BMPs participate in several non-osteogenic developmental processes, like neurogenesis and angiogenesis [9, 16, 37]. The pluripotency factors nanog homeobox (NANOG), octamer-binding transcription factor 4 (OCT4) and SRY (sex determining region Y)-box 2 (SOX2) are known to regulate the processes of self-renewal and differentiation in embryonic stem cells [29, 46]. There is growing evidence that they may play a similar role in

multipotent mesenchymal stem cells. Our experiments intended to examine how the osteogenic markers BMP2 and BMP4 as well as the pluripotency factors NANOG, OCT4 and SOX2 are expressed by ihPDLSCs during cultivation in neurogenic and angiogenic differentiation media.

Material and methods

Isolation and cultivation of ihPDLSCs

Five systemically healthy subjects with a mean age of 44.04 ± 5.73 years (range: 35 to 49 years), who suffered from advanced chronic periodontitis, were selected as donors. Before surgery, all patients received a comprehensive periodontal treatment consisting of professional dental cleaning, oral hygiene instructions and non-surgical periodontal treatment (scaling and root planning). In cases of a residual periodontal defect exhibiting a probing pocket depth > 6 mm, bleeding on probing and a radiographically evident intra-bony component ≥ 3 mm, regenerative periodontal surgery was performed. The granulation tissue preservation technique, comprehensively described by Günay et al. [20], was applied in all cases. Aim of this technique is to preserve as much granulation tissue as possible during mobilization of the mucoperiosteal flaps. The residual granulation tissue was harvested from the bottom of the intra-bony defect and used for the present *in-vitro* investigation. The exposed, defect-related root surfaces were mechanically debrided using sonically-driven scalers (SONICflex, KaVo, Biberach, Germany) and hand curettes. Consecutively, the regenerative procedure consisting of the application of 24 % EDTA gel (PrefGel, Straumann, Freiburg, Germany), irrigation with sterile isotonic sodium chloride solution and application of enamel matrix derivatives (Emdogain, Straumann) was conducted. At the end of surgery, the mucoperiosteal flaps with the adherent granulation tissue were repositioned and fixed with interrupted sutures.

Immediately after harvesting, the granulation tissue was sliced into the smallest pieces possible using sterile scissors and scalpel blades. The enzymatic digestion was performed for 1 hour at 37 °C using alpha minimal essential medium (α-MEM, Gibco, Grand Island, NY, USA) supplemented with 3 mg/ml collagenase type I (Gibco/Life Technologies, Paisley, Scotland) and 4 mg/ml dispase II (Sigma Aldrich, Steinheim, Germany). Afterwards, the mixture was passed through a strainer with a pore size of 70 µm (EASYstrainer, Greiner bio-one,

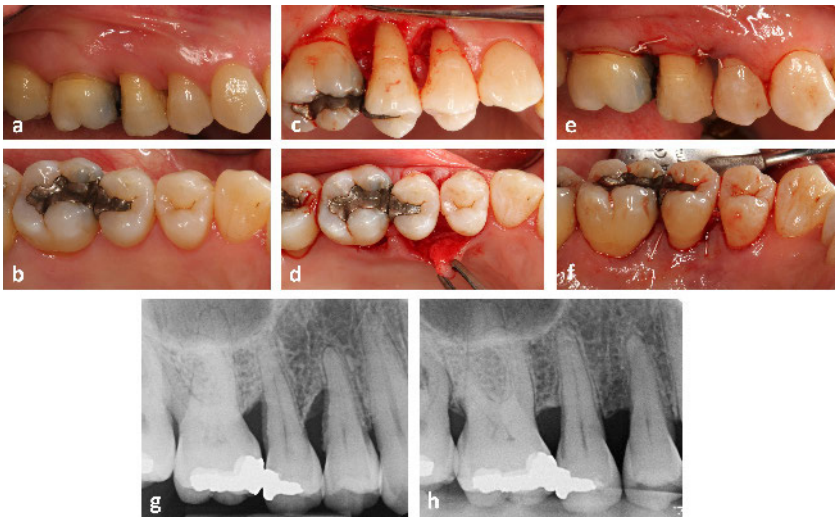


Figure 1a–h Representative case presentation of the granulation tissue preservation technique (GTPT) on teeth 14 and 15: Clinical aspect before (**a, b**) and during surgery (**c, d**): Note the intra-bony defects at the distal areas of teeth 14 and 15 as well as the preserved granulation tissue at the interdental papillae. Clinical aspect after surgery immediately following wound closure with interrupted sutures (**e, f**). Radiological aspect before treatment. Note the calcified deposits and the intra-bony defects at the distal areas of teeth 14 and 15 (**g**). Radiological aspect 16 months after surgery. Note the bone fill at the former intra-bony defects (**h**).

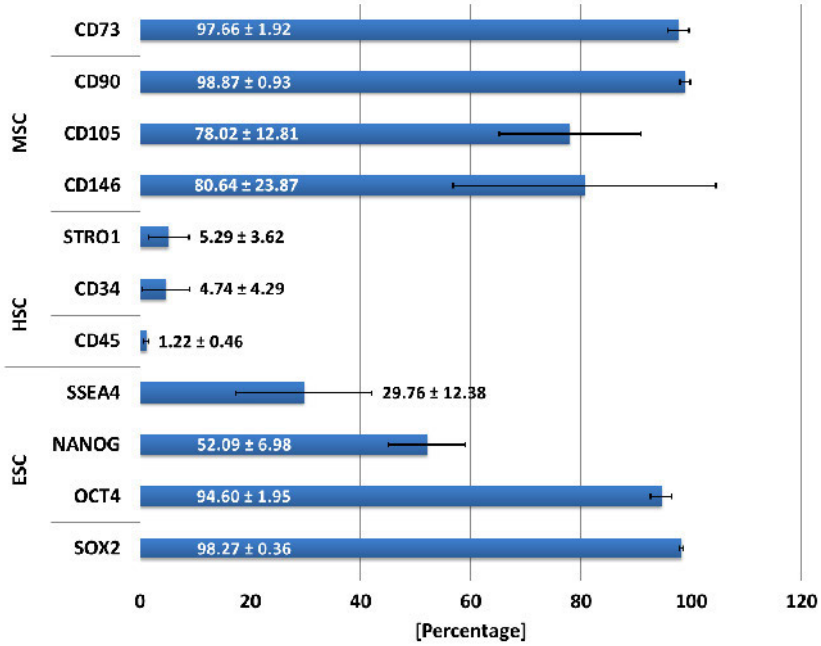


Figure 2 Summarized data of the immunophenotypic characterization using flow cytometry: Expression of markers characteristic for mesenchymal stem cells (MSC: CD73, CD90, CD105, CD146, STRO1), hematopoietic stem cells (HSC: CD34, CD45) and embryonic stem cells (ESC: SSEA4, NANOG, OCT4, SOX2) of all donors (n = 5).

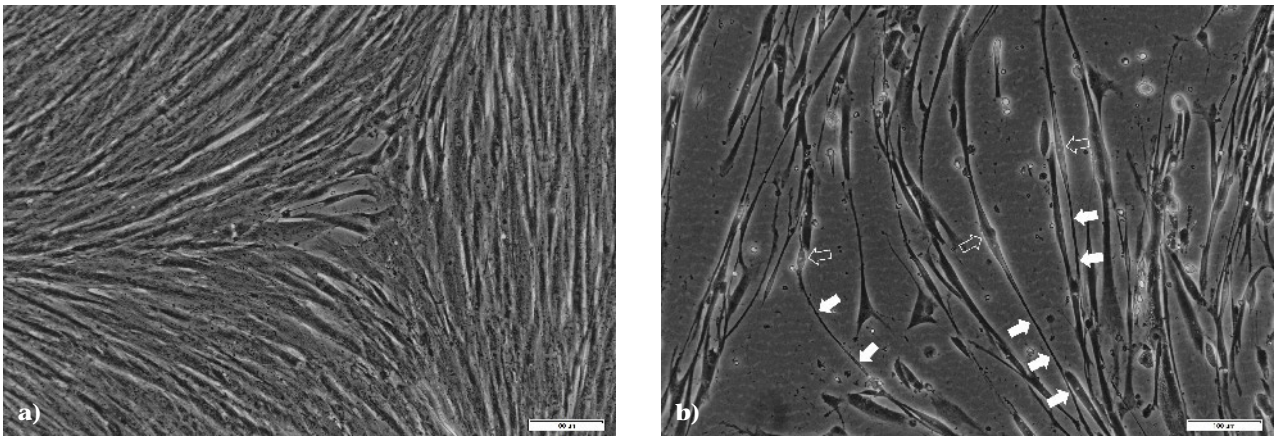


Figure 3a and b Histomorphologic changes occurring during neurogenic differentiation: Representative microscopic images recorded during culture in (**a**) CCM and (**b**) NDM 7 days after seeding. Note the strung-out cell body (open arrow) and the dendrite-like extensions (white arrows) in cells grown in NDM.

Frickenhausen, Germany) and re-suspended in complete culture medium (CCM) consisting of α-MEM supplemented with 15 % fetal 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 resulting single cell suspension was transferred to culture flasks and expanded under humidified atmosphere at 37 °C and 5 % CO_2. Cells of the passages 2–4 were used for the experiments.

The present study was approved by the ethical committee of Hannover Medical School (ethics vote no. 1096). All donors were informed – verbally and in writing – about the intended use of the tissue samples and the goals of the study. All participants signed a consent form.

Characterization of cells using flow cytometry

The cells were seeded into 75 cm^2 culture flasks and expanded in CCM until they reached confluency. Subsequently, the cells were detached from the culture vessel via trypsinization, washed with phosphate buffered saline (PBS) and re-suspended in FACS buffer consisting of PBS, 1 % bovine serum albumin and 0.1 % sodium azide. Each sample contained 1× 10^6 cells/100 µl FACS buffer. Unspecific binding sites (Fc receptors) were blocked through incubation with 1 µg of human IgG (Sigma-Aldrich) for 15 min on ice. For extracellular staining, the cells were incubated for 25 min on ice in the dark with the fluorochrome-conjugated antibodies STRO1-FITC (fluorescein isothiocyanate), CD146-PE (phycoerythrin), CD105-APC (allophycocyanin), CD90-FITC, CD73-FITC, CD45-PE, CD34-APC, and SSEA4-FITC (all from BioLegend, Fell, Germany). For intracellular staining, the cells were fixed using paraformaldehyde-containing buffer (fixation buffer, BD Biosciences, Heidelberg, Germany), permeabilized using saponin-containing buffer (perm/wash buffer, BD Biosciences) and incubated with the fluorochrome-conjugated antibodies NANOG-PE, OCT4-Alexa Fluor 647 and SOX2-PE (all from BD Biosciences) for 25 min on ice in the dark. The flow cytometry analyses were performed using a BD LSR II Flow Cytometer (BD Biosciences). For each sample, 100,000 events were read. The analyses of the raw data were performed using the Summit 5.1 Software (Beckman Coulter, Fullerton, USA). The described procedure was conducted at least thrice for each donor.

Induction of neurogenic differentiation

Cells were seeded into six-well plates coated with 0.1 % gelatin (Sigma-Aldrich) at 1× 10^5 cells/well. Cells were cultivated for 5 weeks in neurogenic differentiation medium (NDM) consisting of neurobasal A medium (Gibco) supplemented with B27 supplement (2 % v/v, 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. Morphological changes towards a neuron-like phenotype were observed using an inverted microscope (Olympus Optical Co. Ltd., Tokyo, Japan). Expression of the neuronal marker proteins neurofilament light polypeptide (NEFL), neural cell adhesion molecule 1 (NCAM1) and enolase 2 (ENO2), of the osteogenic marker proteins BMP2 and BMP4 and of the embryonic transcription factors NANOG, OCT4 and SOX2 were assessed using quantitative reverse transcriptase polymerase chain reaction. These investigations were conducted at days 0, 3, 7, 14, 21, 28 and 35. Cells grown in CCM were used as negative control. NDM and CCM were changed every 2–3 d.

Induction of angiogenic differentiation

Cells (1× 10^5) were seeded into six-well plates coated with collagen I (Santa Cruz Biotechnology, Heidelberg, Germany) and expanded in CCM until they reached confluency. Afterwards, CCM was replaced through the angiogenic differentiation medium (ADM), which consisted of M199 medium (Gibco) supplemented with 5 % FBS,

	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)
Angiogenic	Hs_ANGPT1_1_SG	Angiopoietin 1 (ANGPT1)	QT00046865	NM_001146 (4338 bp) NM_139290 (2379 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_BMP2_1_SG	Bone morphogenic protein 2 (BMP2)	QT00012544	NM_001200 (3150 bp)
	Hs_BMP4_1_SG	Bone morphogenic protein 4 (BMP4)	QT00012033	NM_001202 (1957 bp)
Embryonic	Hs_NANOG_1_SG	Nanog homeobox	QT01025850	NM_024865 (2103 bp) NM_001297698 (2055 bp)
	Hs_POU5F1_1_SG	POU class 5 homeobox 1 (POU5F1)/octamer-binding transcription factor 4 (Oct4)	QT00210840	NM_001173531 (1589 bp) NM_002701 (1430 bp) NM_203289 (2075 bp) NM_001285986 (2300 bp) NM_001285987 (2075 bp)
	Hs_SOX2_1_SG	SRY (sex determining region Y)-box 2 (Sox2)	QT00237601	NM_003106 (2520 bp)

Table 1 QuantiTect primer assays (Qiagen) used for the qRT-PCR analyses

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 endothelial 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). The cells were cultivated for 5 weeks. Morphological changes towards an endothelial cell-like phenotype were observed using an inverted microscope. Quantitative reverse transcriptase polymerase chain reaction was used to assess the expression of the angiogenic marker proteins vascular endothelial growth factor receptor 1 and 2 (VEGFR1, VEGFR2), angiopoietin 1 (ANGPT1), and platelet endothelial cell adhesion molecule 1 (PECAM1), expression of the osteogenic marker proteins BMP2 and BMP4 and expression of the embryonic transcription factors NANOG, OCT4, and SOX2. These investigations were con-

Housekeeping genes	Hs_RRN18S_1_SG	18S ribosomal RNA (RRN18S)	QT00199367	X03205 (1869 bp)
	Hs_ACTB_1_SG	Actin, beta (ACTB)	QT00095431	NM_001101 (1852 bp)
	Hs_B2M_1_SG	Beta-2-microglobulin (B2M)	QT00088935	NM_004048 (987 bp) XM_005254549 (424 bp) XM_006725182 (424 bp)
	Hs_GAPDH_2_SG	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	QT01192646	NM_002046 (1421 bp) NM_001289745 (1513 bp)
	Hs_SDHA_2_SG	Succinate dehydrogenase complex flavoprotein subunit A (SDHA2)	QT01668919	NM_004168 (2803 bp) NM_001294332 (2659 bp) XM_005248329 (2245 bp) XM_005248331 (2151 bp)
	Hs_YWHAZ_2_SG	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta (YWHAZ)	QT02321522	NM_001135699 (3020 bp) NM_001135700 (2974 bp) NM_001135701 (3023 bp) NM_001135702 (3042 bp) NM_003406 (3003 bp) NM_145690 (3077 bp) XM_005251060 (3295 bp) XM_005251061 (3390 bp) XM_005251062 (3165 bp) XM_005251063 (3152 bp)

Table 1: QuantiTect primer assays (Qiagen) used for the qRT-PCR analyses

ducted at days 0, 3, 7, 14, 21, 28 and 35. Cells grown in CCM were used as negative control. ADM and CCM were changed every 2 to 3 d.

Quantitative reverse transcriptase polymerase chain reaction

The expression changes occurring during neurogenic and angiogenic differentiation were investigated on transcriptional level using a two-step reverse transcriptase polymerase chain reaction. Briefly, total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Genomic DNA was eliminated through on-column DNA digestion (RNase-free DNase Set, Qiagen). The RNA concentration was measured using a microplate reader (Synergy H1, BioTek, Bad Friedrichshall, Germany). Synthesis of cDNA was conducted using 1 µg of isolated RNA and the QuantiTect Reverse Transcription Kit (Qiagen). Amplification and real-time quantification of the target cDNA were conducted using the QuantiTect SYBR Green PCR Kit, the QuantiTect Primer Assays (Tab. 1) and the Rotor-Gene Q Cycler (all from Qiagen). The PCR reactions involved an initial incubation at 95 °C for 5 min activating the HotStarTaq DNA polymerase and subsequent 40 cycles consisting of denaturation at 95 °C for 5 sec as well as annealing and extension at 60 °C for 10 sec. A melting curve was used to validate the specificity of the reaction products. Baseline correction, determination of the window of linearity and calculation of the PCR efficiency was performed using the software program LinRegPCR [36]. Normalization was conducted using 2 of the following housekeeping genes: 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). The two most stable housekeeping genes were identified with the software applet geNorm [44] and used for the normalization of the adjusted PCR data. Fold changes in gene expression were calculated using the delta delta CT method [34].

Statistical analysis

The qRT-PCR data recorded during the differentiation experiments were standardized by logarithmic transformation, mean centring and autoscaling [45]. The software program GraphPad Prism 6.0 (GraphPad Software, Inc.; La Jolla, CA 92037 USA) with one-way ANOVA was applied for the statistical analyses. Dunnett's multiple comparison tests were used as post-hoc tests. The main focus was to detect significant differences in gene expression compared to baseline/reference values at day 0. A value of $p \leq 0.05$ was considered statistically significant.

Results

Characterization of cells using flow cytometry

The mesenchymal stem cell markers CD73 (97.66 ± 1.92 %) and CD90 (98.87 ± 0.93 %) were expressed by almost all uninduced ihPDLSCs. CD146 (80.64 ± 23.87 %) and CD105 (78.02 ± 12.81 %) were expressed by the majority of cells. A low expression was observed for STRO1 (5.29 ± 3.62 %). The hematopoietic

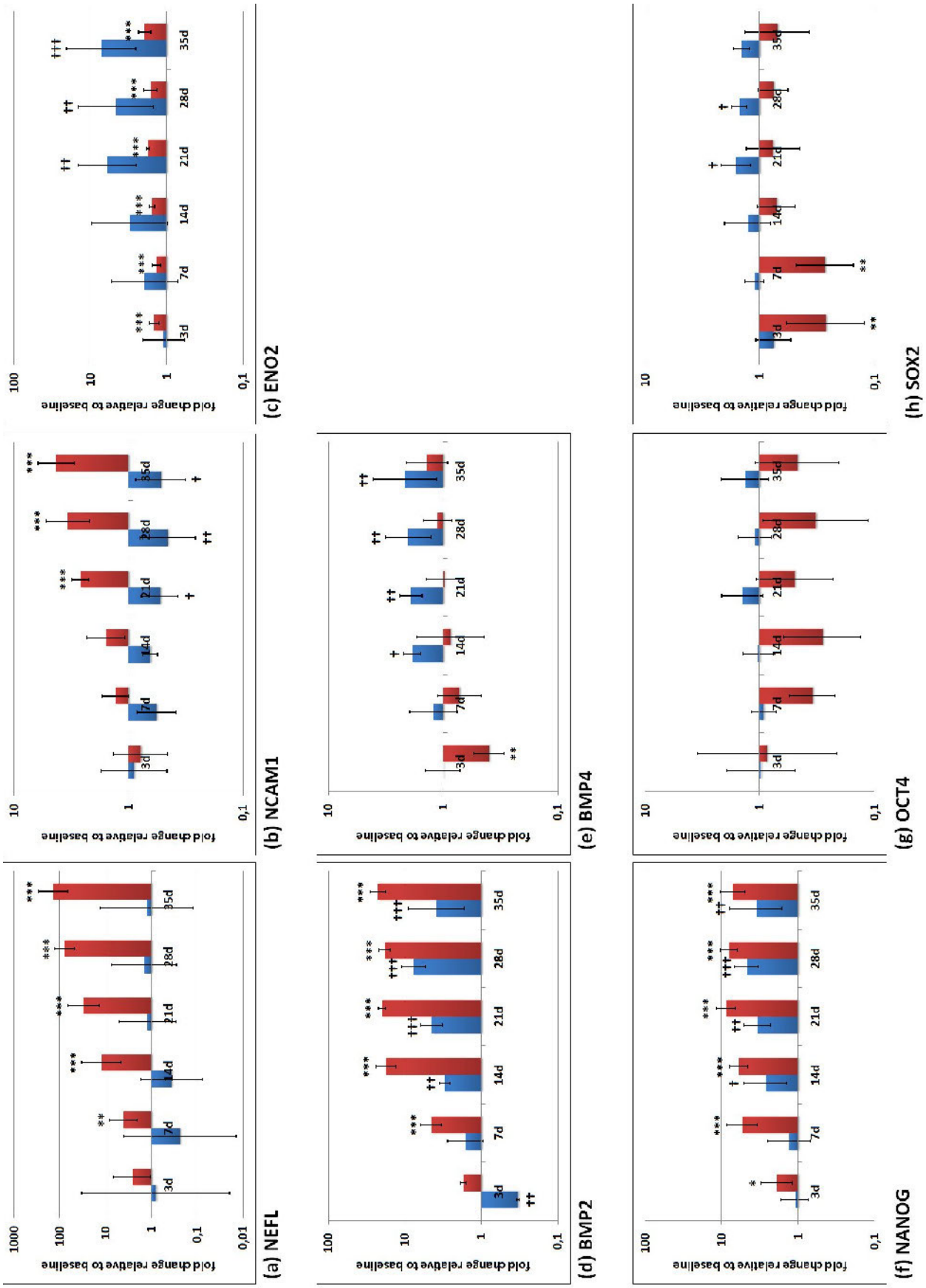


Figure 4a–h Expression of neuronal (NEFL, NCAM1, ENO2), osteogenic (BMP2, BMP4) and embryonic markers (NANOG, OCT4, SOX2) during neurogenic differentiation: The qRT-PCR data are presented as recalculated averages with upper and lower confidence interval (cells grown in NDM: red columns; cells grown in CCM: blue columns). One-way ANOVA with Dunnett's Multiple Comparison Test was used to detect significant differences to the reference value at day 0 (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; †: $p < 0.05$; ††: $p < 0.01$; †††: $p < 0.001$).

stem cell marker CD34 (4.74 ± 4.29 %) showed a low expression as well. Cells expressing the leucocyte common antigen CD45 (1.22 ± 0.46 %) were not detectable in most cases. The embryonic stem cell markers OCT4 (94.60 ± 1.95 %) and SOX2 (98.27 ± 0.36 %) were expressed by almost all cells. NANOG (52.09 ± 6.98 %) and SSEA4 (29.76 ± 12.38 %) showed a medium expression level. The summarized flow cytometry data are given in Figure 2.

Neurogenic differentiation

Cells cultivated in NDM proliferated slowly and showed first morphological changes towards a neuron-like phenotype 3–7 d after seeding (Fig. 3). Thus, cell bodies with elongated shape and dendrite-like extensions occurred. Cells grown in CCM proliferated rapidly and continued showing the fibroblast-specific spindle-shaped morphology. Due to proliferation and multilayered allocation, distinction of cells became more and more difficult at the advanced stages of the experiments. In both media, but in particular in the NDM, a parallel allocation of cells was observed.

Expression of neurogenic markers

In addition to morphological changes, expression of neuron-specific marker proteins was investigated on mRNA level (Fig. 4). Cells grown in NDM showed a continuously increasing expression of NEFL, NCAM1 and ENO2 when compared to cells lysed at day 0. The expression of NEFL was significantly up-regulated at day 7 ($p < 0.01$), 14, 21, 28 and 35 ($p < 0.001$). The expression of NCAM1 was significantly elevated at day 21, 28 and 35 ($p < 0.001$). The expression of ENO2 was significantly increased at day 3, 7, 14, 21, 28 and 35 ($p < 0.001$). In cells grown in CCM, the expression of NEFL did not significantly change, the expression of NCAM1 was significantly down-regulated at day 21 ($p < 0.05$), 28 ($p < 0.01$) and 35 ($p < 0.05$), and the expression of ENO2 was significantly up-regulated at day 21 ($p < 0.01$), 28 ($p < 0.01$) and 35 ($p < 0.001$).

The expression of BMP2 and BMP4 significantly changed in both media. The expression of BMP2 continuously increased in cells grown in NDM with significant values at day 7, 14, 21, 28 and 35 ($p < 0.001$) when compared to day 0. In cells grown in CCM, the BMP2-expression was down-regulated at day 3 ($p < 0.01$) and afterwards up-regulated with significant values at day 14 ($p < 0.01$), 21, 28 and 35 ($p < 0.001$). The BMP4-expression showed a significant increase in cells grown in CCM (day 21, 28 and 35; $p < 0.01$) and a significant decrease in cells grown in NDM (day 3; $p < 0.01$).

Expression of pluripotency markers

The transcription factor NANOG showed a continuously increasing expression in both media. This was significant at day 3 ($p < 0.05$), 7, 14, 21, 28 and 35 ($p < 0.001$) in cells grown in NDM and at day 14 ($p < 0.05$), 21 ($p < 0.01$), 28 ($p < 0.001$) and 35 ($p < 0.01$) in cells grown in CCM. The expression of OCT4 did not significantly change in both media. The expression of SOX2 was significantly down-regulated in cells grown in NDM (day 3 and 7; $p < 0.01$) and up-regulated in cells grown in CCM (day 21 and 28; $p < 0.05$).

Angiogenic differentiation

Cells cultivated in the ADM showed a polygonal endothelial cell-like morphology. This became evident especially at the advanced stages of the experiments (Fig. 5). The allocation of cells changed from a shoal-like to a cobblestone-like pattern. Cells grown in CCM continued showing a spindle-shaped morphology and a shoal-like allocation. The proliferation speed was comparable in both media.

Expression of angiogenic markers

Expression of endothelial cell-specific marker proteins was assessed on the transcriptional level (Fig. 6). A continuously increasing expression of VEGFR1, VEGFR2 and PECAM1 was observed in cells grown in ADM. The expression changes were significant at any time of the assessment. Interestingly, the expressions of VEGFR1 and PECAM1 were also up-regulated in cells grown in CCM. Thus, the expression of VEGFR1 was significantly increased at day 14 ($p < 0.01$), 21, 28 and 35 ($p < 0.001$). The expression of PECAM1 was significantly increased at day 7, 14, 21, 28 and 35 ($p < 0.001$). The expression of ANGPT1 was significantly down-regulated during the entire differentiation experiments in cells grown in ADM ($p < 0.001$) and slightly up-regulated in cells grown in CCM with a significant value at day 35 ($p < 0.05$).

The osteogenic marker proteins BMP2 and BMP4 showed significant expression changes in both media. A continuously increasing expression of BMP2 was observed in both media (significant at any time of the assessment; $p < 0.001$). The expression of BMP4 was significantly down-regulated at day 3, 7, 14, 21, 28 and 35 in cells grown in ADM and significantly up-regulated at day 14 and 35 in cells grown in CCM.

Expression of pluripotency markers

The pluripotency marker NANOG significantly increased in both media ($p < 0.001$ at any time of the assessment). OCT4 did not significantly change in both media. The expression of SOX2 was significantly down-regulated in cells grown in ADM (day 7; $p < 0.05$) and significantly up-regulated in cells grown in CCM (day 21 and 35; $p < 0.01$).

Discussion

Due to their multilineage differentiation potential, mesenchymal stem cells have attracted growing interest in regenerative medicine. Properties of mesenchymal stem cells have been attributed to cell populations isolated from inflamed pulpal [40], gingival [17, 40], and periodontal tissues [30, 31]. The purpose of the present study was to isolate and characterize cells from inflamed granulation tissue of intra-bony periodontal defects. Special focus was given to the neurovascular differentiation potential of the isolated cell populations. The neurogenic and angiogenic differentiation pathways were induced with well-established differentiation media [3, 4]. Changes in the expression of

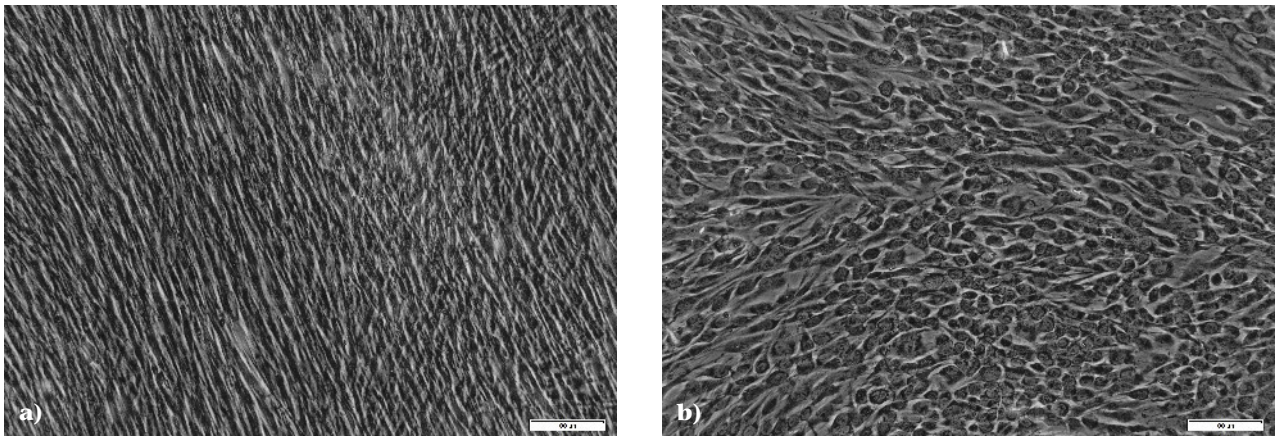


Figure 5a and b Histomorphological changes occurring during angiogenic differentiation: Representative microscopic images recorded during culture in (**a**) CCM and (**b**) ADM 21 days after initiation of the differentiation process. Note the round, polygonal cell morphology and the cobblestone-like arrangement.

the embryonic transcription factors NANOG, OCT4 and SOX2 during these differentiation processes were investigated to provide further insights to their functional role in adult mesenchymal stem cells. Moreover, the functional role of the osteogenic marker proteins BMP2 and BMP4 within the neurogenic and angiogenic differentiation process was evaluated on mRNA level.

Periodontal diseases are mainly caused by anaerobic, gram-negative bacteria, known as periodontal pathogens. Despite the inflamed condition and the presence of periodontal pathogens, harvesting of the periodontal granulation tissues was not associated with any event of microbial contamination during the establishment of our cell cultures. This was due to the non-surgical periodontal pretreatment, which led to a significant reduction of the bacterial load within the periodontal pocket, and due to the supplemented antimicrobials that were used during the enzymatic digestion, cell culture expansion and long-term experiments.

Flow cytometry was used to investigate the expression of epitopes characteristic for mesenchymal stem cells (CD73, CD90, CD105, CD146, SSEA4 and STRO1) and the expression of transcription factors characteristic for embryonic stem cells (NANOG, OCT4 and SOX2). Almost all investigated cells expressed CD73 and CD90 (> 95 %). In addition, the majority of cells expressed CD105 (78 %). CD73, CD90 and CD105 are not only expressed by mesenchymal stem cells, but also by differentiated cells like fibroblasts [28]. Therefore, further markers, like CD146, SSEA4, and STRO1, have been used to prove the presence of mesenchymal stem cells [5, 14, 15, 24, 25]. CD146 (80 %) showed high, SSEA4 (29 %) medium and STRO1 (5 %) low expression in our cell cultures. Recently, comparable results were published for these cell surface molecules [2]. Interestingly, the pluripotency markers NANOG (52 %), OCT4 (94 %) and SOX2 (98 %) were expressed by a large part of our cell populations. This topic will be discussed in more detail at the end of the discussion section. In summary, the flow cytometry data (Fig. 2) suggest that a significant number of mesenchymal stem cells reside in the granulation tissue of intra-bony periodontal defects.

Experiments investigating the neurovascular differentiation potential of ihPDLSCs were used to prove multipotency. Most studies use the osteogenic, adipogenic and chondrogenic pathway to prove multilineage differentiation potential, as proposed by Dominici et al. [12]. Accordingly, Páll et al. [30] demonstrated that cells derived from periodontal granulation tissue are able to generate Alizarin Red S-positive mineralized extracellular matrix, Oil Red O-positive lipid deposits and Alcain Blue-positive chondrogenic micromasses. Aside from osteogenesis, neither adipogenesis nor chondrogenesis is required for the regeneration of tooth-supporting tissues. Therefore, we decided to focus our experiments on the neurogenic and angiogenic differentiation pathways, which are required to warrant the neurovascular supply of the regenerated tissues. During our experiments, we observed the development of a neuron- and endothelial cell-like phenotype (Fig. 3 and 5). Cells grown in NDM exhibited the slowest proliferation rate when compared to cells grown in ADM and CCM. Cells grown in ADM and CCM reached complete confluency on average after 3–7 d. At the same time, there were wide open intercellular spaces in cultures grown in NDM. This phenomenon can be interpreted as prompt modification of the cellular metabolism towards the neurogenic differentiation pathway. In addition, cells grown in NDM immediately started to change their morphological appearance. The elongated cell bodies and the dendrite-like cell protuberances were clearly different from the cell morphologies that were observed in cells grown in CCM. Significant morphological changes induced by ADM were observed later and became obvious after 14–21 d through the development of round-shaped, cobblestone-like arranged cells with a chromatin-rich nucleus. Both differentiation media had a significant impact on the cell

morphology and the pattern of cellular growth. This is an indication that a differentiation process had taken place.

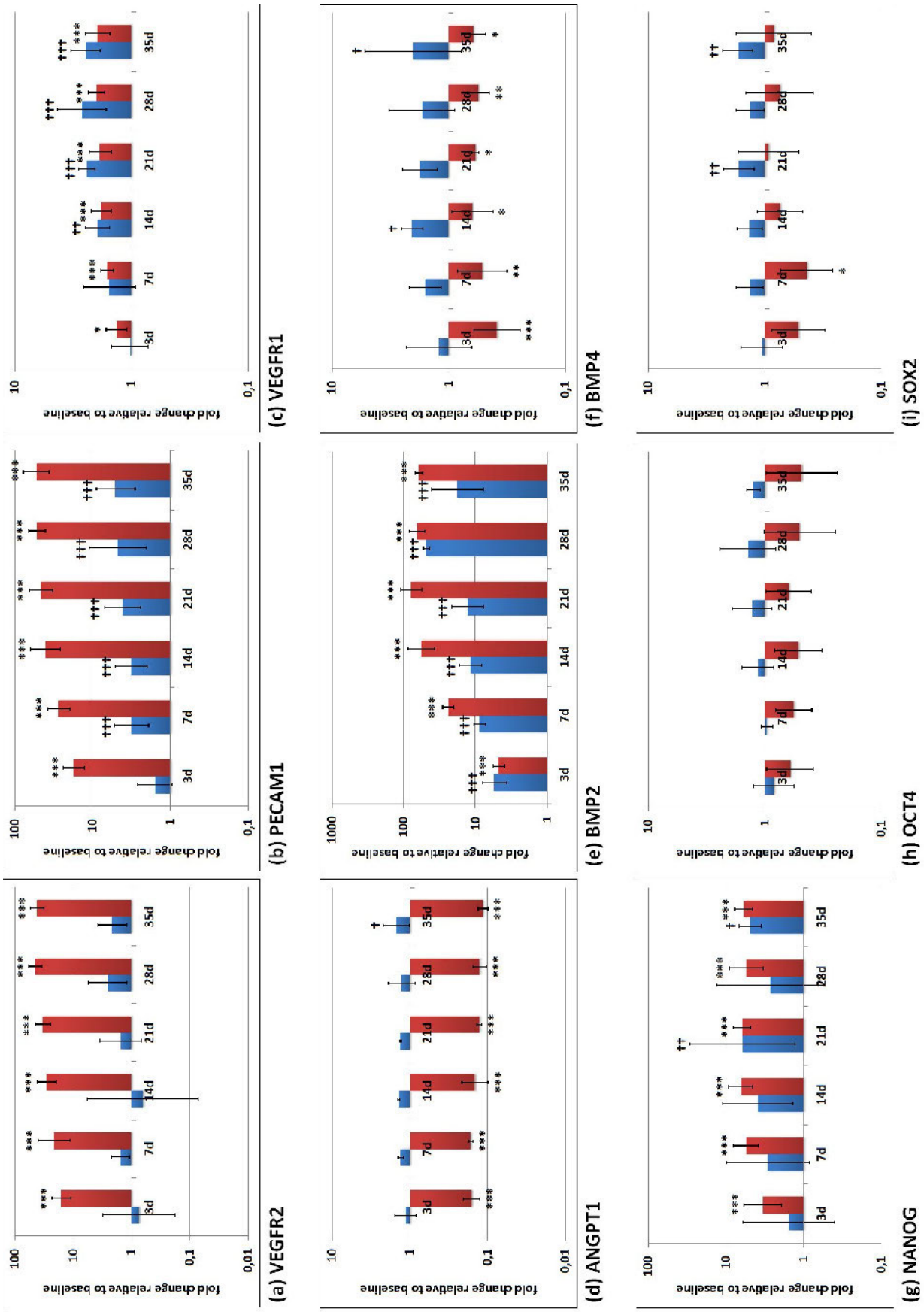
On the transcriptional level, a continuously increasing expression of neuron- (NEFL, NCAM1, ENO2) and endothelial cell-specific marker proteins (VEGFR1, VEGFR2, PECAM1) was observed (Fig. 4 and 6). Interestingly, the expression of ENO2, PECAM1 and VEGFR1 was also increased in cells grown in CCM. These observations suggest that cells grown in CCM show the tendency towards spontaneous differentiation with advanced duration of culture. However, the expression of ANGPT1 was significantly different between cells grown in ADM and CCM, respectively. Whereas a slight increase was observed in cells grown in CCM, a significant decrease was detected in cells grown in ADM. ANGPT1 as an antagonist of ANGPT2 is responsible for the maintenance of vascular quiescence [13]. Branching out of new blood vessels is only enabled when the expression of ANGPT1 is down-regulated, as observed in our experiments when cells were grown in ADM.

Recently, we could show that ihPDLSCs exhibit osteogenic differentiation potential [1]. After induction with osteogenic differentiation medium (ODM), ihPDLSCs showed pronounced matrix mineralization and an up-regulation of alkaline phosphatase and BMP2, which are stage-specific markers characteristic for osteoblastic differentiation. In the present study, we demonstrated that the expression of the osteogenic marker BMP2 is not only increased in cells grown in ODM, but also in cells grown in NDM, ADM and CCM. In cells grown in CCM, this increase can be understood as spontaneous differentiation of ihPDLSCs to osteoblast-like cells, as described by Trivanovic et al. [41]. BMPs are also known to regulate the proliferation and differentiation of neural precursors [37]. Accordingly, Hegarty et al. [21] reported that BMP2 induces neuritogenesis. In addition, BMP2-signaling is involved in vascular development and dysfunction [16]. Benn et al. [6] demonstrated that BMP2 and BMP6 are proangiogenic and induce angiogenesis through binding to specific BMP type I receptors. The results of our study support the hypothesis that BMP2-signaling is involved in neurogenic and angiogenic differentiation.

The expression of BMP4 was significantly down-regulated at the beginning of our neurogenic and angiogenic differentiation experiments. Meyers et al. [27] have shown that an increased expression of BMP4 is associated with reduced neurogenesis and that an inhibition of BMP4-signaling increases neurogenesis. Moreover, BMP4 and VEGF regulate the differentiation of pluripotent stem cells to endothelial cells in a synergistic way [32, 39]. BMP4 is known to convert pluripotent stem cells into mesodermal cells and subsequently VEGF is known to induce the differentiation of endothelial cells. Since ihPDLSCs are cells of mesodermal origin, the expression of BMP4 was not necessarily to be expected and indeed down-regulated during the angiogenic differentiation experiments.

Despite intense research in the field of mesenchymal stem cells, the molecular mechanisms of self-renewal, differentiation and multipotency are not well understood. Pluripotency and self-renewal of embryonic stem cells are regulated by a network of transcription factors, like NANOG, OCT4 and SOX2 [7, 23]. Yu et al. [46] reported that the transcription factors NANOG, OCT4, SOX2 and LIN28 are sufficient to reprogram human somatic cells into induced pluripotent stem cells. This underlines the importance of these factors for the pluripotent state. There is growing evidence that the transcription factors NANOG, OCT4 and SOX2 are not only expressed by embryonic stem cells but also by mesenchymal stem cells [19]. These factors are supposed to regulate the processes of self-renewal, differentiation and multipotency in mesenchymal stem cells [42, 43]. The flow cytometry data of our study revealed that NANOG, OCT4 and SOX2 were expressed by a large part of our heterogeneous cell populations. Therefore, we examined how the expression of these transcription factors was changing during neurogenic and angiogenic differentiation. If the multipotent state is depending on NANOG, OCT4 and SOX2, one would assume that the expression of all these transcription factors is decreasing in the course of differentiation processes. However, the expression of NANOG was significantly up-regulated, the expression of SOX2 was significantly down-regulated (especially at the beginning of the differentiation experiments) and the expression of OCT4 did not significantly change in our study. The large error bars in Figures 4 and 6 reflect that there were distinct inter-individual differences. Interestingly, the expression of OCT4 was significantly down-regulated during both differentiation pathways in 3 donors (with expression patterns of great similarity), almost unchanged in one donor, and clearly up-regulated in one donor (data not shown). Therefore, the most frequently observed decrease in OCT4-expression failed to be statistically significant.

There are several studies investigating the expression of NANOG, OCT4 and SOX2 in mesenchymal stem cells, but only a few assessing the expression changes in the course of *in-vitro* differentiation processes. Greco et al. [19] investigated the expression of NANOG, OCT4 and SOX2 in human bone marrow mesenchymal stem cells using semi-quantitative RT-PCR. They found strong expression of all 3 transcription factors in uninduced cells, but no expression 6 and 12 d following neuronal induction. For OCT4 and SOX2, a similar tendency of decreasing expression could be observed in our study for both, the neurogenic and angiogenic differentiation. Pierantozzi et al. [35] investigated the expression of NANOG, OCT4 and SOX2 in human adult mesenchymal stem cells (bone marrow MSCs, adipose tissue MSCs and cardiac tissue MSCs) using semi-quantitative RT-PCR and immunofluorescence assays. While OCT4 and SOX2 were not detectable at any time of their experiments, NANOG was not expressed by freshly isolated mesenchymal stem cells, but by proliferating cells under *in-vitro* culture conditions. These



(Fig. 1–6; Tab. 1: K. Adam)

Figure 6a–i Expression of angiogenic (VEGFR1, VEGFR2, PECAM1, ANGPT1), osteogenic (BMP2, BMP4) and embryonic markers (NANOG, OCT4, SOX2) during angiogenic differentiation: The qRT-PCR data are presented as recalculated averages with upper and lower confidence interval (cells grown in ADM: red columns; cells grown in CCM: blue columns). One-way ANOVA with Dunnett's Multiple Comparison Test was used to detect significant differences to the reference value at day 0 (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; †: $p < 0.05$; ††: $p < 0.01$; †††: $p < 0.001$).

findings suggest that the expression of NANOG is not required *in-vivo* and only activated when mesenchymal stem cells are grown *in-vitro*. This might be a possible explanation for the continuously increasing expression of NANOG in our study. In addition, Pierantozzi et al. [35] figured out that there was not a direct correlation between the number of NANOG-positive cells and the adipogenic, osteogenic and chondrogenic differentiation potential of the investigated cell populations. Liu et al. [26] investigated the effects of ectopic NANOG and OCT4 overexpression on human bone marrow-derived mesenchymal stem cells. They could show that NANOG and OCT4 overexpression had different effects on adipogenesis. While NANOG overexpression slowed down adipogenesis, OCT4 overexpression improved adipogenesis. Moreover, NANOG and OCT4 overexpression both improved chondrogenesis. These findings suggest that NANOG and OCT4 play a functional role in the *in-vitro* differentiation of mesenchymal stem cells with different functions depending on the differentiation pathway.

To conclude, there are controversial data about the functional role of the transcription factors NANOG, OCT4 and SOX2 in mesenchymal stem cells. However, our data suggest that NANOG and SOX2 are involved in the regulation of multipotency and differentiation, at least under *in-vitro* culture conditions.

Conclusions

The inflamed granulation tissues derived from intra-bony periodontal defects contain cell populations with properties of mesenchymal stem cells. These properties comprise the expression of characteristic cell surface antigens and multilineage differentiation potential. In addition, the pluripotency markers NANOG, OCT4 and SOX2 are expressed by a wide range of the isolated cell populations. In particular NANOG and SOX2 seem to be involved in the processes of self-renewal and differentiation in adult mesenchymal stem cells. Moreover, our *in-vitro* data suggest that BMP2, but not BMP4 is required for the processes of neurogenic and angiogenic differentiation. Future research shall confirm our data on translational level.

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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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