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ORIGINAL ARTICLE

Comparative analysis of proliferative and multilineage differentiation potential of human periodontal ligament stem cells from maxillary and mandibular molars

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Abstract

Background: Clinical experience indicates that wounds in alveolar bone and periodontal tissue heal faster and more efficiently in the maxilla compared with the mandible. Since stem cells are known to have a decisive influence on wound healing and tissue regeneration, the aim of this study was to determine whether differences in proliferation and differentiation of periodontal ligament stem cells (PDLSC) from upper (u-PDLSC) and lower jaw (l-PDLSC) contribute to the enhanced wound healing in the maxilla.

Methods: u-PDLSC and l-PDLSC from the same donor were harvested from the periodontal ligament of extracted human maxillary and mandibular third molars. Cell differentiation potential was assessed by analyzing stem cell markers, proliferation rate, and multilineage differentiation among each other and bone marrow-derived mesenchymal stem cells (MSC). Successful differentiation of PDLSC and MSC toward osteoblasts, adipocytes, and chondrocytes was analyzed via reverse transcriptase-quantitative polymerase chain reaction and histochemical staining (Alizarin Red, Oil Red O, Toluidine Blue).

Results: u-PDLSC and l-PDLSC expressed the MSC-markers $CD73^+$, $CD90^+$, and $CD105^+$ and lacked expression of $CD34^-$ and $CD45^-$. Proliferation was significantly higher in u-PDLSC than in l-PDLSC, regardless of the culture conditions. Osteogenic (ALP, RunX2, and osteocalcin) and chondrogenic (SOX9 and ACAN) related gene expression as well as staining intensities were significantly

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higher in u-PDLSC than in l-PDLSC. No difference in adipogenic differentiation was observed.

Conclusion: u-PDLSC showed a significantly higher proliferative and differentiation potential than l-PDLSC, offering a possible cell-based explanation for the differences in periodontal wound healing efficacy between maxilla and mandible.

KEYWORDS
differentiation, periodontal ligament stem cells, periodontal regeneration, proliferation, wound healing

1 | INTRODUCTION

Clinical experience suggests that defects in alveolar bone and periodontal tissue regenerate faster and more efficiently in the maxilla (upper jaw) than in the mandible (lower jaw). Alveolar bone of the maxilla and mandible already differs in bone tissue composition: the maxilla contains about 46% lamellar bone and 23% bone marrow, while the mandible consists of 63% lamellar bone and 16% bone marrow.[1] Furthermore, there are also differences in the blood supply, innervation, and embryonic development/odontogenesis.[2] Differences between maxilla and mandible are also apparent in the structure of teeth: for example, maxillary and mandibular molars can be distinguished by the ridges, the number of cusps, grooves, and roots.[3] Due to periodontal wound healing being a complex process involving different types of tissues and cells, the mechanisms responsible for the difference in wound healing velocity and efficacy are poorly described in the literature. Our hypothesis is that there might be differences in endogenous stem cells that may influence wound healing parameters: trophic character, recruitment to wounds, and regenerative potential.

In 2004, Seo et al. reported that specific stem cells, which have the potential to form a cementum/periodontal ligament (PDL)-like tissue in vivo, can be isolated from the PDL of extracted third molars. These periodontal ligament stem cells (PDLSC) have been identified as a population of easily accessible stem cells with the capability for self-renewal and differentiation into an osteogenic, cementogenic, chondrogenic, adipogenic, fibrogenic, and myogenic direction.[4–6] The importance of PDLSC in periodontal wound healing and their positive effect on stem cell-based periodontal therapy has already been confirmed in several in vitro and in vivo studies.[7] Various factors have been shown to regulate the stem cell properties of PDLSC, including tissue origin, age of donors, inflammatory condition, culture method, and growth factors.[8] However, it is yet to be known whether the origin from maxilla or mandible affects the stem cell properties of PDLSC.

The aim of this study is to investigate how PDLSC of the maxilla and mandible may influence periodontal wound healing. For this purpose, we compared the stem cell capabilities of PDLSC from both the maxilla and the mandible regarding their respective stem cell markers, proliferation rate, and multilineage differentiation ability. Moreover, we compared them with mesenchymal stem cells (MSC), the natural precursor cells of bone, cartilage, and adipose tissue.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Human third molars, separated into a maxillary and mandibular group according to their origin, were collected from seven patients (three males and four females), between aged 18 and 33 years. For best comparability, only teeth and tissue of patients where both maxillary and mandibular third molars were included in this study.

Under sterile conditions, periodontal ligament was scratched off the middle third of the root and digested in a solution of 1% collagenase type 1 (Worthington, USA) for 1 h at 37°C. The resulting cell suspension was centrifuged for 5 min at 500 *g* and the supernatant was subsequently discarded. Afterward, the cell pellet was resuspended in culture medium, and cells were seeded in a T-75 culture flask (Cellstar, Greiner Bio-One, Germany). Isolated PDLSC were cultured in PDLSC-Medium containing Dulbecco's Modified Eagle Medium (DMEM) high-glucose (Gibco, Germany), 10% fetal calf serum (FCS; Pan-Biotech, Germany), 50 mg/L L-ascorbic-acid (Sigma-Aldrich, Germany), 100 IU/ml penicillin (Gibco, Germany) and 100 μg/ml streptomycin (Gibco, Germany). Strict separation by donor and origin (upper jaw-PDLSC [u-PDLSC] or lower jaw-PDLSC [l-PDLSC]) was ensured.

MSC were isolated from bone marrow of explanted femoral heads (n = 7) according to protocols from Haynesworth et al.[9] and Pittenger et al.[10] as previously described.[11,12]

The MSC-Medium consisted of Mesenpan Basal Medium, 2% FCS, 1% ITS-plus (insulin, transferrin, selenious acid, bovine serum albumin, linoleic acid), 1 nM dexamethasone, 100 μM ascorbic-acid-2-phosphate, 10 ng/ml epidermal growth factor (all Pan-Biotech, Germany), 1.6 mM L-glutamine (Gibco, Germany), 80 IU/ml penicillin (Gibco, Germany) and 80 μg/ml streptomycin (Gibco, Germany).

Cells were cultured in a humidified atmosphere (20% O_2, 5% CO_2) at 37°C. Medium was changed every 3 to 4 days and all cells were tested negative for mycoplasma contamination. All experiments were performed at passage 3.

2.2 | Flow cytometry

MSC, u-PDLSC, and l-PDLSC were analyzed via flow cytometry for specific MSC surface epitopes. Briefly, cells were trypsinized, counted, and kept in flow cytometry buffer (0.09% FCS in phosphate buffered saline (PBS; Gibco, Germany)). Cells were centrifuged at 500 g for 5 min at 4°C and then resuspended in 100 μl flow cytometry buffer with the antibodies. The APC-, PE-, or FITC-isotype controls were diluted with flow cytometry buffer to reach a concentration of 0.2 μg/100 μl, 0.2 μg/100 μl, and 0.5 μg/100 μl. Conjugated antibodies against CD34, CD45, CD73, CD90, and CD105 (eBioscience, Germany) were diluted in flow cytometry buffer at a concentration of 0.5 μg/100 μl, 0.06 μg/100 μl, 0.124 μg/100 μl, 1 μg/100 μl, and 1 μg/100 μl. Then, cells are incubated for 30 min at 4°C, centrifuged for 5 min at 500 g, and the supernatant was removed. Finally, cells were resuspended in 300 μl flow cytometry buffer. Immunophenotype analysis was done with a FACS Canto II cytometer (BD Bioscience, Germany) and at least 10,000 events were measured for each donor.

2.3 | Cell proliferation

To analyze the proliferation rate, PDLSC from maxilla and mandible and MSC were seeded at a density of 10,000 cells/well in 12-well culture plates. Both cell types were cultured in their respective media and cell counts were measured after 1, 3, 5, and 7 days using CASY cell counter (Roche Innovatis, Germany). Doubling times of u-PDLSC and l-PDLSC were calculated according to the following formula: doubling time [h] = $(t_2-t_1)*(\ln(2)/\ln(c_2-c_1))$; ($t_1$ = time point 1, t_2 = time point 2, c_1 = cell count at t_1, c_2 = cell count at t_2). All measurements were performed in triplicate and averages were calculated.

2.4 | Osteogenic, adipogenic, and chondrogenic differentiation

For osteogenic differentiation, cells were seeded in a density of 31,000/cm^2 (MSC) or 10,000/cm^2 (PDLSC) in 24-well culture plates. The osteogenic induction medium consisted of DMEM low glucose (Gibco, Germany), 10% FCS, 100 nM dexamethasone, 10 mM sodium β-glycerophosphate and 0.05 mM L-ascorbic-acid (all Sigma-Aldrich, Germany). Medium exchange was performed 3 times per week.

For adipogenic differentiation, cells were seeded in a density of 80,000/cm^2 (MSC) or 25,000/cm^2 (PDLSC) in 24-well culture plates. Medium was changed twice per week, alternating between induction and maintenance medium. Adipogenic induction medium (AIM) was composed of DMEM high glucose, 10% FCS, 1 μM dexamethasone), 0.2 μM indomethacin, 0.5 mM 3-isobutyl-1-methylxanthine, and 0.01 mg/ml insulin (all Sigma-Aldrich, Germany), while adipogenic maintenance medium (AMM) consisted of DMEM high glucose, 10% FCS and 0.01 mg/ml insulin.

For chondrogenic differentiation, we performed a pellet culture with 250,000 cells (MSC/PDLSC) per 15-ml polypropylene tube. Cells were centrifuged for 5 min at 500 g. The pellets were then cultured in serum-free chondrogenic induction medium, consisting of DMEM high glucose, 5% ITS Plus Premix (BD Biosciences, USA), 100 nM dexamethasone, 0.17-mM L-ascorbic acid, 100 μg/ml sodium pyruvate and 40 μg/ml L-proline (all Sigma-Aldrich, Germany). Each time the medium was changed, 10 ng/ml TGF-β_3 (Thermo Fisher Scientific, USA) was added to the medium. Medium was changed three times per week.

Successful multilineage differentiation of three donors each was analyzed via reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) and specific stainings (each in duplicates) after 7, 14, and 21 days. PDLSC and MSC expansion medium were used as controls.

2.5 | Alizarin Red staining and quantification

Calcium-rich deposits, formed during osteogenic differentiation, were visualized via Alizarin Red staining. The cells were fixed with ice-cold 70% ethanol (–20°C) for 1 h and stained with 40 mM Alizarin Red S (Sigma-Aldrich,

TABLE 1 Primers used for RT-qPCR

Gene	Primer sequence [5′-3′]	Product size [bp]	Annealing temperature [°C]
ALP	FOR: CCG TGG CAA CTC TAT CTT TGG REV: CAG GCC CAT TGC CAT ACA C	89	59
RunX2	FOR: GCA AGG TTC AAC GAT CTG AGA TT REV: AAG ACG GTT ATG GTC AAG GTG AA	74	59
BGLAP/osteocalcin	FOR: GGC AGC GAG GTA GTG AAG AG REV: GAT GTG GTC AGC CAA CTC GT	138	59
PPARγ2	FOR: TGA AGC TGA ACC ACC CTG AGT REV: GCA CGT GTT CCG TGA CAA TC	90	59
FABP4/aP2	FOR: TGG TTG ATT TTC CAT CCC AT REV: TAC TGG GCC AGG AAT TTG AC	114	59
SOX9	FOR: AAG CTC TGG AGA CTT CTG AAC GA REV: GCC CGT TCT TCA CCG ACT T	136	59
ACAN	FOR: TCG AGG ACA GCG AGG CC REV: TCG AGG GTG TAG CGT GTA GAG	85	59
COL1A1	FOR: GGA CAA GAG GCA TGT CTG GTT REV: GAC ATC AGG CGC AGG AA	120	59
GAPDH	FOR: GAA GGT GAA GGT CGG AGT CA REV: AAT GAA GGG GTC ATT GAT GG	108	60

Abbreviation: RT-qPCR, reverse transcriptase-quantitative polymerase chain reaction.

Germany) for 10 min. Unbound Alizarin Red was washed with PBS and microscopic pictures were taken.

For quantification, Alizarin Red was extracted from mineral deposits at low pH using 10% acetic acid and neutralized with 10% ammonium hydroxide afterwards. Absorbance was measured at 405 nm using a microplate reader (Tecan Infinite M200 Plate Reader, Switzerland) and Alizarin Red concentration was calculated using a standard curve. All measurements were performed in triplicate and averages were calculated.

2.6 | Oil Red O staining

Oil Red O staining was used to visualize the lipid vacuoles formed during adipogenic differentiation. Oil Red O solution was prepared according to the manufacturers' instructions (Sigma-Aldrich, Germany). Cells were fixed with cold 50% ethanol (4°C) for 30 min and stained with Oil Red O solution for 10 min. Cell nuclei were counterstained with hematoxylin.

2.7 | Toluidine Blue staining

After 7-, 14-, and 21-days, pellets were fixed with 4% formaldehyde overnight and paraffin embedded. A microtome was used to cut 2 μm thin sections. After deparaffinization with xylene and rehydration, slices were stained with a solution of 1% Toluidine Blue (Sigma-Aldrich, Germany) in 0.1 M sodium acetate buffer to visualize proteoglycans of stem cell-derived chondrocytes.

2.8 | Semiquantitative RT-qPCR

Total RNA was isolated using the NucleoSpin RNA Kit (Macherey-Nagel, Germany), according to the manufacturers' protocol. Reverse transcription of 1 μg RNA was performed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). Relative gene expression (RE) of differentiation-related genes was analyzed in duplicates by RT-qPCR using Power SYBR® Green PCR Master Mix (Applied Biosystems, USA). The following primers were used: alkaline phosphatase (ALP), Runt-related transcription factor 2 (RunX2) and bone γ-carboxyglutamate protein (BGLAP/osteocalcin) for osteogenic differentiation; peroxisome proliferator-activated receptor γ 2 (PPARγ2) and fatty acid binding protein 4 (FABP4/aP2) for adipogenic differentiation; SRY-box transcription factor 9 (SOX9), aggrecan (ACAN) and collagen type 1 α 1 (COL1A1) for chondrogenic differentiation. Primer sequences (Table 1) were determined using the National Center for Biotechnology Information (NCBI) database and synthesized commercially (Eurofins Genomics, Germany).

The following conditions were set in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA): 95°C for 10 min followed by 40 cycles of 95°C for 10 s and 59°C or 60°C for 1 min (Table 1). The relative gene expression was

calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to a housekeeping gene (GAPDH).

2.9 | Statistical analysis

All data were presented as mean ± standard deviation (SD). Differences between MSC, u-PDLSC, and l-PDLSC were analyzed by two-way analysis of variance (ANOVA) followed by Bonferroni multiple comparisons test. Statistical analyses were performed using Prism 9.0 (GraphPad Software Inc., USA). A p value < 0.05 was considered statistically significant.

3 | RESULTS

3.1 | Flow cytometry

To examine the characteristics of cultured PDLSC isolated from maxilla and mandible, their cell surface antigen expression was investigated using flow cytometry and compared with MSC, as shown in Figure 1A. PDLSC were positive for stem cell markers $CD73^+$, $CD90^+$, and $CD105^+$, and negative for the hematopoietic stem cell and endothelial cell surface markers $CD34^-$ and CD45, which conforms with the expression pattern of MSC. PDLSC from maxilla comprised 92.49% ± 11.64%, 96.89% ± 3.08%, and 76.74% ± 21.71% cells staining positive for $CD73^+$, $CD90^+$, and $CD105^+$, respectively, while l-PDLSC were 83.99% ± 15.19%, 86.34% ± 13.88% and 75.40% ± 24.71% positive for $CD73^+$, $CD90^+$, and $CD105^+$, respectively. The overall expression of all three markers was lower in l-PDLSC than in u-PDLSC, although this was not statistically significant due to large variation (Figure 1B).

3.2 | Cell proliferation

Proliferation rate of u-PDLSC, l-PDLSC, and MSC was analyzed in PDLSC-Medium and MSC-Medium. Results for MSC-Medium are displayed in Figure 2A and revealed a higher proliferation rate of PDLSC (day 7: u-PDLSC: 315.890 ± 82.897 cell count/cm^2; l-PDLSC: 159.003 ± 70.447 cell count/cm^2) than MSC (20.711 ± 10.024 cell count/cm^2). Similar results were achieved when the cell types were cultured in PDLSC-Medium (day 7: u-PDLSC: 203.502 ± 60.991 cell counts/cm^2; l-PDLSC: 95.798 ± 77.323 cell counts/cm^2; MSC 20.533 ± 6.066 cell count/cm^2; Figure 2B). In Figure 2C, the cell count of both medium conditions after 7 days is compared, and significant differences could be seen between MSC and u-PDLSC in both medium conditions (both $p < 0.0001$) and between MSC and l-PDLSC when cultured in MSC-Medium ($p = 0.0016$). Comparison of u-PDLSC and l-PDLSC indicated that PDLSC from maxilla have a significantly higher proliferation rate than PDLSC from mandible, regardless of the culture conditions ($p = 0.0111$ (MSC-Medium); $p = 0.0002$ (PDLSC-Medium)). Doubling times (Figure 2D) of PDLSC were calculated and revealed significantly shorter doubling times in u-PDLSC than l-PDLSC when cultured in both PDLSC-Medium (u-PDLSC: 41.20 ± 3.10 h; l-PDLSC: 65.84 ± 16.45 h; $p < 0.0001$) and MSC-Medium (u-PDLSC: 36.85 ± 4.33 h; l-PDLSC: 48.51 ± 11.00 h; $p = 0.0011$).

3.3 | Osteogenic differentiation

The differentiation potential of PDLSC and MSC into osteogenic direction was analyzed via RT-qPCR, Alizarin Red staining, and quantification of staining intensity. Osteogenic differentiation-related gene expression (ALP, RunX2, and osteocalcin) is shown in Figure 3A. In comparison with the control groups, significant upregulation of gene expression could be observed in all cell types (all $p < 0.0001$). ALP reached its highest expression after 7 (MSC) or 14 days (PDLSC), RunX2 after 14 (MSC) or 21 days (PDLSC), and osteocalcin after 21 days (PDLSC and MSC), indicating a successful osteogenic differentiation of PDLSC and MSC. However, MSC appeared to differentiate into osteoblasts earlier than PDLSC.

Comparison of u-PDLSC and l-PDLSC (Figure 3B) revealed significantly higher gene expression levels in u-PDLSC compared with l-PDLSC for ALP after 14 days (u-PDLSC: 8.50 ± 1.42 RE; l-PDLSC: 5.87 ± 0.37 RE; $p = 0.03$), and for RunX2 (u-PDLSC: 5.30 ± 0.80 RE; l-PDLSC: 3.93 ± 0.54 RE; $p = 0.04$) and osteocalcin (u-PDLSC: 6.00 ± 0.83 RE; l-PDLSC: 4.20 ± 0.88 RE; $p = 0.04$) after 21 days. Thus, PDLSC from maxilla displayed a significantly higher osteogenic differentiation-related gene expression than PDLSC from mandible. Calcium phosphate mineral formed during osteogenic differentiation was stained with Alizarin Red (Figure 3C). Cells cultured in osteogenic induction medium showed stronger staining compared with control medium. However, calcium-rich deposits were sparser in PDLSC than in MSC. Quantification (Figure 3D) confirmed a significantly higher count of mineralized nodules during osteogenic induction compared with the control groups (all $p < 0.0001$). In addition, cells from maxilla and mandible stained significantly different ($p = 0.02$). After 21 days of osteogenic differentiation, PDLSC from maxilla (2.00 ± 0.33 mM) had significantly stronger Alizarin Red staining than PDLSC from mandible (1.33 ± 0.20 mM; Figure 3E).

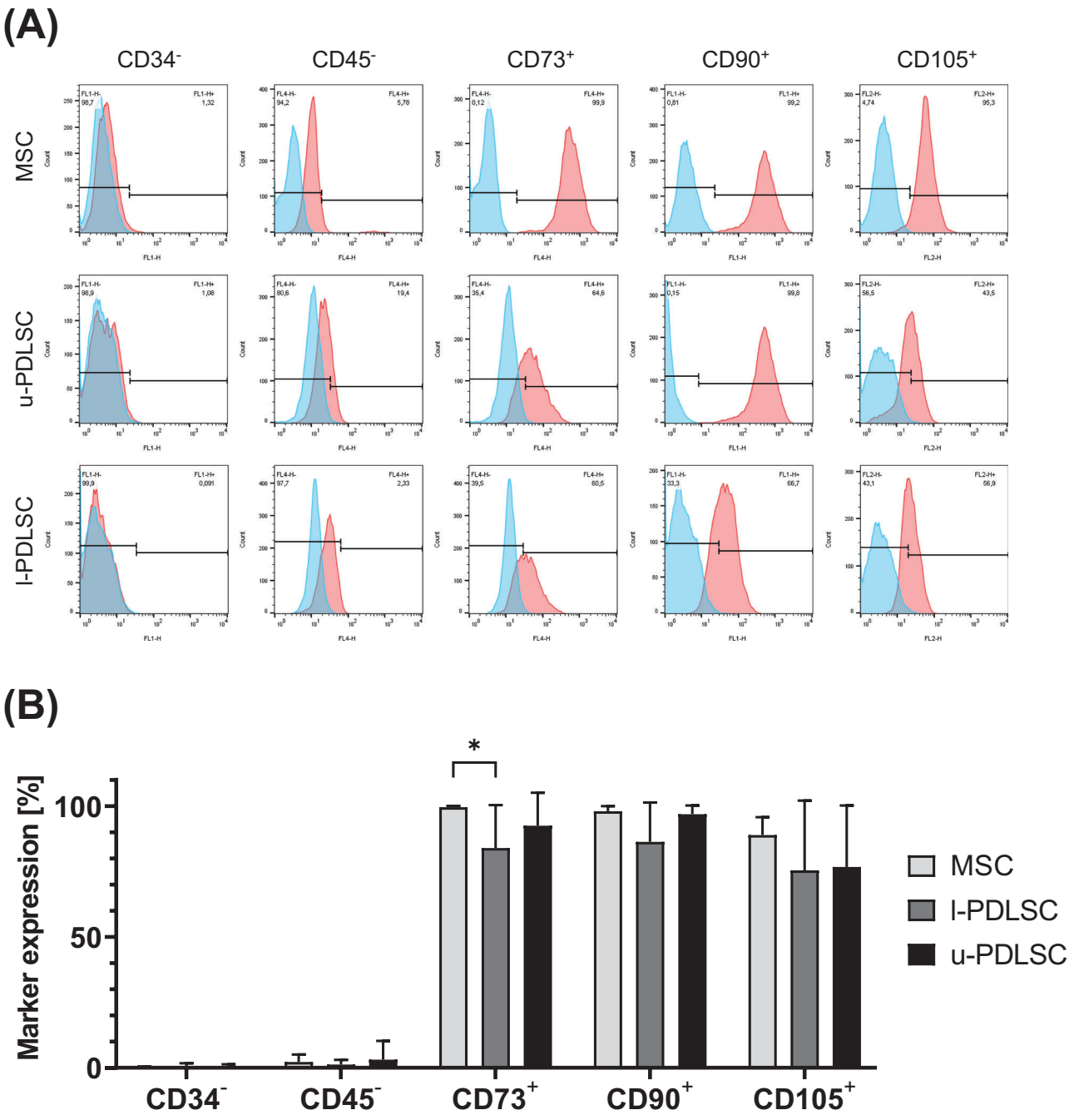

FIGURE 1 Flow cytometric analysis of periodontal ligament stem cells from upper jaw (u-PDLSC), periodontal ligament stem cells from lower jaw (l-PDLSC), and mesenchymal stem cells (MSC). Exemplary flow cytometric analysis of one donor u-PDLSC, l-PDLSC, and MSC for stem cell markers ($CD73^+$, $CD90^+$, $CD105^+$) and hematopoietic stem cell and endothelial cell surface markers ($CD34^-$, $CD45^-$) (**A**). Entire flow cytometric analysis of all donors (**B**). All cell types were negative for $CD34^-$ and $CD45^-$. MSC had a high expression of $CD73^+$ (99.54% ± 0.45%), $CD90^+$ (98.04% ± 1.78%), and $CD105^+$ (88.94% ± 6.30%). u-PDLSC showed a higher expression of $CD73^+$ (92.49% ± 11.64%), $CD90^+$ (96.89% ± 3.08%), and $CD105^+$ (76.74% ± 21.71%) when compared with l-PDLSC (83.99% ± 15.19%, 86.34% ± 13.88%, and 75.40% ± 24.71%, respectively. l-PDLSC, periodontal ligament stem cells from lower jaw; MSC, mesenchymal stem cells; u-PDLSC, periodontal ligament stem cells from upper jaw. *$p < 0.05$; $n = 7$.

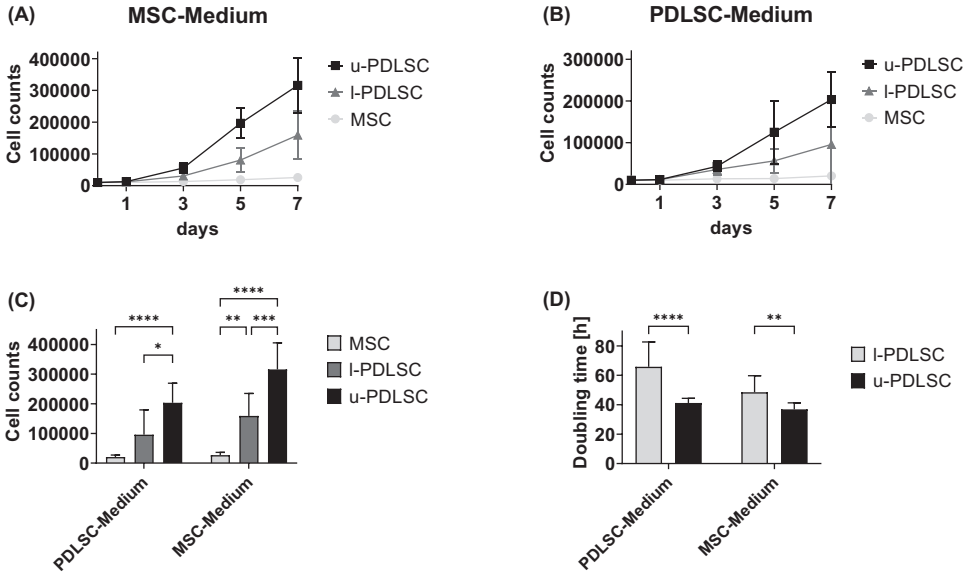

FIGURE 2 Cell proliferation analysis of periodontal ligament stem cells from upper jaw (u-PDLSC), periodontal ligament stem cells from lower jaw (l-PDLSC), and mesenchymal stem cells (MSC). Cell count of u-PDLSC, l-PDLSC, and MSC after 1, 3, 5, and 7 days of culture in MSC-Medium (**A**) or PDLSC-Medium (**B**). In both medium conditions, u-PDLSC did show the highest proliferation rate and MSC the lowest. Comparison of cell counts (**C**) after 7 days revealed significant differences between MSC and u-PDLSC in both medium conditions, between MSC and l-PDLSC in MSC-Medium, and between u-PDLSC and l-PDLSC in both medium conditions; u-PDLSC showed significantly shorter doubling times than l-PDLSC in both medium conditions (**D**). l-PDLSC, periodontal ligament stem cells from lower jaw; MSC, mesenchymal stem cells; u-PDLSC, periodontal ligament stem cells from upper jaw. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; $n = 7$.

3.4 | Adipogenic differentiation

Successful adipogenic differentiation was confirmed via RT-qPCR and Oil Red O staining. Adipogenic differentiation-related gene expression of FABP4 and PPARγ2 is shown in Figure 4A. FABP4 was significantly induced in MSC (31.13 ± 7.88 RE; $p < 0.0001$), u-PDLSC (5.77 ± 2.95 RE; $p < 0.001$), and l-PDLSC (6.23 ± 3.72 RE; $p < 0.01$) at day 21 in comparison with the control groups. After 21 days of adipogenic differentiation, significant induction of PPARγ2 was also present in MSC (3.53 ± 0.99 RE; $p < 0.001$), u-PDLSC (2.20 ± 0.24 RE; $p < 0.0001$), and l-PDLSC (1.90 ± 0.22 RE; $p < 0.01$). The induction of FABP4 and PPARγ2 indicated that PDLSC and MSC had the ability to successfully differentiate into adipocytes. Adipogenic differentiation-related gene expression was significantly higher in MSC than in PDLSC. A comparison of u-PDLSC and l-PDLSC revealed no significant differences in adipogenic differentiation ability (Figure 4B). The Oil Red O staining of lipid vacuoles, which were formed during adipogenic differentiation, are shown in Figure 4C. Lipid vacuoles could first be observed on day 7 in MSC and on day 21 in u-PDLSC, while no lipid vacuoles were detectable in l-PDLSC. Based on the staining, MSC had increased adipogenic differentiation potential than PDLSC, both from maxilla and mandible.

3.5 | Chondrogenic differentiation

Differentiation of PDLSC and MSC toward chondrocytes was evaluated through the quantification of chondrogenic differentiation-related gene expression and Toluidine Blue staining of synthesized proteoglycans. Gene expression of SOX9, ACAN, and COL1A1 is shown in Figure 5A. Gene expression levels peaked after 21 days, displaying significant differences compared with the control groups indicating that PDLSC and MSC underwent chondrogenic differentiation. Comparison of u-PDLSC and l-PDLSC showed significant differences in chondrogenic differentiation-related gene expression (Figure 5B). After 21 days of chondrogenic differentiation, SOX9 expression was higher in u-PDLSC than in l-PDLSC (8.73 ± 0.58 RE vs. 6.13 ± 0.61 RE; $p = 0.0009$). Similarly, ACAN expression was higher in u-PDLSC than in l-PDLSC (5.63 ± 1.23 RE vs. 2.97 ± 0.83 RE; $p = 0.01$), while no significant differences were observed in COL1A1 expression (u-PDLSC: 26.53 ± 4.64 RE; l-PDLSC: 21.23 ± 1.72 RE). Toluidine Blue staining of PDLSC and MSC are shown in Figure 5C. In

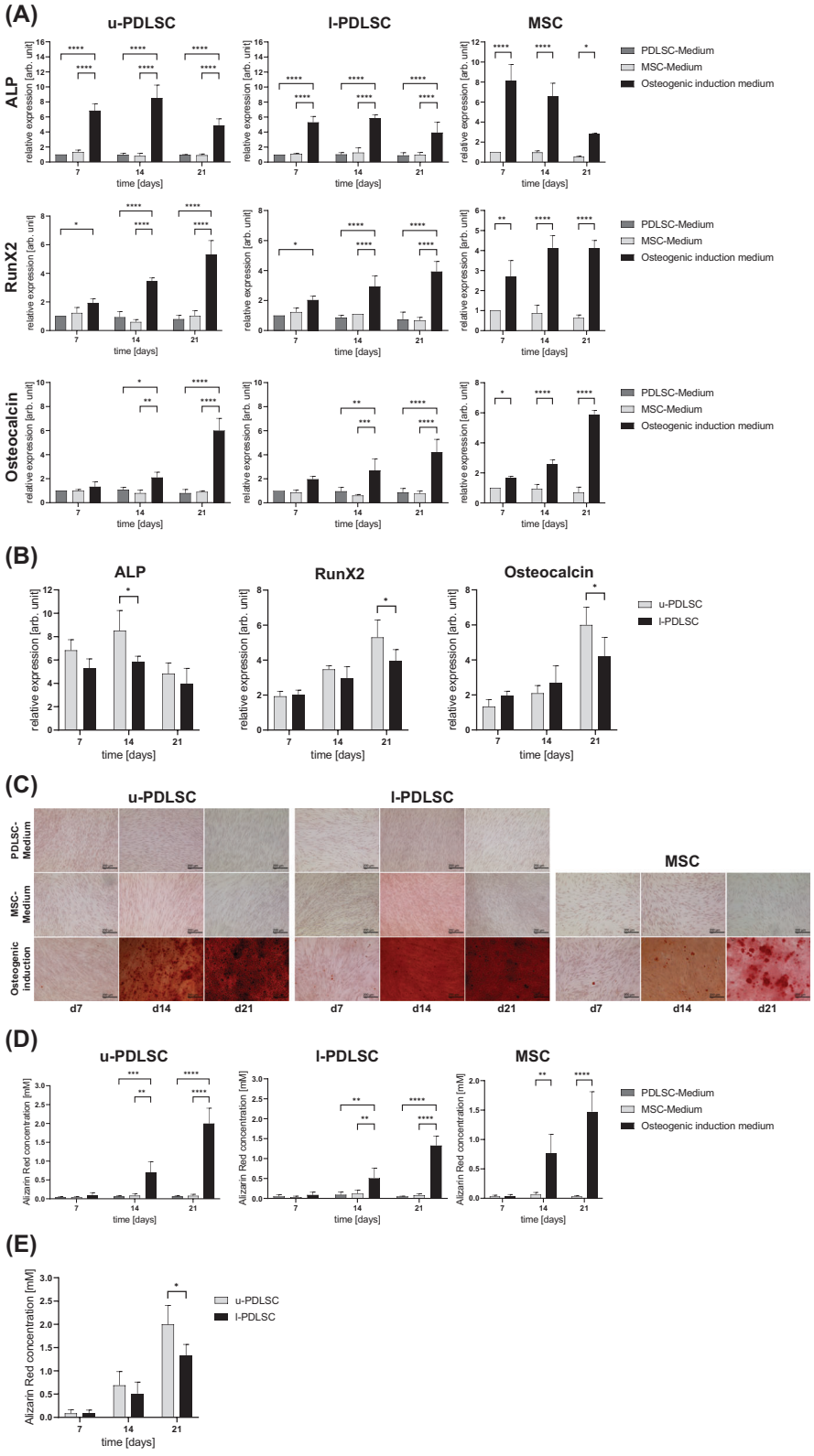

FIGURE 3 Osteogenic differentiation of periodontal ligament stem cells from upper jaw (u-PDLSC), periodontal ligament stem cells from lower jaw (l-PDLSC) and mesenchymal stem cells. (**A**) Osteogenic differentiation-related gene expression of ALP, RunX2, and osteocalcin after 7, 14, and 21 days of culture. Comparison of u-PDLSC and l-PDLSC revealed significantly higher gene expression levels in u-PDLSC compared with l-PDLSC for ALP after 14 days, and for RunX2 and osteocalcin after 21 days (**B**). (**C, D**) Alizarin Red staining of calcium accumulations during osteogenic differentiation and quantification of staining intensity after 7, 14, and 21 days. u-PDLSC showed a significantly higher Alizarin Red concentration after 21 days when compared with l-PDLSC (**E**). l-PDLSC, periodontal ligament stem cells from lower jaw; MSC, mesenchymal stem cells; u-PDLSC, periodontal ligament stem cells from upper jaw. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$; $n = 3$.

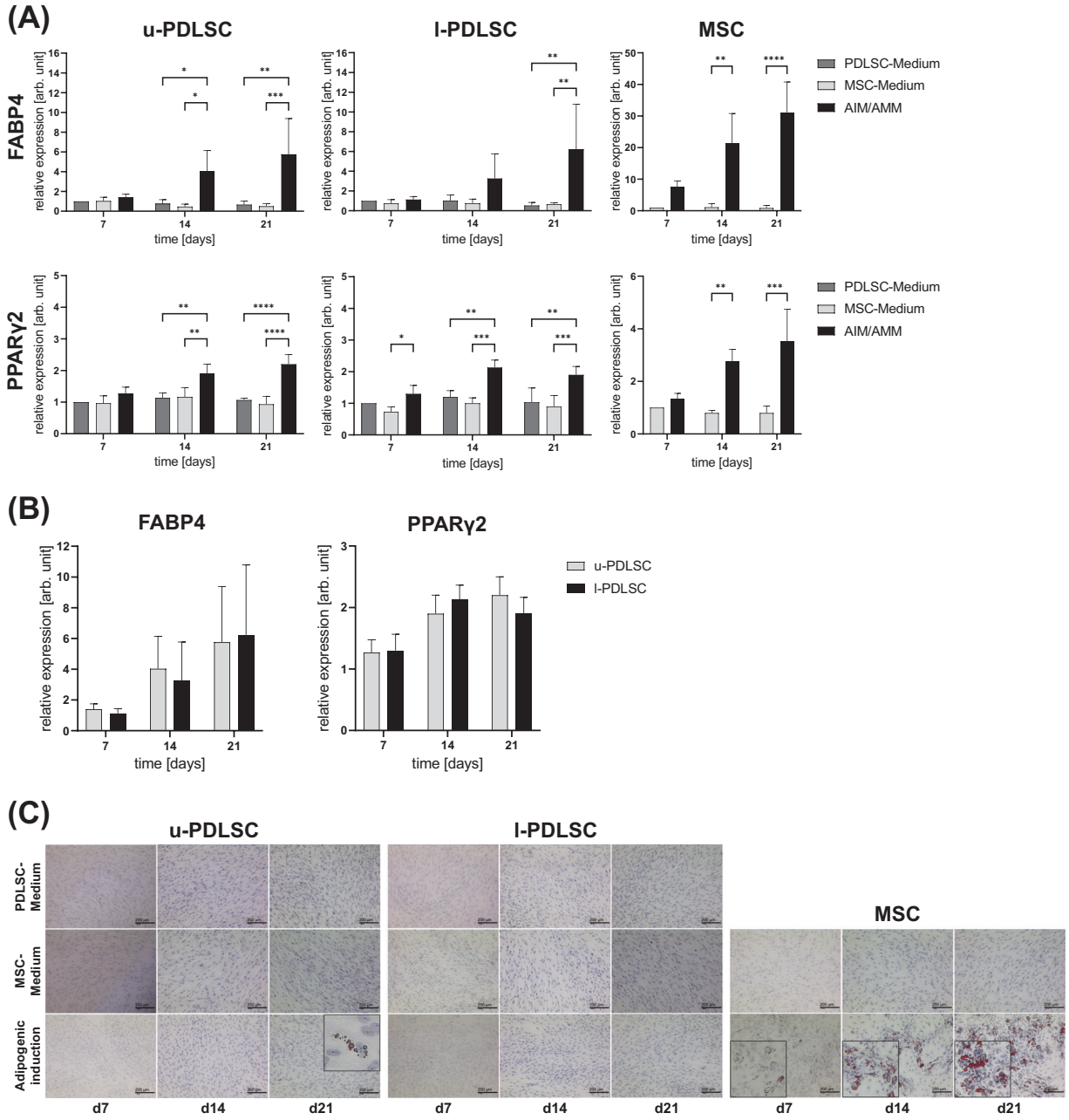

FIGURE 4 Adipogenic differentiation of periodontal ligament stem cells from upper jaw (u-PDLSC), periodontal ligament stem cells from lower jaw (l-PDLSC) and mesenchymal stem cells. (**A**) Adipogenic differentiation-related gene expression of FABP4 and PPARγ2 after 7, 14, and 21 days of culture. Comparison of u-PDLSC and l-PDLSC revealed no significant differences in gene expression levels (**B**). (**C**) Oil Red O staining of lipid vacuole formation after 7, 14, and 21 days of culture. l-PDLSC, periodontal ligament stem cells from lower jaw; MSC, mesenchymal stem cells; u-PDLSC, periodontal ligament stem cells from upper jaw. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; $n = 3$.

comparison with the control groups, more purple-stained proteoglycans were present in the chondrogenic induction group of both cell types. Additionally, pellet formation appeared to be more stable in induction medium than in control media.

u-PDLSC had slightly stronger proteoglycan staining than l-PDLSC, suggesting that PDLSC from maxilla possess greater chondrogenic differentiation potential than PDLSC from the mandible.

4 | DISCUSSION

In the past decades, stem cells and their use in wound healing and tissue engineering, have been at the forefront of scientific research. Due to ease of accessibility and their biological properties, PDLSC play a key role in stem cell-based periodontal wound therapy. Previous studies have demonstrated the low immunogenicity and immunosuppressive function of PDLSC.[13] Therefore, allogenic PDLSC

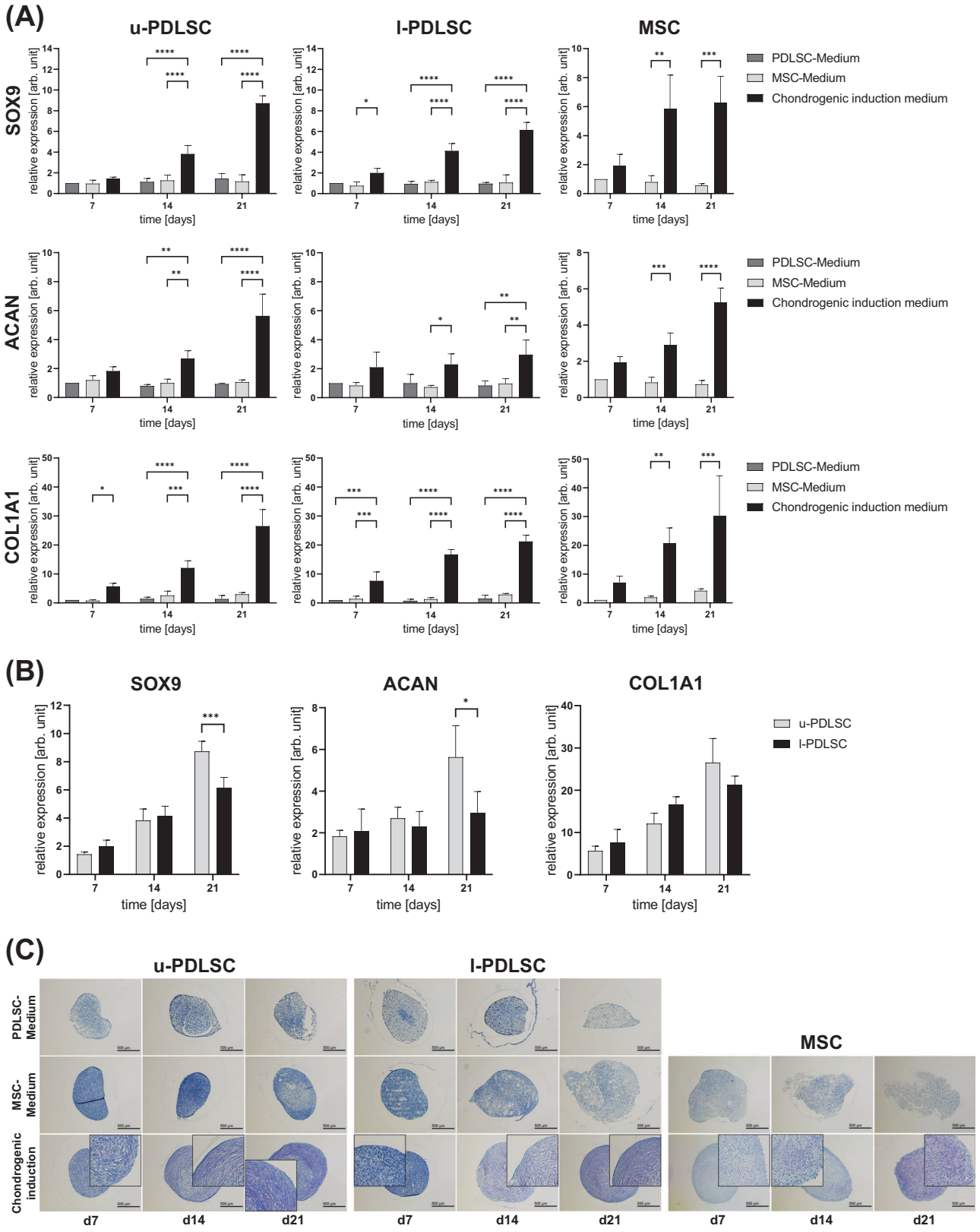

FIGURE 5 Chondrogenic differentiation of periodontal ligament stem cells from upper jaw (u-PDLSC) and lower jaw (l-PDLSC), and mesenchymal stem cells. (**A**) Chondrogenic differentiation-related gene expression of SOX9, ACAN, and COL1A1 after 7, 14, and 21 days of culture. Comparison of u-PDLSC and l-PDLSC revealed significantly higher gene expression levels in u-PDLSC compared with l-PDLSC for SOX9 and ACAN after 21 days (**B**). (**C**) Toluidine Blue staining of proteoglycan formation after 7, 14, and 21 days of culture. l-PDLSC, periodontal ligament stem cells from lower jaw; MSC, mesenchymal stem cells; u-PDLSC, periodontal ligament stem cells from upper jaw. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$; $n = 3$.

may be useful in the treatment of periodontal wounds[14,15] and bone defects.[16] Local application of PDLSC has also been shown to be effective for soft tissue regeneration.[17]

However, the exact mechanisms of periodontal wound healing are unknown, especially concerning the differences between maxilla and mandible. The purpose of this study was to comprehensively compare the proliferative and multilineage differentiation ability of PDLSC from maxilla and mandible to investigate possible reasons for differences in periodontal wound healing velocity and efficacy.

Multiple factors have been identified that influence the stem cell properties of PDLSC: origin from deciduous or permanent teeth,[18,19] donor age,[20–22] culture and isolation methods,[23–25] glucose concentration,[26] and presence of periodontitis.[27] A reduced osteogenic differentiation ability was also found in PDLSC grown in medium containing Porphyromonas gingivalis or *Escherichia coli* lipopolysaccharide.[28,29] Considering these findings, we limited ourselves to only young and healthy donors who had undergone teeth extraction of both maxillary and mandibular third molars. To ensure the best possible comparability, u-PDLSC and l-PDLSC received treatment according to the exact same protocols. This allowed us to reduce intra- and inter-subject variability.

According to the International Society for Cellular Therapy (ISCT), multipotent mesenchymal stromal cells must express $CD73^+$, $CD90^+$, and $CD105^+$, and lack expression of $CD34^-$ and $CD45^-$.[30] Flow cytometry analysis showed that PDLSC and MSC were highly positive for $CD73^+$ and $CD90^+$, but less positive for $CD105^+$. The lower expression of $CD105^+$ in PDLSC has already been described several times and has been associated with different effects on their differentiation ability.[18,31,32] Our results are in accordance with the expression of MSC-markers described in the literature.[8] However, since we did not perform cell sorting, the expression of stem cell markers $CD73^+$, $CD90^+$, and $CD105^+$ under 95% may also be related to a lower number of stem cells in culture. Nevertheless, u-PDLSC consistently showed higher expression of stem cell markers than l-PDLSC, although this was not statistically significant. Since we treated u-PDLSC and l-PDLSC identically, we assume that there are either fewer stem cells or stem cells with a lower proliferative and differentiation ability in PDL of mandibular molars. Other markers such as Stro-1 and CD146 can be used to indicate immature stem cells.[8] In previous studies, $Stro\text{-}1^+/CD146^+$ PDLSC displayed a higher self-renewal ability and osteogenic- as well as chondrogenic differentiation, although only 2.6% of cells isolated from the periodontal ligament were positive for both markers.[33] Nevertheless, PDLSC differ from other MSC by expressing proteins associated with PDL function such as periostin, scleraxis, and α-SMA.[8] Due to their ability to differentiate into cementoblast-like cells in vitro and cementum/PDL-like structure when transplanted into immunocompromised mice, Seo et al. considered PDLSC as a unique population of multipotent stem cells.[4] Therefore, it is not surprising that Tsumanuma et al. were able to show that PDLSC are better suited for periodontal wound healing than bone marrow-derived MSC.[34]

In periodontal wound healing, proliferation and differentiation of resident stem cells are crucial.[35,36] Cell proliferation analysis revealed that PDLSC have a significantly higher proliferation rate when compared with MSC. These results are in line with previous findings from Eleuterio et al.[37] and Lee et al.[38] As we received femoral heads anonymously from the Department of Orthopedic Surgery, the influence of donor age can only be suspected. Since total hip endoprosthesis is performed almost entirely in older patients, with an average age of 69.4 years,[39] we assume that the age of MSC donors may have a negative impact on their proliferative and differentiation ability. When comparing the proliferation rate and doubling time of u-PDLSC and l-PDLSC it was shown that u-PDLSC have a significantly faster growth rate, regardless of the culture conditions.

Osteogenic differentiation of PDLSC and MSC was analyzed via Alizarin Red staining and relative gene expression of ALP, RunX2, and osteocalcin. ALP is one of the most frequently used markers for osteogenic differentiation. It is mainly expressed in the early stages of osteogenic differentiation, while terminal differentiation and matrix maturation are mainly characterized by osteocalcin and mineral deposition.[40] RunX2 is a key transcription factor controlling osteoblast differentiation and bone formation.[41] The expression of RunX2 is upregulated during osteoblast differentiation, peaks in immature osteoblasts, and is downregulated in mature osteoblasts.[42] Our results showed an upregulation of bone-related gene expression and increased amount of calcium deposition during osteogenic differentiation indicating that PDLSC and MSC had the ability to successfully differentiate into osteoblasts. Furthermore, we were able to show significant differences in gene expression levels between u-PDLSC and l-PDLSC, suggesting a better osteogenic differentiation ability of PDLSC from maxilla.

We also demonstrated successful adipogenic and chondrogenic differentiation of PDLSC and MSC in vitro, thus fulfilling the third criteria for defining multipotent mesenchymal stromal cells of the ISCT.[30] Our results also suggested that u-PDLSC have a better chondrogenic differentiation capability than l-PDLSC. Importantly, the pivotal transcription factor in developing and adult cartilage,[43] SOX9, was significantly more induced in PDLSC from maxilla than from mandible. Analysis of adipogenic differentiation ability revealed differences

between PDLSC and MSC, but not between u-PDLSC and l-PDLSC. The reduced adipogenic differentiation ability of PDLSC when compared with other sources of MSC was already described by Szepesi et al.[44] and Xu et al.[33] However, Banavar et al. did not regard this reduced adipogenesis as a deterrent to the use of PDLSC in periodontal wound healing when they considered that periodontal regeneration required predominantly osteogenic potential.[45] Since the periodontium consists of cementum, periodontal ligament and alveolar bone, Liu et al. considered osteogenic, cementogenic, and fibrogenic differentiation to be the most important factors for regeneration of the bone-PDL-cementum complex.[5]

Our results showed that there are indeed differences in the proliferative and multilineage differentiation potential of u-PDLSC and l-PDLSC. Particularly, the significantly improved osteogenic as well as chondrogenic differentiation ability and the significantly higher proliferation rate of u-PDLSC seem to be possible reasons for the more efficient periodontal wound healing in the maxilla. Apart from proliferation and differentiation, further processes such as migration, paracrine effects on other cells, and the immunomodulatory properties of PDLSC are essential in periodontal regeneration.[46,47] The jawbone microenvironment also seems to have a decisive influence on the regenerative capacity of PDLSC.[48]

To our knowledge, for the first time, the present study comprehensively compared proliferative and multilineage differentiation ability of PDLSC from maxillary and mandibular molars in vitro. Accordingly, the underlying mechanisms can only be suspected. We assume that there are indeed intrinsic differences in the stem cell properties of u-PDLSC and l-PDLSC.

To gain further insight into the molecular mechanisms of periodontal wound healing and the differences between PDLSC from maxilla and mandible, further studies need to be conducted, especially in vivo studies to investigate the differences in regenerative potential of PDLSC when transplanted into the recipients' tissue. In particular, the abovementioned processes and the application of u-PDLSC and l-PDLSC in stem cell-based tissue engineering must be addressed.

5 | CONCLUSIONS

Surface epitopes, proliferation rate, and multilineage differentiation ability of PDLSC isolated from human maxillary and mandibular third molars were analyzed and compared with each other. Our findings showed, that u-PDLSC have a significantly higher proliferation rate and a significantly higher osteogenic as well as chondrogenic differentiation ability when compared with l-PDLSC. These results may offer a possible molecular explanation for the differences in periodontal wound healing efficacy between maxilla and mandible.

AUTHOR CONTRIBUTIONS

Project administration and study supervision: Sabine Neuss; study design/conception: Sinan Mert, Hanna Malyaran, Michael Wolf, Sabine Neuss; performing experiments, statistical analysis, and data evaluation: Sinan Mert, Hanna Malyaran; writing, drafting the manuscript, and visualization: Sinan Mert; review and correcting: Sinan Mert, Hanna Malyaran, Rogerio B. Craveiro, Michael Wolf, Ali Modabber, Willi Jahnen-Dechent, and Sabine Neuss.

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INSTITUTIONAL REVIEW BOARD STATEMENT

Collection and usage of periodont alligament from discarded patient samples were approved by the ethics committee for human subjects of the university clinics of RWTH Aachen, Germany (approval number EK374/19). Explanted femoral heads were anonymously provided by the Department of Orthopedic Surgery of RWTH Aachen University Hospital with the approval of the ethics committee for human subjects (EK300/13). This study was conducted in accordance with the Helsinki Declaration of 1975, as revised in 2013. All experiments were carried out in accordance with the relevant guidelines and regulations. Informed consent was obtained from all participants involved in the study.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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