



# Comparative differentiation analysis of distinct oral tissue-derived cells in response to osteogenic stimulation

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## Abstract

**Background** Mixed cell populations from oral tissues may be superior to pure stem cells for regenerative approaches. Therefore, the aim of the present study was to explore the osteogenic potential of mixed cells derived from oral connective tissues compared to alveolar osteoblasts.

**Materials and methods** Primary cells were isolated from the alveolar bone, periodontal ligament and gingiva. Following characterization by colony formation, growth capacity and flow cytometry, all cells were subjected to osteogenic differentiation induction and screened for a large panel of osteogenic markers using western blots, qPCR arrays, and matrix mineralization and alkaline phosphatase quantification.

**Results** Non-induced mixed cells from gingiva showed higher colony formation efficiency but decreased proliferation compared to non-induced periodontal mixed cells, while both entities revealed similar surface markers tested in this setup. Following osteogenic induction, all cell populations individually expressed receptors with distinctively activated downstream effectors. Gene expression of induced periodontal mixed cells was similar to alveolar osteoblasts, but was differently modulated in gingival mixed cells. The latter failed to achieve osteogenic differentiation in terms of matrix mineralization and alkaline phosphatase activity, which was well observed in periodontal mixed cells and osteoblasts.

**Conclusion** Mixed cells from periodontal ligament but not from gingiva feature an inherent osteogenic capacity in vitro. From these results, it can be concluded that periodontal cells do not require further stem cell enrichment in order to qualify for bone regeneration.

**Clinical relevance** Our data contribute to the development of novel cell-based therapies using mixed cells from the periodontal ligament in regenerative periodontics.

**Keywords** Osteoblasts (MeSH-ID D010006) · Periodontal ligament (MeSH-ID D010513) · Gingiva (MeSH-ID D005881) · Cells (MeSH-ID D002477) · Osteogenesis (MeSH-ID D010012)

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## Introduction

Currently, stem cell-based therapies are entering the clinical trial phase [1] and for their successful implementation, it is highly important to unravel which cells may contribute to tissue regeneration. More than a decade ago, mesenchymal stem cells were described to reside in different oral tissues [2, 3], and today, for periodontal regeneration, stem cells from oral connective tissues are considered superior to mesenchymal stem cells from other sources [4–6]. Despite the undeniable body of evidence that oral connective tissues, i.e. periodontal ligament or gingiva, hold progenitor cells, precise isolation and characterization protocols are missing so far [7], and so-called stem cells often are isolated by mere explant culture and/or enzymatic digestion [8]. However, stem or progenitor cell isolation necessarily requires further enrichment by selecting colony-forming or specific epitope-expressing cells [9–11]. Without enrichment, the resulting mixed population has been shown to be predominantly comprised of fibroblasts, while containing less than 1% of stem cells [12]. As a result, a great deal of scientific papers may have used rather mixed cell populations instead of real ‘stem cells’ [13]. Nevertheless, it may be feasible to use mixed cell populations for regeneration therapies [13] because the heterogeneity of differently purified stem cells may impair their therapeutic efficacy and introduce broad variations in the clinical outcome of regenerative approaches [14]. However, mixed cell populations hold the advantage of delivering stem cells together with surrounding non-progenitor cells, which are known to govern the stem cell behaviour [15, 16]. In addition, mixed cells from the periodontal ligament and the gingiva were already found to be capable of a gradual osteogenic differentiation if bone marrow-derived mesenchymal stem cells were present [17]. Moreover, there is evidence that oral fibroblasts even display an osteogenic potential on their own [18–20], emphasizing their usefulness for bone generation in periodontal regeneration. In consequence, transplantation of mixed cells instead of pure stem cells could possibly enhance the therapeutic outcome of regenerative therapies. However, the osteogenic differentiation potential of mixed cell populations from distinct oral tissues is yet to be determined. Hence, the aim of the present study was to investigate the hard tissue-forming capacity of explant culture-derived mixed cells from oral connective tissues and compare them to bone cells. In this context, we hypothesized that mixed cells from the gingiva and periodontal ligament exhibit osteogenic receptors, signalling molecules, genes, matrix calcification and alkaline phosphatase activity comparable to alveolar osteoblasts in response to appropriate biochemical stimulation.

## Materials and methods

### Cell isolation and culture

All experiments were carried out in accordance with the guidelines of the World Medical Association Declaration of Helsinki and were approved by the Committee of Ethics of the Medical Faculty of the Albert Ludwigs University Freiburg, Germany (vote number 516/12). All cells were derived from operative biopsies ( $n = 12$  donors) with informed and written consent. In brief, alveolar osteoblasts were derived from alveolar bone biopsies of patients undergoing corrective osteotomy surgery, and only compact bone parts were used. Periodontal mixed cells were obtained from the ligament tissues of non-carious human premolar or wisdom teeth with healthy periodontium extracted for orthodontic reasons, and the periodontal ligament was scraped off from the middle third of the tooth roots. Gingival mixed cells were derived from healthy gingival connective tissue of donors undergoing gingivoplastic surgery, and tissue fragments were carefully dissected from gingival epithelium. All tissue specimens were rinsed with 10% iodine, thoroughly washed and minced. Tissue fragments were plated as explants in MEMalpha (Lifetechnologies, Darmstadt, Germany) supplemented with 10% foetal bovine serum (Biochrom, Berlin, Germany), 1% glutamax (Life technologies), 1% amphotericin and 1% antibiotics, which was exchanged every 2–3 days until cell outgrowth. Cells were expanded by splitting (osteoblasts passaged (p)4–6 times, periodontal mixed cells p2–4 and gingival mixed cells p3–5), before osteogenic differentiation was induced for 21 days with NH OsteoDiff medium (Miltenyi, Bergisch Gladbach, Germany). All experiments were systematically carried out in replicates for the cells from all donors, except for western blot analyses, which were performed in triplicate by using the cells from two donors each for alveolar osteoblasts and periodontal and gingival mixed cells.

### Colony-forming units and growth curves

For colony-forming unit (CFU) assays, cells were seeded in limiting dilution, i.e. 2 cells/cm<sup>2</sup>, incubated for 14 days, fixed with 4% formaldehyde and stained with 0.1% Azur II (Sigma Aldrich, Taufkirchen, Germany) for 20 min at room temperature, air-dried and photographed with a SZH10 microscope (Olympus, Münster, Germany) equipped with a CCD Colour view III camera and the resulting images were taken using the Cell\* software (both Olympus, Münster, Germany). Images were analyzed using the ImageJ plugin ‘analyze particles’ following threshold adjustment. Stained colonies of  $\geq 50$  cells were scored as CFU-F and counted. CFU-F efficiency was calculated as follows: CFU-F efficiency = (counted CFU-F/cells originally seeded)  $\times$  100. For growth curves, cells were seeded at  $5 \times 10^3$  cells/well in a 24-well plate (duplicates per

time point and donor), and incubated in growth medium for 1, 3, 5, 7, 10 and 14 days. Growth medium was replaced with medium supplemented with 1:10 Alamar blue<sup>®</sup>-dye (Thermo Fisher Scientific, Schwerte, Germany). Cells were incubated for 3 h, supernatant was centrifuged, absorbance was measured in triplicate in an EnSight Multimode Plate Reader (PerkinElmer, Rodgau, Germany), and data were collected with the Kaleido 2.0 software program.

### Flow cytometry

For each run, a total of  $1 \times 10^6$  of non-induced cells were incubated with a final concentration of 5 µg/ml each of fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, peridinin chlorophyll protein complex (PerCP)- or allophycocyanin (APC)-conjugated mouse anti-human CD10, CD13, CD14, CD19, CD34, CD44, CD45, CD73, CD90, CD105, CD146, CD166, HLA-DR, c-kit, Stro1 and alkaline phosphatase antibodies (BD Biosciences, Heidelberg, Germany) or isotype-matched controls, respectively (20 min, 4 °C in the dark). After intense washing and resuspension in phosphate-buffered saline (PBS), flow cytometric analysis was performed using a FACSCalibur (BD Biosciences) flow cytometer. Data were collected and analyzed with CellQuest software (BD Biosciences). For each run, 20,000 cell events were gated and fluorochrome spectral overlap was checked and compensated whenever required.

### Western blot

Cells were lysed using the MemPer Plus kit with Halt<sup>®</sup> protease and phosphatase inhibitor (both Thermo Scientific) according to manufacturer's instructions and quantified using a modified Lowry assay (BCA; Thermo Scientific). Lysates were mixed with Laemmli/DTT buffer, heated and separated on Criterion TGX precast gels (Bio-Rad). After electrophoresis, proteins were transferred to a nitrocellulose membrane using the Transblot Turbo Transfer system (Bio-Rad). The membranes were blocked with 5% bovine serum albumine for 2 h at least, and proteins were detected with appropriate primary antibodies and visualized with the corresponding secondary antibodies using the ECL kit (Thermo Scientific). The antibodies used were as follows: ms SMAD1/2/3, ms TGFBR2, rb EGFR, ms IGF1R antibody (all Santa Cruz Biotechnology, Heidelberg, Germany), rb BMPR1 and rb TGFBR1 antibody (both Abcam, Cambridge, UK), rb phospho-SMAD2 (Ser465/467)/SMAD3 (Ser423/425), rb phospho-SMAD1 (Ser463/465)/SMAD5 (Ser463/465)/SMAD9 (Ser465/467), rb BMPR2, rb p38, rb phospho-p38 (Thr180/Tyr182), ms MEK1, rb phospho-MEK1/2 (Ser217/221), rb ERK1/2 and rb phospho-ERK1/2 (Thr202/Tyr204) antibody (all New England Biolabs, Frankfurt, Germany).

Data were collected with the ChemiDoc XRS+ system and analyzed using the ImageLab v6.0 software (both Bio-Rad).

### qPCR arrays

Gene expression was monitored using the RT<sup>2</sup> Profiler PCR Array technology (QIAGEN, Hilden, Germany). Total cellular RNA was purified using a guanidium–thiocyanate method (RNeasy Mini kit; QIAGEN, Hilden, Germany) and stored at –80 °C. The RNA integrity and quantity were verified using the Experion RNA StdSens chip microfluidic technology according to manufacturer's instructions (Bio-Rad, Munich, Germany), and cDNA was synthesized from 200 ng of total RNA each by using the RT<sup>2</sup> PreAmp cDNA synthesis kit (QIAGEN, Hilden, Germany) in a C1000 Thermal Cycler (Bio-Rad). After genomic DNA elimination and reverse transcription, one quarter of the resulting cDNA was pre-amplified using the RT<sup>2</sup> PreAmp Pathway Primer Mix (QIAGEN). qPCR was performed with the resulting pre-amplicons in duplicate each in a CFX96 cycler (Bio-Rad, Munich, Germany), according to manufacturer's instructions. Genomic DNA contamination, reverse transcription performance and purity, and qPCR efficiency were routinely checked. The amplicons' specificity was checked by melting curve examination. Data were collected with CFX96 Manager Software version 1.0 (Bio-Rad), and the respective genes of interest were normalized to a housekeeping gene pool comprising *ACTB*, *RPL0* and *HPRT*. Data were analyzed and plotted using the RT2 Profiler PCR array data analysis template (<https://www.qiagen.com/de/shop/genes-and-pathways/data-analysis-center-overview-page/>).

### Mineralization assay

Cells were fixed with ethanol, washed and incubated with 40 mM Alizarin red solution (Sigma Aldrich, pH 4.1) for 20 min at RT. After thorough washing, the air-dried specimens were evaluated with a SZH10 microscope (Olympus, Münster, Germany) equipped with a CCD Colour view III camera and the resulting images were taken and analyzed using the Cell\* software (both Olympus, Münster, Germany). For quantification, Alizarin red was extracted with 10% acetic acid (30 min, RT). The solution was covered with mineral oil (Sigma Aldrich) and incubated for 10 min at 85 °C. The absorbance of the supernatant was read at 420 nm in triplicate, and data were collected and analyzed using the Magellan v6.2 software (Tecan, Crailsheim, Austria).

### Alkaline phosphatase assay

Alkaline phosphatase (ALP) enzymatic activity was quantified using p-nitrophenol phosphate (pNPP) as substrate. Cellular proteins were purified using complete Lysis-M buffer

containing protease inhibitor (Roche, Mannheim, Germany) and quantified using a modified Bradford method (Pierce 660 assay; Thermo Scientific). ALP activity was measured in triplicate for each donor and culture condition. In brief, proteins (5  $\mu\text{g}$ ) were mixed with ALP buffer containing 5 mM Mg acetate and 10 mM pNPP, and absorbance at OD = 405 nm was read continuously for 30 min in a microplate reader (Tecan, Crailsheim, Germany). Data were calculated by  $[(\text{OD}_{t_x} - \text{OD}_{t_0}) \times 10^3 \times \text{reaction volume}] / (t_x \times \varepsilon \times \lambda \times \text{sample volume})$  with  $\varepsilon = 18.75 \text{ mM}^{-1} \text{ cm}^{-1}$  and  $\lambda = (\text{OD}_{\text{cal}} - \text{OD}_{\text{H}_2\text{O}}) / \varepsilon \times c$ .

## Statistics

A repeated measures analysis was performed with a linear mixed model for each outcome of interest (growth, normalized protein expression, alkaline phosphatase activity, mineralization). The group effects and differences of least square (ls) means were calculated with their 95% confidence intervals. Several multiple comparisons of ls means in groups were performed and  $p$  values were adjusted for multiple testing by the method of Tukey-Kramer. Calculations were done using PROC MIXED from the statistical software program SAS 9.1.2 (Cary, NC 27513, USA).

## Results

### Characterization of non-induced mixed cells reveals low numbers of stem/progenitor cells

Alveolar osteoblasts and mixed cells from the periodontal ligament and from gingiva were randomly distributed in the culture plates, while no colony formation was observable (Fig. 1a). This observation was substantiated by CFU assays, which revealed a CFU-F efficiency of  $0.5 \pm 1.0\%$  for alveolar osteoblasts and periodontal mixed cells, while the efficiency was increased in gingival mixed cells ( $7.0 \pm 5.8\%$ ; Fig. 1a). All cell populations showed a significant increase in metabolic activity with increased culture duration ( $p < 0.001$  for 3, 5, 7, 10 and 14 days compared to 1 day in all entities (Fig. 1b). However, metabolic activity gain was significantly different for all groups and all time points, with gingival mixed cells showing the highest initial gain ( $p < 0.001$  compared to periodontal mixed cells and alveolar osteoblasts) and periodontal mixed cells showing the highest final gain ( $p < 0.001$  compared to gingival mixed cells and alveolar osteoblasts; Fig. 1b). Further, the time-related proliferation increase, i.e., metabolic activity values normalized to metabolic activity of 1 day, was significantly higher in gingival mixed cells at 3d compared to osteoblasts ( $p = 0.037$ ), while subsequently, both periodontal and gingival mixed cells proliferated significantly more intensely than osteoblasts (5 days:  $p < 0.001$  periodontal

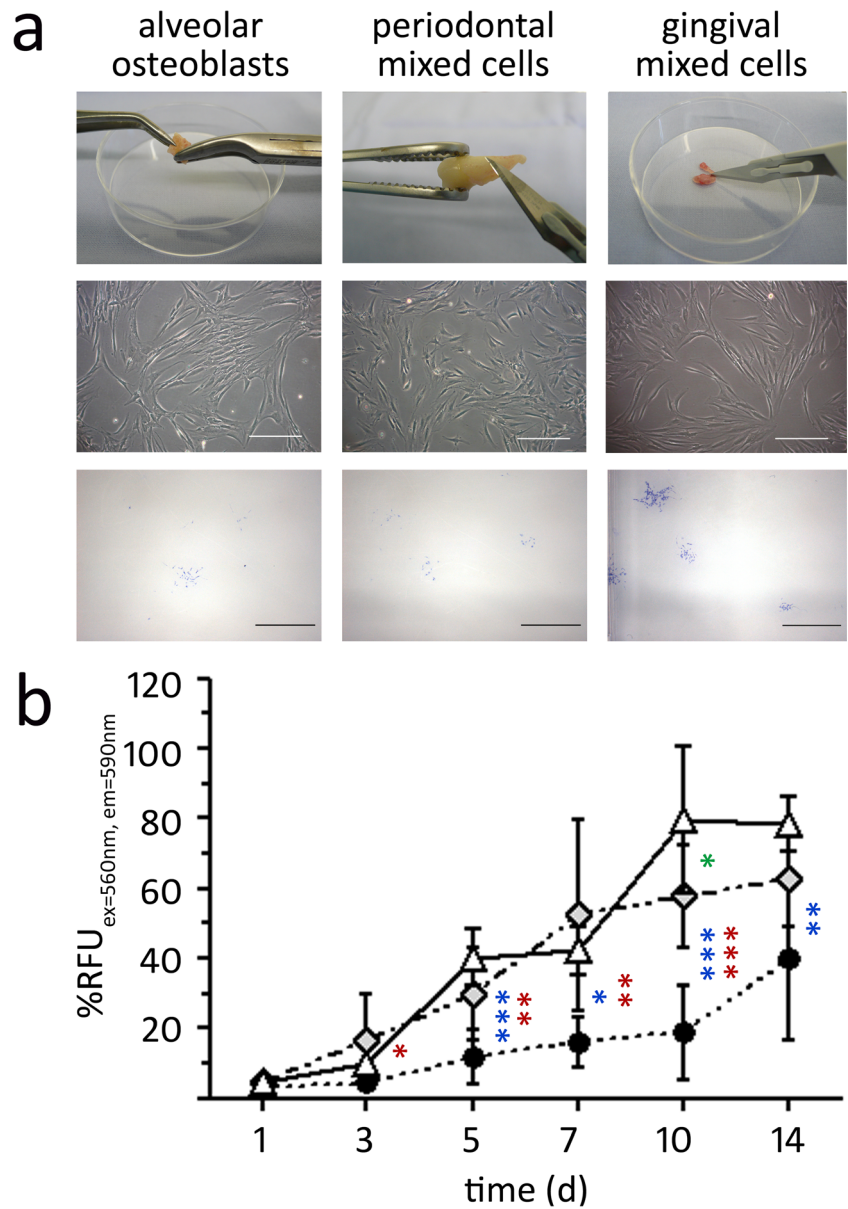
mixed cells vs. osteoblasts,  $p = 0.003$  gingival mixed cells vs. osteoblasts; 7 days:  $p = 0.02$  periodontal mixed cells vs. osteoblasts,  $p = 0.001$  gingival mixed cells vs. osteoblasts; 10 days:  $p < 0.001$  both periodontal and gingival mixed cells vs. osteoblasts). Additionally at 10 days, periodontal mixed cells had a significantly elevated metabolic activity increase compared to gingival mixed cells ( $p = 0.014$ ), while the increase at 14 days was significant only in periodontal mixed cells compared to osteoblasts ( $p = 0.003$ ; Fig. 1b). With regard to surface marker expression, we found that non-induced alveolar osteoblasts strongly expressed CD13, CD44, CD73, CD90, CD105 and CD166, while the expression of CD146 and membrane-bound ALP was low (Fig. 2a, Table 1). Further, the expression of CD14, CD19, CD34, CD45, HLA-DR, c-kit and Stro1 was virtually absent, and the expression of CD10 was moderate in osteoblasts (Fig. 2a, Table 1). In comparison, non-induced periodontal and gingival mixed cells showed similar surface epitopes and highly expressed CD13, CD44, CD73, CD90, CD105 and CD166, respectively (Fig. 2b, c, Table 1). Similar to osteoblasts, periodontal and gingival mixed cells negatively expressed CD14, CD19, CD34, CD45 and HLA-DR, but both showed an intense expression of the fibroblast marker CD10 (Fig. 2b, c, Table 1). The percentage of cells with membrane-bound ALP was very low in periodontal (Fig. 2b, Table 1) and gingival mixed cells (Fig. 2c, Table 1). Regarding putative stem cell markers, periodontal mixed cells expressed intermediate levels of CD146, while the expression of Stro1 and c-kit was very low (Fig. 2b, Table 1). Of note, only  $0.06 \pm 0.03\%$  of periodontal mixed cells were double-positive for Stro1 and CD146. In comparison, gingival mixed cells expressed only low levels of CD146, and the percentage of Stro1- and c-kit-positive cells was close to zero (Fig. 2c, Table 1). These data suggest that mixed cell populations from periodontal ligament and gingiva mainly comprise fibroblasts together with very low numbers of stem/progenitor cells.

### Mixed periodontal and gingival cells individually express biomolecules associated with responsiveness to osteogenic induction

Western blot analyses revealed that all cell types expressed similar levels of BMPR1, which were elevated following 21 days of osteogenic induction (Fig. 3a, Table 1). While appearing generally weaker in soft tissue-derived cells, BMPR2 proteins were increased in gingival mixed cells following osteogenic induction in contrast to alveolar osteoblasts and periodontal mixed cells (Fig. 3a). The expression intensity of TGFBR1 was comparable to non-induced controls, while appearing slightly decreased in induced mixed cells from the periodontal ligament (Fig. 3a). Similarly, TGFBR2 was slightly decreased in all cell types under study following 21 days of osteogenic induction (Fig. 3a). While the expression of IGF1R



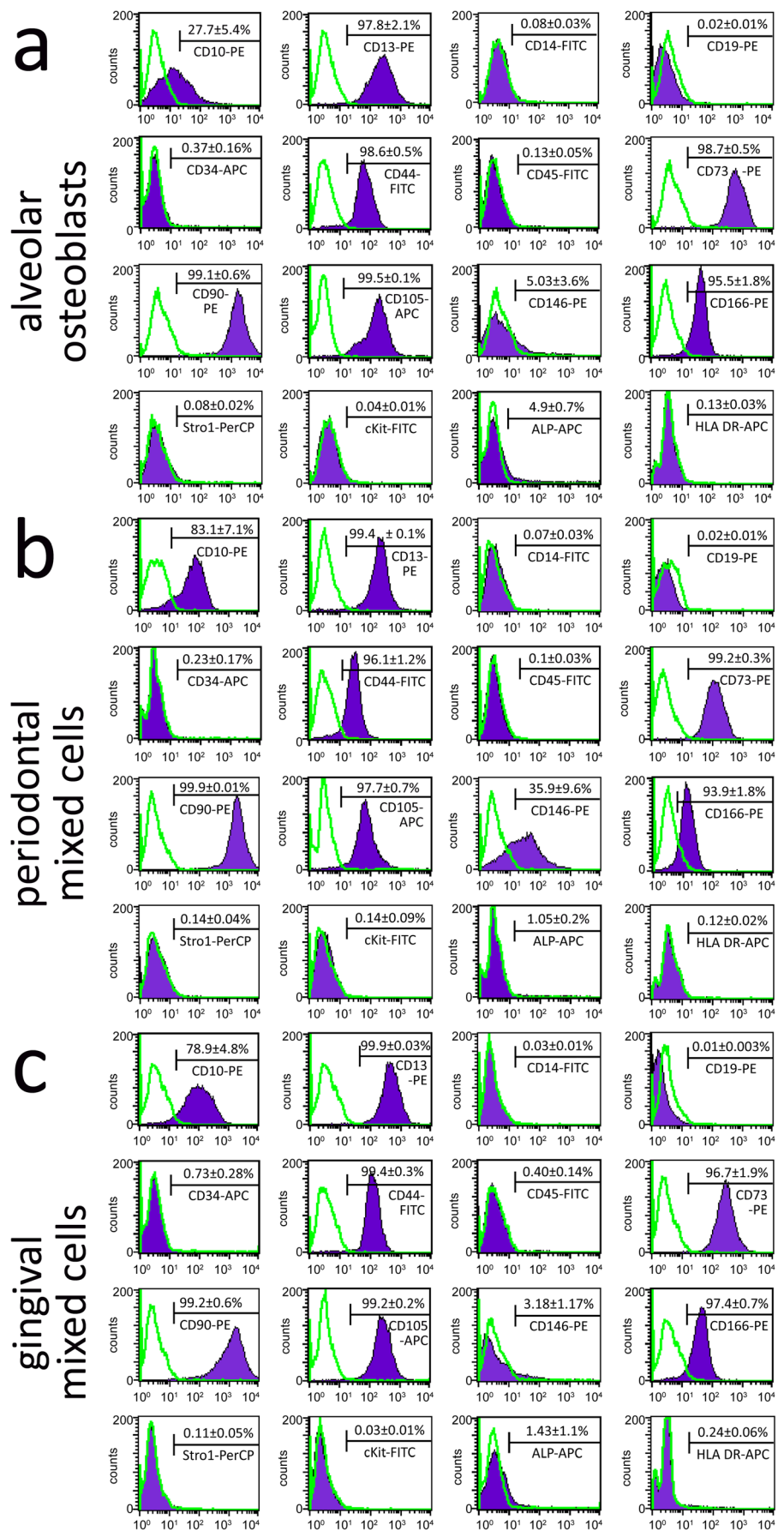
**Fig. 1** Isolation, colony formation and growth of human alveolar osteoblasts and mixed cells from periodontal ligament and gingiva. **a** After removal of tissue impurities like bone marrow or gingival epithelium, cells were isolated from compact alveolar bone, periodontal ligament, or gingival connective tissue, respectively. All cells appeared equally distributed throughout the petri dish (scale bar = 50  $\mu$ m), and virtually no colonies were visible for osteoblasts and periodontal mixed cells, while single colonies emerged in gingival mixed cell populations (scale bar = 5000  $\mu$ m). **b** All cell populations showed increased metabolic activity by time, while periodontal (white triangles connected by a solid line) and gingival mixed cells (grey diamonds connected by a dashed line) proliferated significantly more than osteoblasts (black circles connected by a dotted line; blue = periodontal mixed cells vs. osteoblasts, red = gingival mixed cells vs. osteoblasts, green = periodontal vs. gingival mixed cells)



was clearly stronger in non-induced osteoblasts, the opposite trend became obvious both for mixed cells from the periodontal ligament and gingiva (Fig. 3a). The expression of EGFR was strong and quite uniform for osteoblasts and gingival mixed cells irrespective of osteogenic induction, while appearing decreased in induced mixed cells from the periodontal ligament (Fig. 3a). SMAD molecules were strongly expressed in alveolar osteoblasts and gingival mixed cells, but were low in periodontal mixed cells, with no significant expression changes following osteogenic induction in all cell types (Fig. 3b). MEK1/2 was intensely expressed in alveolar osteoblasts and gingival mixed cells, while only faint bands appeared for periodontal mixed cells which remained unchanged by osteogenic induction (Fig. 3b). The expression of ERK1/2 was not affected by osteogenic induction in

osteoblasts, but was obviously decreased in mixed cells from the periodontal ligament ( $p = 0.020$ ) and gingiva ( $p = 0.003$ , Fig. 3b). Changes in p38 expression following osteogenic induction were found non-significant for all cell types under study (Fig. 3b). Regarding the activation status of downstream signalling effectors, we found only weak signals for phosphorylated MEK1/2, which appeared stable in induced osteoblasts as well as in induced periodontal and gingival mixed cells (Fig. 3b, Table 1). Phosphorylated ERK1/2 was robustly detectable in non-induced controls, but significantly downregulated for all cell types following osteogenic induction (Fig. 3b,  $p = 0.007$  for alveolar osteoblasts,  $p = 0.005$  for periodontal mixed cells and  $p < 0.001$  for gingival mixed cells, respectively). Phosphorylated p38 was most strongly expressed in periodontal mixed cells, while it remained unchanged in

**Fig. 2** Immunophenotypic characterization of osteoblasts and mixed cells from periodontal ligament and gingiva. Flow cytometry screening of mesenchymal surface epitopes associated with hard tissue cells revealed that similar to alveolar osteoblasts (a), periodontal (b) and gingival mixed cells (c) expressed CD13, CD44, CD73, CD90, CD105 and CD166, while lacking CD14, CD19, CD34, CD45 and HLA-DR. a Alveolar osteoblasts expressed intermediate levels of CD10, low levels of CD146 and ALP, while Stro1 and c-kit-expression were absent. b The percentage of CD10-positive cells was high in periodontal mixed cells that expressed intermediate levels of CD146 with very low levels of ALP, Stro1 and c-kit. c Gingival mixed cells expressed comparably high levels of CD10, but low levels of CD146, while the expression of ALP and Stro1 was very low and c-kit was absent



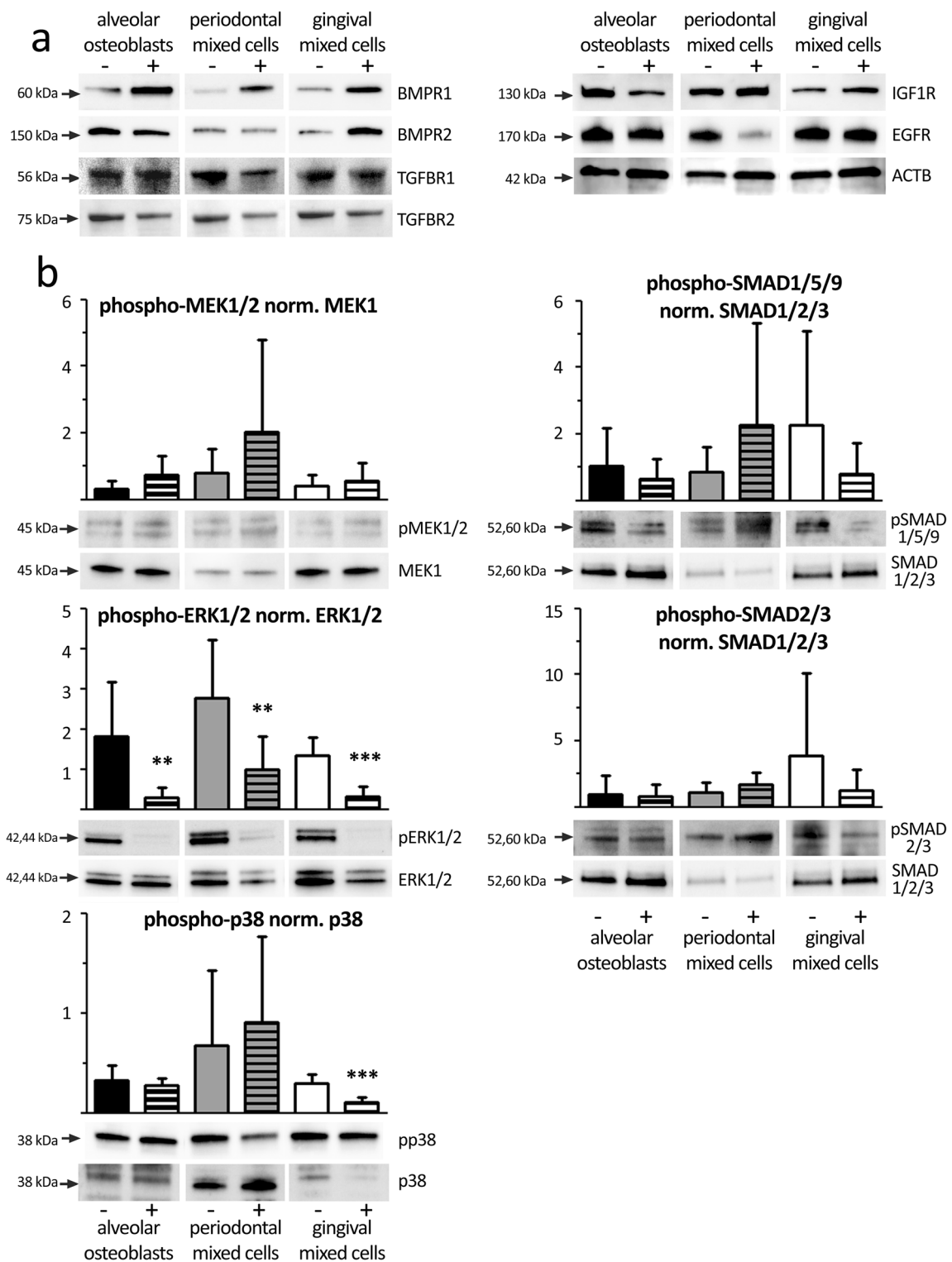
**Table 1** Performance of mixed cells from the periodontal ligament and gingiva compared to osteoblasts

	Osteoblasts		Periodontal cells		Gingival cells	
CFU-F	-		-		+/-	
Flow cytometry						
CD10	+		++		++	
CD13	+++		+++		+++	
CD14	-		-		-	
CD19	-		-		-	
CD34	-		-		-	
CD44	+++		+++		+++	
CD45	-		-		-	
CD73	+++		+++		+++	
CD90	+++		+++		+++	
CD105	+++		+++		+++	
CD146	+/-		+		+/-	
CD166	+++		+++		+++	
Stro1	-		-		-	
c-kit	-		-		-	
HLA-DR	-		-		-	
ALP	+/-		-		-	
Growth	+++		+		++	
	Non-induced	Induced	Non-induced	Induced	Non-induced	Induced
Receptors and signaling						
BMPR1	+	+++	-	+	+/-	++
BMPR2	++	++	+/-	±	+/-	++
TGFBR1	+	+	++	+	+	+
TGFBR2	+	+	+	+	+	+
IGF1R	++	+	+	++	+/-	+
EGFR	+++	++	++	-	++	++
SMAD1/2/3	+	++	+/-	±	+	++
Phospho-SMAD2/3	+	+/-	±	+	+	-
Phospho-SMAD1/5/9	±	+/-	±	+	+	+/-
MEK1/2	+	+	+/-	+/-	+	+
Phospho-MEK1/2	-	+/-	-	+/-	-	-
ERK1/2	+	+	+	+/-	++	+
Phospho-ERK1/2	+	-	++	-	+	-
p38	+	+	+	+/-	+	+
Phospho-p38	+/-	±	+	++	+/-	-
Matrix mineralization	+++	-	++	-	+/-	-
Alkaline phosphatase activity	++	-	+++	-	-	-

Individual behaviour of all cell populations under study with regard to colony formation, surface marker expression, growth, protein expression, matrix mineralization and alkaline phosphatase activity (details: see text). For gene expression results, please see Suppl. Table 1

induced osteoblasts. Although only low amounts of active p38 were found in non-induced gingival mixed cells, it was significantly decreased following osteogenic induction ( $p = 0.027$ ). The changes in SMAD protein phosphorylation were found non-significant for all cell types; however, both active SMAD1/5/9 and active SMAD2/3 appeared decreased or constant, respectively, in alveolar osteoblasts and gingival

mixed cells undergoing osteogenic differentiation, and showed an upward trend in periodontal mixed cells. To estimate the involvement of the pathways under study, ratios of active/non-active downstream signalling effectors were calculated. For all cell types, changes in phosphorylated SMAD2/3 normalized to SMAD1/2/3 and phosphorylated SMAD1/5/9 normalized to SMAD1/2/3 were non-significant (Fig. 3b).



**Fig. 3** Osteogenesis-related receptor (a) and downstream signaling effector expression and activation (b) in oral cells. **a** Western Blot analyses showed that all cells under study expressed similar levels of BMPR1 and TGFBR2 under control conditions (-), which were simultaneously increased or decreased, respectively, if cells were cultured in osteogenic differentiation medium (+). However, differences were obvious for BMPR2, TGFBR1, EGFR and IGF1R expression. The housekeeping protein ACTB was expressed uniformly for all cell types irrespective of osteogenic induction. **b** Analysis of phosphorylated mitogen-activated protein kinases (bar graphs,

y-axis: ratio of normalized volume intensities, x-axis: cell types and culture conditions) revealed that phosphorylated MEK1/2 was increased in induced osteoblasts while rather decreased in periodontal and gingival mixed cells. Phosphorylated ERK1/2 was decreased in all cell types following osteogenic induction, while phosphorylated p38 was differentially regulated. While phosphorylated SMAD1/5/9 and phosphorylated SMAD2/3 appeared decreased in induced alveolar osteoblasts and gingival mixed cells, both were rather increased in periodontal mixed cells following osteogenic induction (norm. = normalized to)



Similarly, phosphorylated MEK1/2 normalized to MEK1/2 showed an upward trend in all cell types (Fig. 3b). However, phosphorylated ERK1/2 normalized to total ERK1/2 was significantly decreased in induced osteoblasts ( $p = 0.003$ ), and induced mixed cells from the periodontal ligament ( $p = 0.005$ ) and gingiva ( $p < 0.001$ , Fig. 3b) following osteogenic induction. Again, phosphorylated p38 normalized to total p38 was significantly decreased only in induced gingival mixed cells ( $p < 0.001$ , Fig. 3b).

### Gene expression profile reflects the differential osteogenicity of periodontal and gingival mixed cells

We were next interested in the expression of genes related to osteogenesis. In alveolar osteoblasts, the vast majority of genes investigated was regulated significantly both in biological and statistical terms following osteogenic induction, i.e. showing a  $\geq 1.5$ -fold up- or downregulation, respectively, with  $p < 0.05$  (Fig. 4a, Suppl. Table 1). Regarding osteogenic soluble factors, the gene expression of the following markers was found to be significantly upregulated in alveolar osteoblasts following osteogenic induction: *BGN*, *BMP6* and *TGFB1*; while *BMP4*, *FGF2* and *TGFB3* appeared significantly decreased (Fig. 4a, Suppl. Table 1). Further, mRNA expression of some osteoblast-featured receptors was regulated in induced osteoblasts, i.e. *EGFR*, *FLT1*, *IGF1R* and *TGFB1R* were decreased and *FGFR1* together with *TGFB2R* were increased (Fig. 4a, Suppl. Table 1). Transcripts of the respective downstream effectors involved in osteogenesis, i.e. *SMAD1* and *SMAD3*, were downregulated in alveolar osteoblasts after 21 days of osteogenic induction (Fig. 4a, Suppl. Table 1). Further, the gene expression of the following transcription factors and differentiation markers associated with osteogenesis appeared significantly increased: *BGLAP*, *RUNX2*, and *OSX* (Fig. 4a, Suppl. Table 1).

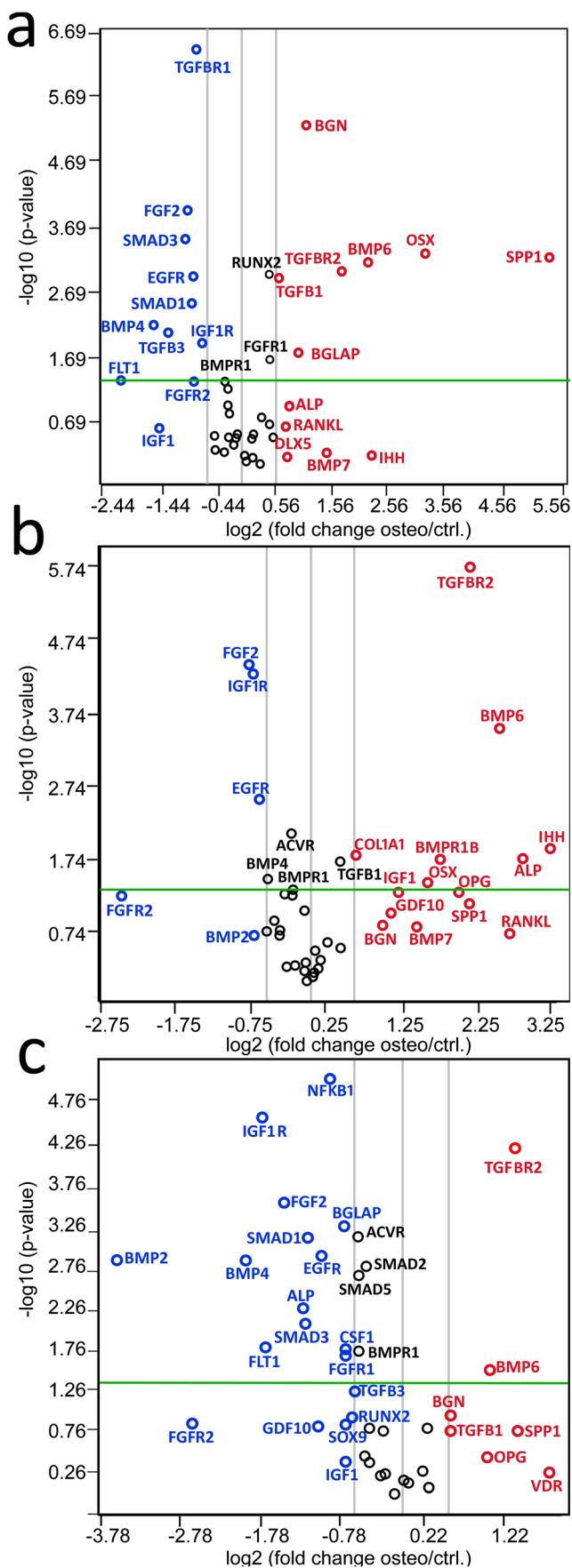
Induced periodontal mixed cells showed a fairly equal gene expression pattern particularly for positively regulated genes (Fig. 4b, Suppl. Table 1). Here, even more genes encoding for osteogenic soluble factors were modulated, with *BMP6* and *TGFB1* being significantly upregulated, and *BMP4* and *FGF2* being downregulated (Fig. 4b, Suppl. Table 1). Of note, the gene expression of molecules that govern osteoclast regulation, i.e. *RANKL* and *OPG*, was meaningfully increased compared to non-induced periodontal mixed cells (+ 6.1 and + 3.9-fold, respectively) without reaching statistical significance (Suppl. Table 1). Comparable to induced alveolar osteoblasts, the gene expression of *TGFB2R* was significantly upregulated, while the transcription of *EGFR* and *IGF1R* was downregulated in induced periodontal mixed cells (Fig. 4b, Table 1). Further, the gene expression of *BMP1B* was significantly increased, but the gene expression of *ACVR* and *BMP1A* was decreased (Fig. 4b, Suppl. Table 1). While no significant expression changes were found for *SMAD* genes, the

transcription of *ALP* and *IHH* was tremendously increased, and *OSX* and *COL1A1* genes were significantly stronger expressed upon osteogenic induction (Fig. 4b, Suppl. Table 1).

With respect to transcripts showing similarity to alveolar osteoblasts, mixed cells from gingiva displayed preference to the spectrum of downregulated genes following osteogenic induction. Except for *BMP2* and *CSF1* gene expression, the regulation pattern in gingival mixed cells of osteogenic soluble factors nearly matched with that observed for alveolar osteoblasts (Fig. 4c, Suppl. Table 1). Comparable to alveolar osteoblasts and periodontal mixed cells, the gene expression of *TGFB2R* was significantly increased while the gene expression *EGFR* and *IGF1R* was decreased in induced gingival mixed cells (Fig. 4c, Suppl. Table 1). Further, the gene expression of *FLT1*, *ACVR* and *BMP1A* was decreased in induced gingival mixed cells comparable to the other cell types, whereas *FGFR1* transcription was decreased only in gingival mixed cells (Fig. 4c, Suppl. Table 1). Apart from *SMAD4*, the expression of all other *SMAD* genes under study was significantly decreased in gingival mixed cells following osteogenic induction (Fig. 4c, Suppl. Table 1). Gingival mixed cells showed some distinctive features with regard to the gene expression of osteogenic transcription factors and differentiation markers, which is substantiated by a significant downregulation of *NFKB1*, *ALP* and *BGLAP* (Fig. 4c, Suppl. Table 1). Of note, the expression of osteogenic hallmark genes such as *RUNX2*, *OSX* and *SPP1* appeared either clearly weaker or inverse in gingival mixed cells (Suppl. Table 1).

### Oral cells show an individual matrix mineralization capacity

After 21 days of osteogenic induction, mineralization events were found in all cell types in a gradual manner, i.e. osteoblasts showed the most intense and uniform alizarin red stainings, followed by periodontal mixed cells with a patchy alizarin red staining and finally, by gingival mixed cells which showed the lowest staining intensity (Fig. 5a). This observation was confirmed by alizarin red quantification, which revealed that induced alveolar osteoblasts ( $p < 0.001$ ) and periodontal mixed cells ( $p < 0.0001$ ) showed significant matrix mineralization compared to controls (Fig. 5b). Although a clear trend in matrix mineralization was observable for gingival mixed cells, it was not significant compared to matched controls (Fig. 5b). Consequently, intercellular comparison revealed significantly more calcium deposits in induced osteoblasts ( $p = 0.0002$ ) and periodontal mixed cells ( $p = 0.0406$ ) compared to gingival mixed cells, while osteoblasts and periodontal mixed cells showed fairly equal levels of matrix mineralization (Fig. 5b). With respect to intracellular alkaline phosphatase (ALP) activity, we found that control osteoblasts showed some baseline ALP activity, which was significantly



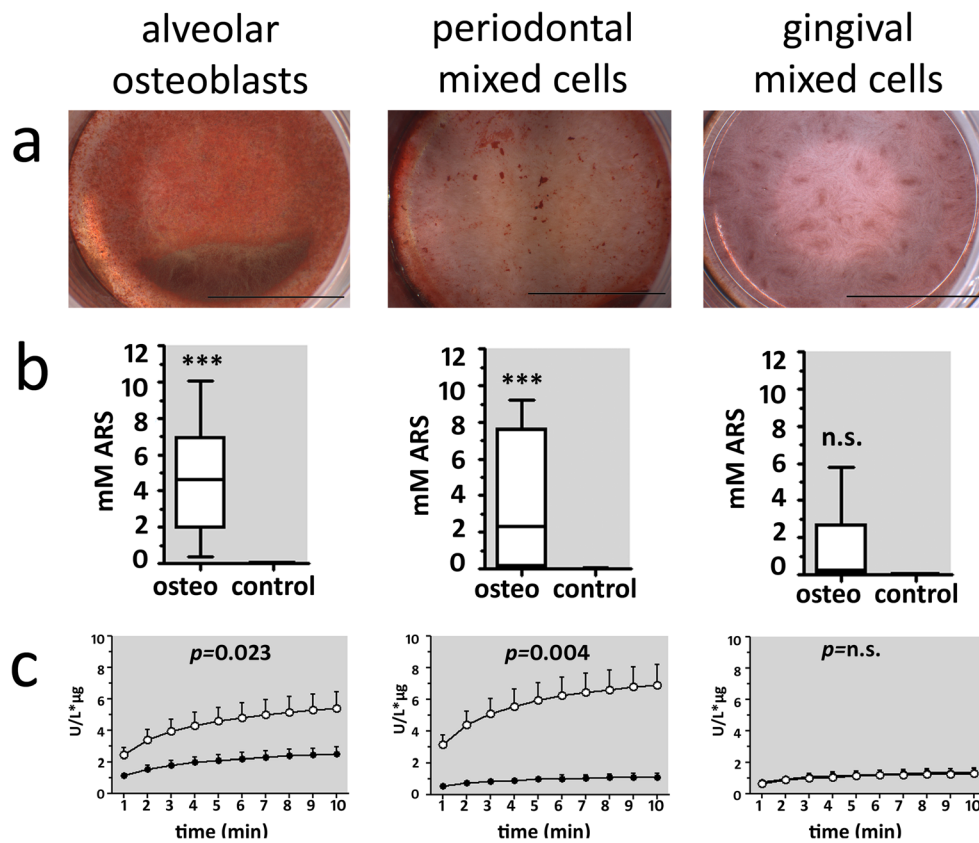
**Fig. 4** mRNA profile of osteogenic marker genes in alveolar osteoblasts (**a**) and periodontal (**b**) and gingival mixed cells (**c**). Volcano plots of mRNA expression following osteogenic induction display statistical significance ( $y$ -axis) versus fold regulation change ( $x$ -axis) compared to matching controls (middle black line) with 1.5 as fold-change cutoff (vertical black lines on the left and right) and  $p < 0.05$  as the  $p$  value cutoff (horizontal green line). **a** In osteoblasts, a broad panel of genes associated with osteogenesis was significantly upregulated following osteogenic induction, i.e. *TGFBR2*, *TGFBI*, *BGN*, *BMP6*, *OSX*, *SPP1* and *BGLAP*, while *TGFBR1*, *EGFR*, *IGF1*, *FGFR2*, *FLT1*, *FGF2*, *IGF1*, *BMP4*, *SMAD1* and *SMAD3* were significantly downregulated. **b** In periodontal mixed cells, similar genes associated with osteogenesis were in part significantly upregulated. **c** mRNA expression in gingival mixed cells resembled the osteoblast pattern in particular with respect to down-regulated genes. Common for all cell types, *TGFBR2* was significantly upregulated, while *EGFR*, *IGF1*, *SMAD1* and *SMAD3* were significantly downregulated following osteogenic induction

increased upon osteogenic induction ( $p = 0.023$ , Fig. 5c, compare black with white dots). This significance also applied for periodontal mixed cells ( $p = 0.004$ , Fig. 5c), while gingival mixed cells showed no elevation of their ALP activity (Fig. 5c). These data suggest a differential osteogenic capacity for the cells from the alveolar bone or from periodontal ligament and gingiva, respectively, in response to osteogenic induction.

## Discussion

Since mixed cells from the periodontal ligament and the gingiva prove promising for the regeneration of periodontal hard tissues, i.e. bone and cementum, they were characterized and tested for their osteogenic differentiation capacity in this study. We found that periodontal and gingival mixed cell populations comprise only low numbers of stem/progenitor cells, as substantiated by minute percentages of colony-forming and/or Stro1- and c-kit-positive cells. While mixed cells from the periodontal ligament displayed an osteogenic differentiation capacity comparable to osteoblasts, gingival mixed cells did not achieve in vitro hard tissue formation. Both mixed cell population showed some unique features: a great deal of non-induced periodontal cells positively expressed CD146, while induced gingival cells showed enhanced expression of BMP receptors, decreased levels of active p38 kinase, inverse regulation of some osteogenic marker genes and no remarkable matrix mineralization or alkaline phosphatase activity.

If dealing with mixed cells, the precise nature and composition of the cell population is unknown. The phenotypic characterization in the present study revealed an epitope spectrum that is in line with current reports [12, 21, 22], emphasizing the very low percentage of stem/progenitor cells within the mixed cell populations. Stro1 is considered the ‘gold standard’ marker for stem cells from oral tissues including dental pulp, periodontal ligament, gingiva and bone marrow [2]. Originally known as marker for stem cells with cardiomyogenic potential [23],



**Fig. 5** Osteogenic differentiation visualized by Alizarin red stains (a) and quantification (b) as well as alkaline phosphatase activity (c). **a** Osteogenic induction yielded intensely coloured Alizarin red stains (ARS) in alveolar osteoblasts, followed by periodontal mixed cells, and, to a lower extent, by gingival mixed cells. **b** Quantification of extracted ARS revealed that in osteoblasts and periodontal mixed cells, the increase in ARS was significant following osteogenic induction, while gingival mixed cells showed less calcium deposits (box plot graphs, y-axis: ARS

level [mM], x-axis: osteogenic induction vs. controls). **c** Alkaline phosphatase line graphs show that following osteogenic induction (white dots representing means ± s.e.m.), the enzymatic activity was significantly increased in alveolar osteoblasts and periodontal mixed cells compared to non-induced controls (black dots), while being stable in gingival mixed cells irrespective of osteogenic induction (i, y-axis: ALP activity [U/L × µg], x-axis: time [min])

CD117/c-kit has been used to isolate progenitors from dental pulp [24] and gingiva [25]. CD90, also known as Thy-1, is highly expressed by several cell types including fibroblasts, as well as immune and neuronal cell lines [26, 27], and in our hands by all kinds of oral tissue-derived cells, including oral fibroblasts [17] and both Stro1-positive and Stro1-negative dental pulp cells (unpublished data). CD146 is a cell adhesion molecule that labels endothelial cells and pericytes which co-express CD73, CD90 and CD105 [28, 29]. The latter maintain the hematopoietic stem cell niche and act in a pro-angiogenic manner, while exhibiting a limited multipotency [14]. In periodontal ligament cells, CD146 is accepted as a marker for stem/progenitor cell enrichment only if co-expressed with Stro1 [30, 31], and in gingiva-derived cells, CD146 expression is associated with the absence of Stro1 [32]. In this regard, the finding that high percentages are positive for CD146 suggests that mixed cells could contribute to tissue regeneration, but also implies that CD146 together with CD73, CD90 and CD105 are unspecific stem cell markers in the oral tissue context. The immunophenotyping results, together with the low colony

efficiency, suggest that periodontal and gingival mixed cells are comprised of fibroblasts rather than progenitor cells.

Following osteogenic induction, the expression pattern of the receptor and downstream effector configuration of periodontal and gingival mixed cells points to a major role of non-canonical signaling pathways in oral cells [33]. Since SMAD signaling is predominant during osteogenic differentiation in long bone-derived osteoblasts [34, 35], the present findings disclose a unique feature of oral cells which may be attributed to their developmental origin as neural crest descendants [36, 37]. Given the individual downstream effector activation patterns in each cell type under study, it is not surprising that the expression of osteogenic genes and the final differentiation performance are distinctive. In the present study, gene expression was measured after 21 days, and time-dependent variations in mRNA transcription may account for some counter-regulation of the investigated biomolecules at the mRNA level compared to the corresponding protein status. Nevertheless, both protein and gene expression analysis showed disparities between gingival and periodontal

mixed cells, and a main finding is that gingival mixed cells fail to accomplish osteogenic differentiation in response to biochemical induction. Of note, gingival cells were previously found to be superior to periodontal cells in terms of hard tissue formation in the presence of mesenchymal stem cells [17]. This means that gingival cells are able to adopt an osteogenic phenotype, following interaction with mesenchymal stem cells, despite their failure of hard tissue formation in response to biochemical induction. The discrepancy between the previously published observations and the present findings suggests that (i) gingival mixed cells alone may not be useful for bone and/or cementum regeneration, because (ii) bona fide cell-to-cell interactions with mesenchymal stem cells are obviously required for triggering their osteogenesis [17]. More importantly, our results show that mixed periodontal cells are in fact capable of hard tissue formation to comparable degrees as osteoblasts, which renders them an attractive cell source for bone and cementum regeneration. Periodontal mixed cells are easily available from extracted teeth, and their use holds great advantages compared to stem cells from the periodontal ligament for the following reasons: (i) no depletion of the initially small cell numbers derived from the very small periodontal ligament biopsies is required for stem cell enrichment, with the consequence that (ii) the time span of in vitro cell manipulation that precedes therapeutic administration is considerably reduced, (iii) thereby decreasing the incidence of undesired genetic and epigenetic changes [38] together with cell senescence [39].

Taken together, our work emphasizes the importance of a precise characterization of oral tissue-derived cell populations [40], and provides a methodological platform in doing so. The present study further reveals that periodontal and gingival mixed cells react individually to osteogenic induction with regard to receptors and signaling effectors equipment as well as mRNA transcription, and the degree of matrix mineralization. By delivering insights in the markers that mixed cell populations express in response to osteogenic triggers, our results provide the basis for the decision-making in future research strategies and clinical therapies, because they provide first evidence that mixed cells from the periodontal ligament are suitable for periodontal regeneration purposes. This knowledge will contribute to anticipate the clinical outcome of hard tissue-inducing strategies, and opens the road for the development of novel cell-based therapies using mixed cell populations.

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## Compliance with ethical standards

**Conflicts of interest** The authors declare that they have no competing interests.

**Ethical approval** All procedures performed in studies involving human participants were in accordance with the ethical standards of the Committee of Ethics of the Medical Faculty of the Albert Ludwigs University Freiburg, Germany, and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

**Informed consent** Informed consent was obtained from all individual participants included in the study.

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