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The Role of Cell Surface Markers and Enamel Matrix Derivatives on Human Periodontal Ligament Mesenchymal Progenitor Responses In Vitro

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Introduction

In humans, periodontitis is a pervasive, chronic infectious disease of the soft and hard-tissues supporting the teeth. This disease is characterized by the loss of both the hard and soft tissues (e.g. the periodontal tissues) that anchors the tooth in the jaws. Conventional periodontal therapy involves debridement of the root surface to induce local healing by repair pathways. However, regeneration of bone and cementum with a periodontal ligament (PDL) remains a challenge. Recently, the PDL has been shown to be of critical importance in the regenerative process [1] by providing mesenchymal stem/stromal cells (MSC) located within the PDL [2] that contribute to regeneration of the destroyed tissues [3].

Cells of the PDL represent a heterogeneous population [4] and it is not known which subsets of PDL cells are specifically involved in wound healing. However, it is well established that the PDL contains a cell population with MSC properties, such as self-renewal, clonal expansion and multiple lineage differentiation. Furthermore, these MSC-like cells of PDL origins display similarities with bone marrow derived MSC (BMSC), dental pulp stem cells and dental follicle stem cells [5]. The absence of a single specific MSC marker makes analysis of PDL progenitors more difficult, requiring instead the use of a combination of cell surface markers for their identification. Among these, CD146/MUC-18 is one the most employed. CD146 is located on endothelium, smooth muscle, Schwann cells, in some

neoplasms and is considered as one of the key markers of perivascular-, multipotent-progenitor cells (e.g. pericytes) in human connective tissues [6] including the PDL [3, 7, 8]. While the precise function of CD146 is not known, it has been linked to various cellular processes including cell adhesion, cytoskeletal reorganization, cell shape, migration, and proliferation through its capacity for transmembrane signaling [9]. Several studies demonstrated that CD146 positive (+) cell populations from numerous connective tissue sites exhibit MSC potential [10–15]. Because the CD146(+) population is not homogenous, attention has been paid to further refine CD146(+) cell subsets. When associated with the stem cell marker STRO-1, CD146 has been shown to identify PDL cell populations with MSC-like properties [2, 8] involved in regenerating periodontal tissues [3]. Others cell surface markers have been proposed to locate precursors cells within the human PDL, including CD106 (VCAM-1) [16] and the tissue non-specific alkaline phosphatase (TNAP) [17], recently shown to be identical to the MSC antigen 1 (MSCA-1), known to be expressed in human BMSC [18]. To date, the behavior of specific subsets of PDL cells have not been fully characterized and the role of these populations during periodontal healing warrants further elucidation. The differentiation capacity of PDL subsets during the regeneration process also remains unclear.

Multiple treatment modalities have been deployed in the treatment of periodontal defects including bone grafts or bone-substitutes, the use of barrier membrane and biological mediators. One of the goals of periodontal regenerative therapy, and especially the use of bioactive factors, is to trigger specific populations of PDL progenitor cells that would result in optimal periodontal regeneration. One biological mediator called Enamel Matrix Derivatives (EMD) is composed of immature porcine enamel matrix, rich in amelogenin protein, but that also contain bone morphogenetic proteins (BMP) –2 and –7 [19]. EMD has been used to treat infrabony defects and based upon observations from various animal models, EMD has been suggested to enhances PDL cell proliferation, migration and osteo-cementogenic differentiation [20]. Although EMD and the BMPs it contains has been shown to target MSC [21–23], the mechanisms of their action on PDL MSC progenitors is controversial.

In this study, cells were recovered from the PDL of 6 donors and their CD146, CD106 and MSCA-1 cell surface expression analyzed. To decipher the effects of EMD on the *in vitro* behavior of PDL progenitor cells, we used pure recombinant sources of amelogenin and BMP2/7.

Materials and Methods

Materials

Sources and concentrations of manufactured antibodies and reagents are summarized in table 1. Recombinant poly(His) tagged mouse 180 amino acid amelogenin, rp(H)M180 [24] was used at 5 µg/mL. Preparation of the STRO-4 monoclonal antibody anti-heat shock protein 90β, has been recently described [25]. All other reagents were from Sigma (St Louis, MO, USA).

HPDL cells isolation and cell culture

Human PDL cells (hPDL) were isolated from non-impacted premolars extracted for orthodontic reasons obtained from six healthy donors (four female, two male; age range 13–16 years). PDL tissue was separated from the surface of the mid-third of the root and cells were recovered and cultured in growth medium (GM: α -Minimum Essential Medium (MEM) Glutamax + 10% Fetal Calf Serum [FCS]) as previously described [19]. The use of human subjects conformed to standard practices established at the University of Toulouse and regulated by the French Ministry of Health. Written informed consent was obtained from the patient's parents. A declaration of biological collections has been made to the French Ministry of Superior Education and Research.

Flow cytometry assay and cell enrichment

Single-suspensions of hPDL cells in fluorescence-activated cell sorting (FACS) buffer (2% FCS/phosphate buffered saline [PBS]) were pelleted at 500 rpm for 5 minutes. Cells were incubated with selected antibodies at the concentrations stated in table 1 and/or ELF97 phosphatase substrate (1/50), on ice for 30 min [26, 27], followed by ten min incubation with 12 μ M levamisole in the case of ELF97 experiments [27, 28]. After two washes, 7-aminoactinomycin D (7-AAD) was added for cell death exclusion. Acquisition of 10,000 events was performed on a LSR-2 or using a FACSCalibur cytometer (BD Biosciences). For cell selection, single-suspensions of hPDL cells (3.5×10^7) in FACS buffer were pelleted then incubated with CD146 and MSCA-1 conjugated antibodies on ice for 30 min. Cell selection was performed using FACS Aria cell sorter (BD Biosciences). Selected fractions were subsequently cultured in GM for expansion.

Cell proliferation assay

To measure cellular division rates (doubling time, DT) during the course of the expansion, hPDL cells were initially plated at 1500 cells per cm^2 , cultured in GM and counted every 48 hrs during 6 days. DT (in hours) was calculated using the following formula: $DT = 48 \cdot \ln(2) / \ln(n_2/n_1)$ (where n_2 is the counting time performed 48 hrs later than the previous interval, n_1). The effect of agonists or vehicle on hPDL cells growth was monitored by quantifying DNA synthesis [26]. hPDL cells were seeded (1000 cells/ cm^2) in GM, allowed to attach for 12 hours and synchronized in Dulbecco's Modified Eagle Medium (DMEM) alone for additional 24 hrs. Cultures were subsequently maintained in GM or in 0.5%FSC/DMEM alone (control) [29] or in 0.5%FSC/DMEM supplemented with agonists as follows: rhBMP2/7, EMD +/- rhNoggin, rp(H)M180, dexamethasone (10 nM) or rp(H)M180+rhBMP2/7, for 60 and 120 hrs. At the end of the experimental period, the total DNA of each well was quantified using Qbit dsDNA assay kit (Invitrogen) following the manufacturer instructions.

Cell cycle experiment on live cells

hPDL cells were seeded at 5000 cells per cm^2 , attached, starved for 24 hrs and challenged as described above (see *Cell Proliferation Assay*). After 24 hrs, Hoechst 33342 (1.25 $\mu\text{g/mL}$) was added for 1 hr in medium. Cells were recovered and used in FACS experiments.

Colony-forming units-fibroblastic assay

hPDL cells were plated at 10 cells/cm² and cultured for 11-days in GM. Cells were fixed for 15 min in 100% methanol before being stained with 0.1% Toluidine blue for 20 min. Colonies were counted only when they were greater than 50 cells in size and were not in contact with another colony.

In vitro wound healing experiment

hPDL cells (5000/cm²) were seeded in 6-well plate or on glass slide chambers and cultured in GM reach to 70–80% confluence. After 24 hrs serum starvation, monolayer cells were scratched using a pipette tips to make a 2-mm wide cell-free strip. A linear mark was made with a scalpel to localize the center of the wound before the cells were rinsed with PBS. Cultures were incubated with agonists or vehicle in the same conditions that are described in *Cell Proliferation Assay* (see above). After 36 hrs, the cells were fixed in 3.7% paraformaldehyde for 15 min. In order to quantify the wound filling cells, samples grown in plastic plates were stained for 30 min with a solution of 5µg/mL of Hoechst 33342 in PBS. Nuclei were photographed using fluorescent illumination and counted in a 0.75 mm² area adjacent to the scalpel mark, using Image J software (NIH, USA). For confocal microscopy analysis, hPDL cells seeded in glass chambers were treated as described below (see “Immunofluorescence Studies”).

Cell differentiation

For osteogenic, chondrogenic and adipogenic differentiation, 70–80% confluent hPDL cells were cultured as previously described [19]. Chondrocyte pellets were fixed in 3.7% paraformaldehyde, embedded in paraffin and 4 µm-thick sections were performed. To test the effect of agonists on osteogenic differentiation, 70–80% confluence hPDL cells were maintained in differentiation medium (D-MEM with 2% FCS, 50 µg/mL L-ascorbate 2-phosphate) alone (control) or supplemented with agonists as follows: rhBMP2/7, dexamethasone (10nM), EMD +/- rhNoggin, rp(H)M180 or rp(H)M180+rhBMP2/7, for various time as indicated. Cells were used for immunofluorescence, dot blotting, FACS experiment or biochemical studies, as described.

Quantification of alkaline phosphatase (AP) activity and total protein content

After 2-, 5- and 10-days in differentiation medium, cultures were rinsed with PBS and scraped to disrupt the cells into RIPA buffer. AP activity was determined using the SensoLyte® pNPP Alkaline Phosphatase Assay Kit (Anaspec, Fremont CA, USA) according to the manufacturer's instructions. Total protein was quantified with the Bio-Rad protein assay kit (Bio-Rad, Marne-La-Coquette, France). AP activity was expressed as nanomole of paranitrophenol per microgram of protein. Absorbance was measured with a spectrophotometer (Bio-Rad 3550 microplate Reader).

Mineralization assay

After 21days in differentiation medium supplemented with 10 mM β-glycerophosphate, hPDL cells were rinsed with PBS. Calcium deposits were stained with Alizarin Red (AR) as previously described [30], and the samples were photographed. Cells were treated with 10%

(w/v) cetylpyridinium chloride in 10 mM sodium phosphate for 15 min to extract the bound dye. Calcium concentration was determined by measuring absorbance at 562 nm with a spectrophotometer (Bio-Rad 3550 microplate Reader).

Dot blot analysis

Osteogenic and adipogenic differentiation of hPDL cells was confirmed by osteopontin (OPN) and peroxysome proliferators-activated receptors- γ (PPAR γ) expression respectively (normalized to β actin expression), using dot blot methodology. A known concentration of proteins from cell lysates was spotted to a Millipore PDFV membrane (Millipore Molsheim, France). OPN, PPAR γ and β actin expression were assayed using WesternDot 625 Goat Anti-Mouse Western Blot Kit (Invitrogen) and the membrane was photographed.

Immunofluorescence studies

For confocal microscopy analysis, cells were seeded on glass slide chambers and cultured as indicated. Cells were fixed in 3.7% paraformaldehyde for 15 min, blocked in 5% goat serum for 30 min and incubated with the indicated primary antibody for 1 hr following by incubation for 1 hr after washing in Dylight 549 goat anti-mouse IgG and Dylight 647 goat anti-rabbit IgG (Invitrogen). Detection of AP activity was performed with ELF97 endogenous phosphatase detection kit according to the recommendations of manufacturer. Cell nuclei were stained by Hoechst 33342 (5 μ g/mL) for 30 min. In negative controls, primary antibodies were replaced by an appropriate isotype-matched negative antibody IgG. Fluorescence staining was visualized by confocal microscopy (Zeiss LSM 710).

Histochemical and immunohistochemical analysis

The detection of the collagen II in chondrocyte pellets and Alcian blue staining were performed as previously described [19]. For visualization of lipid droplets, hPDL cells were cultured with adipogenic medium, fixed in 3.7% paraformaldehyde for 15 min, and rinsed in 60% Isopropanol. Oil Red O (RO) was added to the cells for 10 min and the sample was washed and microphotographed. Equal volumes of 60% isopropanol were subsequently added to the culture dishes to destain the fixed cells. The solution containing the RO was collected, and absorbance was measured at 510 nm using a spectrophotometer (Bio-Rad 3550 microplate Reader).

Statistical analysis

Results were expressed as mean $\pm$ SEM of at least triplicate values. Comparative studies were performed by non-parametric Mann-Whitney test. Correlations were analyzed by Spearman's test. Statistical significance was considered when $p < 0.05$.

Results

Characterization of hPDL cells

We analyzed a panel of 13-cell surface markers for their expression on donor hPDL cells (Supplemental data 1, Fig A). The staining pattern for each of the hematopoietic markers (79a, 45, 14 and HLADR), CD34, and ELF97 was negative, whereas the expression levels

for non-hematopoietic cell markers (CD90, 73, 105, and STRO-4) were uniformly positive (>95% of positive cells) with the exception of three markers, CD146, CD106, and MSCA-1, whose expression showed marked variability between donors (32% $\pm$ 18); (7% $\pm$ 5); (4% $\pm$ 2) respectively (Fig. 1A and Supplemental data 1, Fig B). Multicolor FACS-analysis experiment indicated marked differences between CD146(+) MSCA-1(+), CD146(+) CD106(+) and CD106(+) MSCA-1(+) hPDL cell subsets among the donors ((1.6% $\pm$ 1.3); (1.7% $\pm$ 0.9); (0.4% $\pm$ 6.2) respectively, Fig. 1B and Supplemental data 1, Fig B). Growth rates demonstrated a wide range of values from among hPDL cell samples (doubling time (DT): 45.4 hrs $\pm$ 3.8, Fig 1C). In addition, we observed variation in the ability of these hPDL cell cultures to form clonogenic clusters (CFU-F: 19.4 $\pm$ 10.4, Fig. 1D). We observed variability in their ability to differentiate toward multiple mesenchyme-derived cell types, such as cells producing a calcified matrix, chondrocytes or adipocytes, as demonstrated by specific staining (Fig. 1 E–G and Supplemental data 1, Fig C) and their expression of genes indicative of these differentiated cell states (e.g. OPN, collagen II and PPAR- γ , respectively) (Fig. 1H and Supplemental data 1, Fig D). Intriguingly, the Spearman's test showed a positive correlation between the percentages of CD146(+), CD146(+) MSCA-1(+) and CD146(+) CD106(+) hPDL cells with their DT ($p=0.019$, $p=0.005$, $p=0.005$, respectively), as well as their potential to form colonies ($p=0.005$, $p=0.019$, $p=0.001$, respectively), to form calcified matrices ($p=0.019$, $p=0.005$, $p=0.005$, respectively) and lipid droplets ($p=0.001$, $p=0.005$, $p=0.005$, respectively).

hPDL cell proliferation

The hPDL cells were stimulated for 60- and 120-hrs with agonists or vehicle as indicated (Fig. 2A). Total DNA was significantly increased in a time-dependent manner with rp(H)M180, EMD $\pm$ rhNg, rp(H)M180+rhBMP2/7 or GM (1.7-, 2-, 1.9, 1.8- and 3.5-fold-increase at 120-hrs, respectively) but not with rhBMP2/7 alone, dexamethasone or the negative control (0.5% FCS). The response to mitogenic induction was correlated with the initial percentage of CD146(+) marked hPDL cells in the sample ($p<0.02$ for all mitogenic agonists).

Cell-cycle analysis showed that stimulation by rp(H)M180, EMD $\pm$ rhNg, rp(H)M180+rhBMP2/7 or GM for 25 hours significantly increased the percentage of hPDL cells in S-phase and G2/M-phase compared to dexamethasone or 0.5% FCS (Fig. 2B and Supplemental data 2, Fig A). The use of rhBMP2/7 resulted in the weak up-regulation of the percentage of hPDL cells in S-phase alone. Moreover, treatment for 25 hrs with rp(H)M180, EMD $\pm$ rhNg, rhBMP2/7, rp(H)M180+rhBMP2/7 or GM significantly increased the percentage of CD146(+) hPDL cells compared to control, while the MSCA-1(+) and CD106(+) sub-populations remained constant (Fig. 2C).

The effect of mitogens on the distribution of dividing cells among the CD146(+) hPDL cell population was assayed by multicolor flow cytometric analysis (Fig. 2D and Supplemental data 2, Fig B). After 25-hr-treatment by rp(H)M180, EMD $\pm$ rhNg or rp(H)M180+rhBMP2/7, the CD146(+) subset contained more cells in G2/M phase than the CD146(–) population. In contrast, when similar cultures were maintained in GM alone, the percentage of cells in G2/M phase was balanced among CD146(+) and CD146(–) hPDL

cells. Although devoid of effects on cell division, rhBMP2/7 altered the proportion of cells in G2/M phase in favor of CD146(+) cells. No significant change to the distribution of G2/M phase cells marked as MSCA(+) and CD106(+) or the MSCA(−) and CD106(−) counterparts was observed for these culture conditions (data not shown). The use of rhNg did not diminish the effect of EMD on DNA synthesis, suggesting the effects of EMD on cell expansion and the increase of CD146(+) hPDL cells were not dependent on BMP in the EMD preparations.

hPDL cell behavior in the in vitro wound healing assay

A scratch test model for wound healing was used to investigate the ability of hPDL cells to fill a defined cell-free area after 36 hrs in selected agonists or vehicle as indicated in figure 3A and Supplemental data 2C. Wound filling was significantly accelerated by treatment with rp(H)M180, rhBMP2/7, EMD +/- rhNg, rp(H)M180+rhBMP2/7 or GM, compared to dexamethasone or 0.5% FCS (5.6-, 2.6-, 7.8-, 5.5, 7.6- and 7.3-fold-increase, respectively). The addition of rhNg to the EMD-containing medium did not alter the effect on wound filling. In all instances, cell responsiveness to fill the void correlated with the initial proportion of CD146(+) in the culture ($p < 0.02$ for all).

Next, we focused on CD146, CD106 and MSCA-1 expression in untreated (0.5% FCS) or EMD-treated cultures using the wound healing assay. Confocal microscopy analysis showed intense expression of CD146 in cells lining the wound borders and in migrating cells, an outcome that was especially noticeable in EMD-treated hPDL cells compared to controls (Fig 3B). MSCA-1(+) cells and CD106(+) were mainly localized at the rear of the wound edge, while some CD146(+) MSCA-1(+) and CD146(+) CD106(+) cells were identified among the migrating cells in cultures receiving EMD.

hPDL cell differentiation

To investigate the potential of hPDL cells to respond to selected agonists or vehicle control to undergo osteoblastic or cementoblastic differentiation, we assessed alkaline phosphatase (AP) activity and calcium deposition. After 5-days of stimulation, AP-activity was significantly increased in treated hPDL cells compared to unstimulated cultures (Fig. 4A). At day-10, AP activity was significantly greater after induction with EMD or rp(H)M180+rhBMP2/7 (3.3- and 3.2-fold increase, respectively) than values obtained for cells stimulated with dexamethasone or rhBMP2/7 (2.6- and 2.4 fold increase, respectively). The use of rp(H)M180-treatment alone exhibited a weak response (1.6-fold increase compared to control), but one that was statistically significant ($p = 0.01$). Incubation of hPDL cells with rhNg partially reversed the EMD-induced rise in AP activity after a 10-day period of stimulation ($p = .004$), suggesting that the AP-activity induced by EMD treatment was partially mediated through the BMP axis. At day-21, calcium deposits were significantly increased in agonist stimulated-hPDL cells compared to untreated (Fig. 4B). Alizarin Red staining was significantly greater with EMD or rp(H)M180+rhBMP2/7 stimulation (4.2- and 3.8-fold increase, respectively) compared to dexamethasone, rhBMP2/7, or rp(H)M180 (3.1-, 2.7- and 1.6-fold increase, respectively). The addition of rhNg to the medium also partially inhibited the EMD-induced matrix mineralization in hPDL cell cultures, confirming the involvement of BMPs in these responses ($p = 0.004$) (Fig. 4B). The Spearman's test

indicated that the agonist efficiency correlated with the initial proportion of CD146(+) hPDL cells contained in the culture ($p < 0.05$ for all effective agonists).

To investigate the expression of cell surface markers among the hPDL cell sub-populations during their osteo/cementoblastic differentiation, the changes in the expression of CD146, CD106, MSCA-1 markers, and extent of AP expression was analyzed by multicolor flow cytometric analysis (Fig. 5A–E and supplemental data 3).

After 2 days of stimulation by various agonists, the proportion of CD146(+) MSCA-1(–) ELF97(–) population was significantly greater in rp(H)M180, rhBMP2/7, or EMD conditions than observed for these cells when challenged with dexamethasone or in control conditions (Fig. 5A). The percentage of CD146(+) CD106(+) MSCA-1(–) cells was higher in all treated cell cultures than in untreated cells and in starting cultures (day 0) (Fig. 5B). The CD146(+), MSCA-1(+), ELF97(–) phenotype was enhanced by treatment with rp(H)M180, rhBMP2/7, dexamethasone or EMD when compared to controls (Fig. 5C).

After 5 days of agonist stimulation, the yield of CD146(+) MSCA-1(–) ELF97(–) cells was dramatically decreased in untreated- and dexamethasone-treated cultures compared to their representation in the original cell population. In contrast, the CD146(+) MSCA-1(–) ELF97(–) phenotype were retained in cells treated with rhBMP2/7 alone (Fig. 5A). The proportion of CD146(+) CD106(+) MSCA-1(–) hPDL cells was greater in cultures treated with either EMD or rhBMP2/7 compared to the values observed for the cells at the start of culture or for control cultures (Fig. 5B). The percentage of CD146(+) MSCA-1(+) ELF97(–) cell subset was significantly higher after treatment with rhBMP2/7, dexamethasone or EMD compared to starting or control cultures (Fig. 5C). After 5 days of treatment, it was noticeable that a percentage of ELF97(+) cells expressed MSCA-1 or CD106, but not CD146 (Fig. 5D, 5E).

After 10 days in differentiation medium, the ratio of CD146(+) MSCA-1(–) ELF97(–) cells was reduced in all cultures except those treated with rhBMP2/7 (Fig. 5A). The percentage of CD146(+) CD106(+) MSCA-1(–) cells was only sustained in rhBMP2/7 stimulated cells (Fig. 5B). The proportion of the CD146(+) MSCA-1(+) ELF97(–) subset was higher in cells treated with EMD, dexamethasone and rhBMP2/7 than in those treated with rp(H)M180 or in control cultures (Fig. 5C). After 10 days, EMD induced the greatest number of hPDL cells that displayed the CD146(–) MSCA-1(+) ELF97(+) and the CD146(–) CD106(+) ELF97(+) phenotype. These levels were followed in descending order by dexamethasone, rhBMP2/7, and rp(H)M180 treatment, when compared to unstimulated control cells (Fig. 5D;E). These latest data confirm the effect of agonists on AP activity (see above) and the lack of CD146 expression by the differentiated, ELF97(+) cells.

During the course of EMD-stimulation we used confocal microscopy to follow the distribution of CD146, CD106, MSCA-1 expression, and AP activation (e.g. ELF97 staining) in hPDL cells cultures (Fig. 5F; G). Cells expressing the CD146 marker only were identified in untreated and 2 day-treated cultures, while few CD146(+) MSCA-1(+) ELF97(–) and CD146(+) CD106(+) ELF97(–) cells were distinguishable (Fig. 5 F i, ii and G i, ii). After 5 days of treatment, numerous CD146 (+) cells expressed the MSCA-1 marker but lacked expression of ELF97 (Fig. 5 F iii). Conversely, sites of activated AP were only

found in cells that were CD146(–) MSCA-1(+), where these same cells undertook cell spreading (Fig. 5 F iv). CD106 expression was located both on ELF97(+) and ELF97(–) marked hPDL cells, while CD106(–) ELF97(+) cells could also be identified (Fig. 5 G iii). Finally, 10 day-treated cultures exhibited numerous CD146(–) MSCA-1(+) cells strongly stained with ELF97 (Fig. 5F v), whereas few CD106(+) ELF97(+) cells could be identified (Fig. 5G iv).

hPDL cell enrichment

Next, we sought to compare the *in vitro* behaviour of hPDL cells based on their expression of either CD146 and/or MSCA-1 cell surface markers. Cell-sorting experiment were conducted to obtain cell fractions expressing either CD146(–) MSCA-1(–) (~85% of the cell fraction) or CD146(+) MSCA-1(–) (~78% of the cell fraction) or CD146(–) MSCA-1(+) (~80% of the cell fraction) or CD146(+) MSCA-1(+) (~93% of the cell fraction) from the hPDL cell cultures. Despite many attempts, cells from CD146(–) MSCA-1(+) fraction did not attach after sorting, while cell fractions expressing other combinations of cell markers consistently plated and grew in culture. Surprisingly, although the proportion of CD146(–) MSCA-1(–) and CD146(+) MSCA-1(–) cell fractions were equally maintained during expansion, cells from the CD146(+) MSCA-1(+) fraction tended to loose (by ~20-fold) their MSCA-1 expression and shifted towards the CD146(+) MSCA-1(–) phenotype (Fig. 6A).

Interestingly, treatment of the cells expressing CD146(–) MSCA-1(–), CD146(+) MSCA-1(–) and CD146(+) MSCA-1(+) markers with dexamethasone, rhBMP2/7 or EMD for 4-days strongly increased the yield of CD146(+) MSCA-1(+) cells, and the extent of the escalation was proportional to the initial percentage of CD146(+) in the culture (Fig. 6B). CD146(+) enriched cell fractions exhibited lower DT (Fig. 7A) and higher clonogenic, osteogenic, adipogenic and chondrogenic potential than the CD146(+) depleted subsets (Fig. 7B–H and supplemental data 4, Fig. A). Proliferation (Fig. 7I), *in vitro* wound healing (Fig. 7J), and differentiation experiments (Fig. 7K–L) performed on selected CD146(–) MSCA-1(–), CD146(+) MSCA-1(–) and CD146(+) MSCA-1(+) expressing hPDL cells confirmed the effect of agonists on unfractionated/parental hPDL cells. Furthermore, the addition of rp(H)M180 to media containing rhBMP2/7 was seen to exert a synergistic effects on both AP activity and calcium deposition for CD146(+) enriched fractions only. Similarly to the proliferation and scratch test experiments, the differentiation assays demonstrated the superior responsiveness of CD146(+) enriched fractions to agonists in contrast to the CD146 depleted cell fractions.

Discussion

For many years considerable efforts have been made towards developing strategies for the regeneration of tooth-supporting tissues destroyed by periodontal disease. Evidence has shown that the periodontal ligament (PDL) contains a heterogeneous mix of progenitor cells that represents the main source for the healing of those tissues destroyed by periodontitis [2]. The present study highlights the usefulness of cell surface markers, especially CD146, to

identify the cell subset within the heterogeneous PDL progenitor population that may be triggered by biological agents as part of therapeutic periodontal regeneration.

To help improve patient care, it would be informative to identify markers for those patients who may respond favorably to regenerative therapeutic interventions. In this study, several diverse human PDL cell preparations were characterized according to published criteria [31]. Similar to the variable response rates observed for clinical regeneration, we also observed noticeable variations in the responsiveness of the hPDL cells from a range of patients to divide, migrate, and to differentiate. Previous studies have reported on the impact that senescence or gender can exert on the *in vitro* behavior of BMSCs [32]. Here, the PDL progenitor cells were recovered from a cohort of subjects from the same gender that were of similar age and health profiles. Therefore, the differences observed among the PDL cells in previous studies with respect to their responses to various therapeutic agents may be explained by variation among these individuals. It is recognized that the proportion of MSC-like cells in dental pulp varies among 16–18 year-old subjects [33], but such age related variation has not been reported for hPDL progenitor cells. The capacity of the hPDL to contribute cells with MSC-like potential was highly correlated with the percentage of CD146 positive cells in the culture, as previously reported [8, 34]. CD146 is a recognized cell marker for endothelial and progenitor cells and it has also been described as a pericyte/perivascular associated marker [9, 35]. Accumulating evidence suggest that pericytes or subsets of perivascular cells may be the origin for MSC-like populations in different tissues [6], where CD146(+) cells are located around the vessels in healthy and regenerating PDL [3]. Our findings confirm previous data demonstrating the presence of MSC-like cells in CD146(+) PDL population [1] that may confer to the PDL the ability to regenerate periodontal tissues.

The second part of this paper aimed to decrypt the *in vitro* effects of selected biological factors previously suggested for periodontal regeneration. Emdogain is a commercial product routinely employed in periodontal surgery that contains enamel matrix derived proteins (EMD), as well as BMP-2 and -7. However, the outcome for EMD use *in vivo* can be unpredictable and subject-dependant [1, 17, 36]. Although EMD was shown to induce cell proliferation and differentiation in several mouse and human MSC *in vitro* models [37–42], little is known regarding the use of EMD on multipotent cells of PDL origin. Our results confirm that EMD enhances hPDL cell proliferation, migration and mineralized-tissue cell-type differentiation, as recently reported [20]. More importantly, we provide evidence that the EMD efficiency is correlated with the proportion of CD146(+) cells available in the culture.

The proliferation experiments we performed suggest that the mitogenic effects of EMD are not mediated by rhBMP2/7, but rather by amelogenin, the principal component of Emdogain [43]. Amelogenin protein, such as rp(H)M180, has recently been shown to enhance the growth rate for human PDL cells and BMSCs [23, 44, 45]. Samples containing more CD146 marked cells respond by proliferation not only to rp(H)M180, but to all mitogens tested [13, 14]. Moreover, since both EMD and rp(H)M180 exhibited similar effects on hPDL cells wound healing it suggests that EMD induced cell migration is mediated by amelogenin and not another growth factor contaminant in the EMD. In accord with these observations,

several papers report similar enhanced wound filling by EMD [46–48] or amelogenin [44] using similar *in vitro* models. Here, we demonstrated that wound healing by CD146(+) cells is also likely mediated by amelogenin. Despite BMP-2 being shown to promote BMSC cell migration [49], the effects of rhBMP2/7 heterodimer on hPDL cells wound filling observed here are weak and do not seem to be involved in the cell motility EMD-induced since soluble BMP antagonist did not alter the response of the hPDL cells. Similarly with our proliferation experiments, the efficiency in wound filling for EMD or rp(H)M180 is correlated with the percentage of CD146 positive hPDL cells. Interestingly, the use of dexamethasone didn't enhanced the hPDL cell proliferation, as reported for dental pulp cells [50].

We observed that once the PDL progenitor cells underwent proliferation/migration to the site of injury, a proportion of them differentiate along osteogenic or cementogenic lines, as shown by the alkaline phosphatase and mineral deposition in culture. We postulate that these or similar events occur *in vivo* and result in periodontal tissue regeneration. Our results confirm the previously reported induction of AP activity and enhanced mineralization in the presence of EMD [51–53]. However, these EMD effects are mainly mediated by contaminating BMPs contained in the EMD preparation since soluble decoy proteins that oppose BMP signaling ablated the response. Similar results have been reported for human dental follicle cells [19]. Furthermore, in this study we showed that the capacity of EMD and BMP2/7 to induce osteo/cementogenic differentiation in the treated cell population was correlated with the percentage of CD146(+) hPDL cells. Indeed, the effect of BMPs on the differentiation of human MSC is already well documented [54]. Despite the fact that we did not obtain a pure CD146(+) hPDL cell population, the data strongly suggests that BMP2/7 triggers the CD146(+) hPDL cells to differentiate toward the osteo/cementogenic phenotype, while maintaining a pool of undifferentiated and/or early committed mesenchymal cells, in contrast to the canonical dexamethasone-based osteogenic medium as already reported in dental pulp cells [50]. The use of rp(H)M180 produced a positive effect on AP activity and mineralization of hPDL cells as reported [55], but plays only a modest role in the EMD-induced cell differentiation when compared to BMP2/7 [56]. Amelogenin was previously shown to reduce osteoclastogenesis, and to enhance mouse periodontal cell proliferation and migration [57]. Thus, amelogenin may improve the *in vivo* effect of EMD activity on mineralized PDL tissue formation by acting not only on the differentiation state of stromal progenitors, but also by reducing bone/cementum resorption by inhibiting osteoclast function. Furthermore, our data demonstrated that rp(H)M180 synergizes with BMPs to promote the differentiation of osteogenic/cementogenic progenitor from CD146(+) enriched preparations. Interestingly, amelogenin was recently been shown to directly bind BMP-2 and to enhance BMP mediated EMD efficiency [58].

Having identified that the *in vitro* efficiency of select known therapeutic agents is correlated with the proportion of CD146(+) cells within PDL cultures, we next aimed to investigate how these agents alter the proportion of specific surface markers in hPDL cells. Our data provide evidence that the cell division induced by EMD or rp(H)M180 is associated with an increase in the percentage of CD146(+) cells in the cultures. However, the increase in the number of CD146(+) hPDL cells was also reported for non-mitogenic molecules BMP-4

[59] and BMP2/7, suggesting that the regulation of CD146 expression may occur independent of cell division. Interestingly, treatment with rp(H)M180, rhBMP2/7 and EMD unbalanced the percentage of dividing cells in favor of CD146(+) subset rather than to favor their CD146(-) counterpart. In contrast, the mitotic effect delivered by GM resulted in cell markers becoming equally distributed between both CD146(-)/(+) populations. These findings clearly highlight the specific effects of EMD to increase the yield of CD146(+) cells as well as the percentage of dividing cells in the CD146(+) population mediated through the actions of amelogenin and BMP2/7.

The wound healing experiments confirmed that EMD and rp(H)M180 act as molecules to recruit CD146(+) cells. Regardless of the culture conditions, following 36 hrs after injury, the majority of the wound border and filling cells were CD146(+) while MSCA-1(+) and CD106(+) cells remained at the rear of the wound edge. Interestingly, when treated with EMD, some CD146(+) CD106 (+) and CD146(+) MSCA-1(+) hPDL cells were also found among the wound filling cells. Collectively, these findings may indicate that EMD exerts a positive action perhaps by recruiting progenitors to fill the wound.

In this study, we identified that the hPDL cells expressing the CD146 marker represent the population of PDL cells that contain MSC-like cells that may differentiate during periodontal tissue regeneration. This CD146(+) population is, by itself, a heterogeneous population that can be further fractionated into two cell subsets expressing either CD146(+) and CD106(+) or CD146(+) and MSCA-1(+). The percentage of these two hPDL subpopulations is small in undifferentiated cultures but it correlates well with their stem cell-like potential. More importantly, the number of CD146(+) with CD106(+) and CD146(+) with MSCA-1(+) hPDL cells was rapidly and transiently changed using agonists that induce differentiation.

We observed during the mineralized-tissues cell-type differentiation an early increase of the percentage of CD146(+) with CD106(+) cells that was not obtained by EMD, amelogenin or dexamethasone, in contrast to rhBMP2/7. This observation suggests that one specific effect of BMPs is to maintain the CD146(+) CD106(+) phenotype, but we have yet to determine the differentiation potential of this minor CD146(+)CD106(+) cell sub-population. Interestingly, with all agonists we observed an increase of the percentage of CD106(+) cells with AP activation that do not express the CD146 marker (e.g., the CD146(-) CD106(+) ELF97(+) profile), during differentiation conditions. Although previous studies have reported that the CD106/VCAM-1 marker can be used to identify MSC-like populations in several connective tissues, including the PDL [16], their role to mark undifferentiated stromal cells remains controversial [60]. Whereas CD106/VCAM-1 expression was shown to be down-regulated during osteogenic differentiation, our findings identified CD106 as being up-regulated during differentiation of hPDL cells, as previously reported for human osteoblasts [61]. Consequently, CD106 may be useful to identify multipotent hPDL cells (and perhaps MSC in general) but only if combined with the use of the CD146 marker.

The CD146(+)CD106(+) expression profiles observed during differentiation did not overlap with those of CD146(+) MSCA-1(+), suggesting that these two distinct hPDL cell subsets may have distinct roles during differentiation. The MSCA-1 marker was recently shown to

be identical to TNAP, an isoenzyme known to be expressed by committed osteo/cementogenic progenitors throughout their differentiation as mineralized-tissue cell-types [62]. We show in this study that the percentage of hPDL cells expressing the CD146(+) MSCA-1(+) is transiently increased during their differentiation toward mineralized-tissue cell lineages. These data suggests that hPDL cells expressing the CD146(+) MSCA-1(+) markers may indicate an early commitment to osteo/cementogenic fates as previously shown for markers consisting of STRO-1(+) with alkaline phosphatase (+) in the case of BMSC cells [62]. Finally, we identified a CD146(–) MSCA-1(+) ELF97(+) hPDL cell population after 5 days in differentiation conditions, especially in cell treated by EMD, that proved to be the more effective differentiation agent used herein. Accordingly, we hypothesized alkaline phosphatase (+) cells that acquire the ability to mineralize an extracellular matrix, loose their CD146 expression, and probably their MSC-like properties at the same time.

Conclusion

The data presented in this paper aids in defining the *in vitro* behavior of human PDL progenitors and their progeny during the biological events involved in periodontal wound healing. We identified the effect of EMD on hPDL cells sub-populations by using their main components, amelogenin protein and BMP2 and 7 on the PDL progenitors. This knowledge can be used to improve the involvement of endogenous progenitor cells with MSC-like characteristics that are resident in the PDL to contribute to periodontal healing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. hPDL cells *in vitro* features

Cells from six donors were analyzed for their cell surface markers, growth rate, CFU-F ability, and multilineage differentiation. (A): Distribution of CD146, CD106 and MSCA-1 expression and AP activation level (indicated by ELF-97 staining). (B): Percentage of CD146(+) MSCA-1(+), CD146(+) CD106(+) and CD106(+) MSCA-1(+) among donor cells (C): Doubling time (D) colony number and multilineage differentiation (E–G) among donor cells are shown. hPDL cells were cultured with or without osteogenic, adipogenic or chondrogenic media (OM, AM, CM respectively) as described in *Methods*. Note the variability in calcium deposit, lipids droplets and proteoglycan formation between the hPDL cells preparations. (H) Dot blot and immunohistochemistry (IHC) were used to confirm the

osteogenic, adipogenic and chondrogenic potential among sample differentiation by their expression of OPN, PPAR γ and Col II, respectively. OPN and PPAR γ expression was normalized on β Actin expression. Mouse IgG isotype-matched was used in negative control (Iso) for IHC experiment. n=6. *: $p<.05$. Abbreviations: CFU-F, colony-forming units-fibroblastic; AP, alkaline phosphatase; hPDL cell, human periodontal cell; OPN, osteopontin; PPAR, Peroxisome Proliferator-Activated Receptor-gamma; Col II, type II collagen; OD, optical density; β act, beta-actin. Bar = 300 μ m.

Figure 2. Growth rate, cell-cycle and cell surface markers expression analysis during the cell-cycle

(A): Growth rate analysis of hPDL cells, as measured by DNA content. Following 60 and 120 hours, the cells were incubated with rp(H)M180 or EMD+/- rhNoggin, or rp(H)M180+rhBMP2/7 or 10% FCS to enhance cell proliferation compared to Dex, rhBMP2/7 or the control (vehicle; 0.5% FCS). Graph shows the mean $\pm$ SEM; $n=6$. *: $p < .05$. (B): Cell cycle analysis of hPDL cells after 25 hour of selected treatments. Percentage of cells in S phase and G2/M stage was significantly higher with treatment by rp(H)M180, EMD+/- rhNoggin, rp(H)M180+ rhBMP2/7 or 10% FCS stimulated cultures compared to values observed for Dex, or control (vehicle; 0.5% FCS). RhBMP2/7 weakly enhanced the S phase but not G2/M cell ratio compared to control. (C): Expression of CD146, CD106 and MSCA-1 cell surface markers in hPDL cells. The number of CD146(+) cells was

significantly higher after induction for 25 hours by treatment with rp(H)M180, rhBMP2/7, EMD+/- rhNoggin, rp(H)M180+ rhBMP2/7 or 10% FCS when compared to Dex treatment or control in contrast to CD106 and MSCA-1 expression that were largely unchanged. (D): The percentage of G2/M hPDL cells in CD146(+) and CD146(-) population was analyzed by FACS multicolor analysis based on CD146 and Hoechst 33352 staining as described in *Methods*. After treatment for 25 hours with rp(H)M180, EMD+/- rhNoggin, rhBMP2/7 or rp(H)M180+ rhBMP2/7, the yield of G2/M cells in CD146(+) hPDL cells was significantly higher than in CD146(-) hPDL cells, whereas the same cell markers were equally distributed in hPDL cells treated with 10% FCS. Graphs show the mean +/- SEM; n=3. *: $p<.05$. Abbreviations: hPDL cell, human periodontal ligament cell; EMD, enamel matrix derivative; rp(H)M180, recombinant poly (Histidine) Mouse 180; NG, Noggin; rhBMP2/7, recombinant human bone morphogenetic protein 2/7 heterodimer; Dex, dexamethasone.

Figure 3. Cell number and phenotype in selected treatment in *In vitro* wound healing experiment
 (A): hPDL cells response to selected treatments in an *in vitro* wound healing scratch assay. The number of wound-filling cells was significantly greater after rhBMP2/7, rp(H)M180, EMD +/- rhNoggin, rp(H)M180+rhBMP2/7 and 10% FCS treatment for 36 hours compared to the response of cells treated with Dex or control (vehicle; 0.5 % FCS. Incubation of hPDL cells with rhNoggin (Ng) did not inhibit the EMD-induced wound healing response. Graph shows the mean $\pm$ SEM; n=6. *: $p < .05$. **: $p < .01$. Abbreviations: hPDL cell, human periodontal ligament cell; EMD, enamel matrix derivative; rp(H)M180, recombinant poly (Histidine) Mouse 180; Ng, Noggin; RhBMP2/7, recombinant human bone morphogenetic

protein 2/7 heterodimer; Dex, dexamethasone. Bar = 50 μ m. (B, i–iv): Confocal analysis of CD146 (magenta), MSCA-1 (green) and CD106 (orange) expression in hPDL cells measured during *in vitro* wound healing assay after stimulation by PBS (i, iii) or EMD (ii, iv) for 36 hours. CD146 was strongly expressed in lining and filling cells especially in EMD treated cultures. CD146(–) MSCA-1(+) (green arrows) and CD146(–) CD106(+) (yellow arrows) cells were located primarily at the rear of the wound edge, while some CD146(+) MSCA-1(+) (white arrows) and CD146(+) CD106(+) (cyan arrows) cells were identified among the filling cells. Nuclei were counterstained with Hoechst 33352 (blue). Negative control (Iso) was performed by replacing the primary antibody with an appropriate isotype-matched negative antibody Ig. n=3.

Figure 4. hPDL cells mineralized-tissue cell-type differentiation: AP activity and calcium deposition

(A): AP activity in hPDL cells after 2, 5 and 10 days in the presence of differentiation medium with various agonists as indicated. After day 5, stimulation with rhBMP2/7, rp(H)M180, Dex, EMD or rp(H)M180+rhBMP2/7 significantly enhanced AP activity in a time-dependent manner compared to control treatment (vehicle, FCS 2%). After 10 days-incubation, AP activity was increased by rp(H)M180, Dex, rhBMP2/7, EMD and rp(H)M180+rhBMP2/7 agonists. Incubation of hPDL cells with rhNoggin (Ng) partially inhibited the EMD-induced AP activity. (B). Quantification of mineral salts produced by hPDL cells after 21 days of stimulation in the presence of differentiation medium and agonist or vehicle as described in *Methods*. At day 21, calcium deposits by hPDL cells stimulated with rp(H)M180 were moderate, showing greater values when stimulated with Dex, rhBMP2/7 alone, and the greatest deposit when treated with EMD or rp(H)M180+rhBMP2/7. Incubation of hPDL cells with rhNoggin (Ng) incompletely inhibited the EMD-induced mineralization. Graphs show the mean $\pm$ SEM; n=6. *: p<.01

compared to control °: $p < .05$ compared with rhBMP2/7 or Dex, §: $p < .05$ compared to rp(H)M180. Abbreviations: hPDL cell, human periodontal ligament cell; AP, alkaline phosphatase; EMD, enamel matrix derivative; rp(H)M180, recombinant poly (Histidine) Mouse 180; rhBMP2/7, recombinant human bone morphogenetic protein 2/7 heterodimer; Dex, dexamethasone.

Figure 5. hPDL cells mineralized-tissue cell-type differentiation: cell phenotypes changes (A–E): Changes in hPDL cells subsets surface markers percentage during the course of biomineralized-tissue cell-type differentiation, analyzed by multicolour FACS experiment. (A): Yield of CD146(+) MSCA-1(–) ELF97(–) cells decreased with extent of all differentiation. The decay of CD146(+) MSCA-1(–) ELF97(–) surface markers occurred after 2 days in Dex-treated or in untreated cultures, and after 5 days in EMD and rp(H)M180 conditions. The decreased ratio of cell expressing CD146(+) MSCA-1(–)(ELF97(–) surface markers was not significant with rhBMP2/7 treatment. (B): The proportion of cells

expressing CD146(+) CD106(+) MSCA-1(-) surface markers was transiently increased after 2 days-stimulation compared to values for cells at the start of culture and was notably maintained in rhBMP2/7 condition. (C): Percentage of CD146(+) MSCA-1(+) ELF97(-) cells was higher after 2-days in stimulated cultures than in untreated cultures and increased after 5-days of stimulation with EMD, Dex or rhBMP2/7 treatment. (D-E): Percentage of CD146(-) MSCA-1(+) ELF97(+) cells (D) and CD146(-) CD106(+) MSCA-1(+) cells (E) was significantly increased after 5-days in treated or in untreated cultures. At day 10, treated cultures displayed higher CD146(-) MSCA-1(+) ELF97(+) and CD146(-) CD106(+) MSCA-1(+) population ratios than untreated cultures. Graphs show the mean; n=6. *: p<.05 compared to starting point (Day 0) °: p<.05 compared to control (2% FCS). (F): Confocal analysis of CD146 (magenta), MSCA-1 (green) and ELF97 (yellow) staining of hPDL cells after 2-, 5- and 10-days in the presence of differentiation medium +EMD. In untreated and 2 day-treated cultures (i;ii), CD146(+) cells were identified, although some CD146(+) MSCA-1(+) cells were also distinguishable; no cell was stained with ELF97. More numerous CD146(+) MSCA-1(+) ELF97(-) cells (iii, white arrow) and some CD146(-) MSCA-1(+) ELF97(+) cells (iv, red arrow) were identified after treatment for 5-days. When EMD was added for 10-days in differentiation medium, numerous CD146(-) MSCA-1(+) cells with strong ELF97 staining were identified in the cultures (v, cyan arrows). (G) Confocal analysis of CD146 (magenta), CD106 (green) and ELF97 (yellow) staining of hPDL cells after 2-, 5- and 10-days in the presence of differentiation medium + EMD. (i;ii) CD106 expression was detectable on few CD146(+) cells after 2-day stimulation by EMD, without ELF97 staining. (iii): After 5 day-treatment by EMD, CD106 expression was located on ELF97(+) (white arrow) as well on ELF97(-) (red arrow) cells, whereas CD106(-) ELF97(+) cells were identified (blue arrow). (iv): In 10-days treated culture, the majority of ELF97(+) cells was not stained by CD106. Nuclei were counterstained with Hoechst 33352 (blue). Negative control (Iso) was performed by replacing the primary antibody with an appropriate isotype-matched negative antibody Ig. n=4. Abbreviations: hPDL cell, human periodontal cell; EMD, enamel matrix derivative; rp(H)M180, recombinant poly (Histidine) Mouse 180; AP, alkaline phosphatase; RhBMP2/7, recombinant human bone morphogenetic protein 2/7 heterodimer; Dex, dexamethasone. Bar = 50 µm.

Figure 6. Phenotype stability of hPDL cell selected-fractions

CD146(-) MSCA-1(-), CD146(+) MSCA-1(-) and CD146(+) MSCA-1(+) hPDL sub-populations were enriched by cell selection, plated for 4-days in growth medium and phenotyped. (A): CD146(-) MSCA-1(-) and CD146(+) MSCA-1(-) fraction globally retained their phenotype during expansion (~ 83 and 73% resp.). However, the subset expressing both CD146 and MSCA-1 surface markers dramatically lost their MSCA-1 expression and revealed a cell number yield similar to the CD146(+) MSCA-1(-) fraction (B): Each of three cell fractions distinguishable expression of cell surface markers were subjected to differentiation medium (DMEM, 2%FCS, AA (50 µg/mL)) with or without agonists, as indicated. After 4-days, the percentage of cells expressing both CD146 and MSCA-1 markers increased in all treated cultures compared cells from the starting point (day 0), and was greater in CD146(+) cell-enriched fraction than in CD146(+) cell-depleted fractions. The addition of EMD to the differentiation medium appeared to be associated with a greater response. Abbreviations: hPDL cell, human periodontal cell; EMD, enamel matrix derivative; AA, ascorbic acid.

Figure 7. The *in vitro* behavior of hPDL cell-selected fractions

The *in vitro* features of each hPDL cell fraction distinguishable by surface markers were characterized. (A–H): CD146(+) MSCA-1(–) and CD146(+) MSCA-1(+) expressing subsets of cells exhibited lower doubling time (A), and greater numbers of colony fibroblastic forming-units (CFU-F) (B), as well as multipotency (C–H) compared to CD146(+) depleted cell fraction. (I): Growth rate analysis of enriched subsets of hPDL cell as measured by DNA content. Cells were Incubated for 120-hours with rp(H)M180, or EMD +/- rhNg, or rp(H)M180+rhBMP2/7, or 10% FCS to enhance cell proliferation compared to treatment

with Dex, or rhBMP2/7 or the control (vehicle; 0.5% FCS). Expansion of the CD146(+) enriched fractions (the CD146(+) MSCA-1(-) and CD146(+) MSCA-1(+) fractions) was significantly stronger than the CD146(+) depleted fraction. (J): The wound filling response of hPDL cell fractions challenged with selected treatments. Incubation for 36 hours with rp(H)M180, rhBMP2/7, EMD +/- rhNg, rp(H)M180+rhBMP2/7, or 10% FCS enhanced the wound-filling cell number compared to treatment with Dex or a control (vehicle; 0.5% FCS). The response of the two CD146(+) enriched fractions was significantly greater than the response observed for the CD146 depleted fraction. (K -L): The differentiation of hPDL cell selected subsets was measured by their expression of alkaline phosphatase (AP) activity (K) and calcium deposition (L) in various media and supplements for 10- and 21-days respectively. Stimulation with rhBMP2/7, rp(H)M180, Dex, EMD +/- rhNg or rp(H)M180+rhBMP2/7 significantly enhanced AP activity and calcium deposition in all fractions compared to control (vehicle; 2% FCS). The response of the two CD146(+) enriched fractions was significantly greater than values observed for the CD146(+) depleted fraction. Cells treated with EMD or rp(H)M180+rhBMP2/7 exhibited the strongest treatment. Rp(H)M180 and rhBMP2/7 exerted a synergistic effect on AP activity and calcium deposition for only the two CD146(+) enriched fractions. Incubation of hPDL cells with rhNoggin partially inhibited the EMD-induced AP activity and the calcium deposition. Graph depicts the mean +/- SEM; n=3 for each fraction. *: p<.05 compared to control °: p<.05 compared to CD146(+) depleted fraction. Abbreviations: hPDL cell, human periodontal ligament cell; AP, alkaline phosphatase; EMD, enamel matrix derivative; rp(H)M180, recombinant poly (Histidine) Mouse 180; Ng, recombinant human Noggin; RhBMP2/7, recombinant human bone morphogenetic protein 2/7 heterodimer; Dex, dexamethasone.

Table 1

Sources and use of recombinant proteins, antibodies and reagents.

Materials	Manufacturer	Concentration use
Recombinant human (rh) protein heterodimer $\alpha 2(\beta 1)-\alpha 2(\beta 2)$	R&D System Inc. (Minneapolis, MN, USA).	100 ng/mL
Rh Noggin	R&D System Inc. (Minneapolis, MN, USA).	500 ng/mL
Rh TGF- $\beta 3$	R&D System Inc. (Minneapolis, MN, USA).	10 ng/mL
Monoclonal anti-osteopontin antibody (ab)	R&D System Inc. (Minneapolis, MN, USA).	1/500 (v/v)
Monoclonal anti-peroxysome proliferators-activated γ ab	R&D System Inc. (Minneapolis, MN, USA).	1/500 (v/v)
Enamel Matrix Derivative (EMD)	Straumann (Basel, Switzerland). Developmental Hybridoma Study Bank (Iowa City, IO, USA).	50 μ g/mL
Monoclonal anti-collagen II unconjugated ab	Developmental Hybridoma Study Bank (Iowa City, IO, USA).	1/100 (v/v)
Monoclonal anti CD106 unconjugated ab		1/100 (v/v)
7-amino actinomycin D	Ebiosciences (Paris, France).	1/10 (v/v)
Monoclonal anti CD 14, 34, 45, 73, 79a, 90, 105, HLA-DR, isotype matched negative control abs	Ebiosciences (Paris, France).	1/10 (v/v)
Monoclonal anti CD146 and anti MSCA-1 conjugated abs	Miltenyi Biotec (Paris, France).	1/10 (v/v)
Monoclonal anti MSCA-1 unconjugated ab	Miltenyi Biotec (Paris, France).	1/100 (v/v)
Rabbit anti CD146 unconjugated ab	Anaspec (Fremont CA, USA).	1/100 (v/v)
Rabbit and mouse IgG unconjugated ab	Serotec (Cergy Saint-Christophe, France).	1/100 (v/v)
Monoclonal anti CD106 conjugated ab	BD-Biosciences (Bedford, MA)	1/10 (v/v)
ITS-Premix	BD-Biosciences (Bedford, MA)	50 mg/mL
ELF97 endogenous phosphatase detection kit	Invitrogen (Carlsbad, CA, USA).	1/50 (v/v)
Recombinant poly(His) tagged mouse <u>180</u> amino acid amelogenin (rp(H)M180)	Home-made material	5 μ g/mL
Monoclonal anti STRO-4 unconjugated ab	Home-made material	1/10 (v/v)
Penicillin, streptomycin, phosphate buffer saline, α and D-MEM, fetal calf serum, Hoechst 33342	Invitrogen (Carlsbad, CA, USA).	