ABCG2 Is a Selectable Marker for Enhanced Multilineage Differentiation Potential in Periodontal Ligament Stem Cells

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Periodontal ligament stem cells (PDLSCs) provide an important source for tissue regeneration and may become especially useful in the formation of osteogenic seeds. PDLSCs can be cultured, expanded, and differentiated in vitro; thus, they may be applied in the long-term treatment of the defects in the dental regions. Here we studied numerous potential markers allowing the selection of human PDLSCs with a maximum differentiation potential. We followed the expression of the ATP-binding cassette subfamily G member 2 (ABCG2) membrane transporter protein and isolated ABCG2-expressing cells by using a monoclonal antibody, recognizing the transporter at the cell surface in intact cells. The expression of the ABCG2 protein, corresponding to the so-called side-population phenotype in various tissue-derived stem cells, was found to be a useful marker for the selection of PDLSCs with enhanced osteogenic, chondrogenic, and adipogenic differentiation. These findings may have important applications in achieving efficient dental tissue regeneration by using stem cells from extracted teeth.

Introduction

THE PERIODONTAL LIGAMENT (PDL) is a soft connective tissue with a physiological role to fix the tooth into the alveolus. In addition to its anchoring function, this tissue has an important role in the homeostasis and regeneration of the periodontium [1,2], which is critical in the cases of tooth loss, chronic periodontitis, or deep intraosseous defects [3,4]. There is a continuous clinical need to find cellular therapies for the regrowth of the attachment apparatus destroyed as a consequence of periodontitis. Such a process requires new connective tissue to attach to the root surface, including the regeneration and insertion of periodontal fibers into newly formed cementum [5].

The surgically removed wisdom teeth may provide a large number of cells that can be easily isolated from the tooth surface and expanded in in vitro cultures. The PDL contains heterogeneous cell populations, predominantly fibroblasts and a small subset of cells with self-renewing and clonogenic ability. These latter cells are called periodontal ligament stem cells (PDLSCs). These progenitor cells are both capable of differentiating into osteoblasts, cementoblasts, or fibroblasts, and produce the extracellular matrix of the PDL [6,7]. According to the data in the literature, the osteoblastic

and cementoblastic phenotype is based on the expression of alkaline phosphatase (ALP), osteopontin (OPN), osteocalcin (OCN), osterix (OSX), and cementum protein 1 (CEMP1) [7–9]. Cells derived from PDL also possess of mesenchymal stem cell (MSC)-like features, that is in vitro osteogenic, adipogenic, and chondrogenic differentiation potential; the expression of MSC markers (STRO-1, CD13, CD29, CD44, CD73, CD90, CD105, and CD166); and the lack of expression of hematopoietic markers.

Although there were several attempts to find a unique cell surface marker (CD106, CD146, SSEA4, and STRO-1) [10–12] to identify a subset of PDL cell population with enhanced multilineage differentiation capacity, these efforts were unsuccessful to candidate for regenerative therapy application as yet.

A potential approach to identify such multipotent-tissue-derived stem cells is to look for the so-called side-population (SP) cells. These cells have been identified based on their low-level staining by the Hoechst 33342 fluorescent dye, due to the active dye extrusion by the ATP-binding cassette subfamily G member 2 (ABCG2) protein, expressed at a higher level in these cells [13]. During the past few years SP cells were identified in numerous normal and cancerous tissues, representing early progenitors or stem

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cells [14–16]. It has been shown that the PDL also contains an ABCG2-expressing SP [17] but functional data for the differentiation of these SP cells have not been reported as yet. Ninomiya et al. [18] suggested an elevated bone differentiation capacity for rat PDLSCs showing SP features, although in this case the dye extrusion was ABCB1 dependent.

Based on these studies, the selection of human PDLSCs expressing ABCG2 may help to identify a multipotent stem cell population for therapeutic applications. It is important to note that a selection based on the use of DNA-binding dyes, potentially causing major genetic alterations, does not allow a further clinical utilization of these cells. Therefore, we have used a specific antibody-based sorting method to enrich ABCG2-expressing SP cells, applicable for stem-cell-based therapy, without the use of potentially toxic fluorescent dyes. Here we demonstrate the successful sorting and detailed characterization of these cells, and the relationship between ABCG2 expression and an increased bone-forming ability of the selected PDLSCs.

Materials and Methods

Cell isolation and culture

Work with human PDLSCs was performed with the permission of the ethical committee of the Hungarian Medical Research Council (ETT). The donors gave written permission for the utilization of the removed tissues. We have isolated and characterized several samples ($n=7$) from different donors (aged 17–35) with variable ABCG2 expression.

Most of the reagents were purchased from Life Technologies Japan Ltd. (Tokyo, Japan), all others as indicated. The teeth were rinsed in phosphate-buffered saline (PBS) and scraped with scalpel. Enzymatic digestion was performed with 0.1% w/v collagenase type I (Sigma-Aldrich, St. Louis, MO) at 37°C for 1 h. Digestion was stopped by adding identical volume of MSC expansion medium [Dulbecco's modified Eagle's medium/nutrient mixture F-12 (DMEM/F-12), 10% v/v fetal bovine serum, and 1 ng/mL fibroblast growth factor-2 (FGF-2)]. The digested cell suspension was centrifuged at 800 g for 10 min, washed with PBS, and resuspended in MSC expansion medium. Initially, cells were plated at a density of $2 \times 10^5/\text{cm}^2$. Following selection for plastic adherence, PDLSCs were subcultured once a week at a density of $4 \times 10^3/\text{cm}^2$.

Human embryonic stem cell line 9 (HUES9) was a kind gift of Douglas Melton, Harvard University, Cambridge, MA. The HUES culture conditions were used as described in detail by Apáti et al. [19].

Flow cytometry

Expression of the cell surface markers was assessed by flow cytometry, as described previously [20]. All antibodies were purchased from BD Biosciences (Erembodegem, Belgium). In the case of using unlabeled primary antibodies (ABCG2, ALP, and HLA-G), the cells were subsequently stained with fluorescent-conjugated, isotype-matched secondary goat anti-mouse antibodies with shaking in a 37°C water bath. For maximum cell surface labeling of ABCG2 with the conformation-sensitive 5D3 monoclonal (IgG2b) antibody, the specific ABCG2 inhibitor Ko143 (Sigma-Aldrich) was added. 7-Amino-actinomycin D (7-AAD) or

TO-PRO-3 iodide was used for the exclusion of dead cells. All control cells were stained with the corresponding isotype controls. The measurements were done in a 4-color FACS-Calibur flow cytometer (BD Biosciences); data were analyzed with CellQuest Pro software (BD Biosciences). For cell sorting 1×10^6 cells were labeled with the anti-ABCG2 antibody as described previously. Cell-surface-located ABCG2-positive and -negative cells were analyzed and sorted by using FACSaria flow cytometer (BD Biosciences).

For DyeCycle Violet (DCV) staining, the cells were incubated with 2 μM DCV for 30 min with shaking in a 37°C water bath. To inhibit ABC transporters, 5 μM Ko143 (Sigma-Aldrich) or 10 μM verapamil (Sigma-Aldrich) was added. DCV was excited at 405 nm by ultraviolet laser and its dual wavelengths were detected using 450/40 (DCV-Blue) and 603/48 (DCV-Red) filters in an Attune Acoustic Focusing Cytometer (Life Technologies Japan Ltd.).

STRO-1 immunohistochemistry

Cells were fixed with 4% w/v paraformaldehyde, blocked against nonspecific staining with 1% w/v bovine serum albumin and 4% v/v nonimmune serum, and incubated with primary mouse anti-STRO-1 monoclonal antibody or mouse IgM isotype control for 1 h at room temperature. AlexaFluor 546 secondary antibody was applied for fluorescent detection, and nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI).

Cell proliferation

Proliferation of different PDLSC isolates was compared using a cellular viability assay, based on the reduction of the nonfluorescent resazurin (Sigma-Aldrich) dye to fluorescent resorufin. Cells were seeded in growth medium at a density of $500/\text{cm}^2$ in 96-well microplates. At each time point (from day 1 to 7), one microplate was measured as described previously [20].

Colony-forming unit–fibroblast assay

To assess the colony-forming efficiency of PDLSC samples, cells were seeded at a density of 400/10 cm of culture dishes in MSC growth medium. After 14 days, the cells were fixed with 4% w/v paraformaldehyde and stained with 0.04% v/v crystal violet. Aggregates of 50 or more cells were scored as a colony-forming unit–fibroblast (CFU-F) colony. Colony-forming efficiency was determined by the number of colonies relative to the total number of seeded cells in each plate.

Osteogenic, chondrogenic, and adipogenic differentiation

For osteogenic differentiation the cells were kept in an osteogenic medium (StemPro Osteogenesis Differentiation Kit). After 7 days of induction, differentiated and undifferentiated samples were collected to test ALP activity. Following cell lysis with 0.1% w/v Triton X-100 solution diluted in ALP buffer [50 mM TRIS, 150 mM NaCl, and 10 mM MgCl_2 (pH 9.5)] the enzyme activity was detected with p-nitrophenyl phosphate (pNPP) in final concentration of 50 mM dissolved in ALP buffer. After 1 h of incubation at room temperature, the supernatants were transferred to a 96-

well plate and fluorescence was read at 405 nm in a plate reader (PerkinElmer, Waltham, MA). The data were normalized based on total protein concentrations determined with the Lowry method.

After 14 days the cells were fixed and stained with 2% w/v Alizarin red at pH 4.2. To quantify the mineralization, the stain was dissolved with a solution containing 10% acetic acid and 20% methanol. Fluorescence was read at 450 nm in a plate reader. Calcium deposition was also measured with Calcium (CPC) LiquiColor test (Stanbio Laboratory, Boerne, TX), according to the manufacturer's instructions.

For chondrogenic differentiation 2×10^5 PDLSCs were plated in 5 μ L culture medium in a 24-well cell culture dish for 1 h and then chondrogenic medium (StemPro Chondrogenesis Differentiation Kit) was added and changed twice a week for 14 days. For histological examination, the pellets were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 5- μ m sections. Newly synthesized glycosaminoglycans were evaluated by hematoxylin and eosin staining, following deparaffinization.

For adipogenic differentiation the cells were kept in an adipogenic medium (StemPro Adipogenesis Differentiation Kit) for 14 days and then fixed and stained with oil red O. To quantify the lipid accumulation, the stain was dissolved with 100% isopropanol and the fluorescence was read at 490 nm.

Gene expression analysis

Total RNA was isolated from undifferentiated and differentiated cells and cDNA was synthesized as previously described [20]. The expression levels of OCT4, SOX2, NANOG, ALP, ABCG2, CEMP1, and the peroxisome-proliferator-activated receptor gamma (PPAR γ) gene known as an adipocyte marker were measured using TaqMan reagents. The expression of OCN, OSX, and SOX9 mRNA was determined by using Power SYBR Green reagents in StepOne Plus qPCR instruments (Life Technologies Japan Ltd.). The list of primers and assays is shown in Supplementary Table S1 (Supplementary Data are available online at www.liebertpub.com/scd).

Statistical analysis

Gene expression levels were calculated with $2^{(-\Delta Ct)}$ method relative to GAPDH as a reference. The data are presented as the mean $\pm$ SD. Student's *t*-test was used for the comparison of the sorted cell populations.

Results

Characterization of PDLSC isolates

The cells isolated from PDL as described in the "Materials and Methods" section are most probably close relatives of the MSCs, with variable differentiation potential in different tissues. To establish these features we performed a detailed characterization of these cells. The basic characterization of the cell surface markers was performed by quantitative flow cytometry.

As shown in Supplementary Table S2, in accordance with the data in the literature, we found that all PDLSC isolates expressed in 100% the general MSC cell surface markers (CD13, CD29, CD44, CD73, CD90, and CD105). The endothelial progenitor marker CD146 (MCAM and MUC18)

was expressed in a highly variable proportions (4%–62%), and the CD106 protein (VCAM-1), again an endothelial progenitor marker, was expressed in 0%–48% on the cell surface of the cultured PDLSCs. All the markers identifying hematopoietic or endothelial cell type (CD14, CD34, CD45, CD117, CD133, CD31, CD144, and CD309) were practically absent, or expressed at a low level (HLA-DR).

The potential MSC marker STRO-1 was also measured on the surface of cells by flow cytometry, and was found in <1% in all PDLSC isolates (Supplementary Table S2). Immunohistochemical staining of STRO-1 was also performed, in order to confirm the flow cytometry measurements (Supplementary Fig. S1A, B).

To examine the presence of potential multipotent/pluripotent stem cells in the preparations, we investigated the expression of a glycolipid, the stage-specific embryonic antigen-4 (SSEA4) marker on the cell surface, as well as the expression of mRNA for OCT4, NANOG, and SOX2 in the cell populations. We found that only a low percentage (0%–7%) of PDLSCs expressed the SSEA4 marker, and the mRNA levels for the pluripotency markers were negligible (ΔCt values were 6.68–10.01), as compared with the embryonic stem cell line HUES9 (ΔCt values 0.68–2.93) (see Supplementary Fig. S2).

The ABCG2 (MXR/BCRP) multidrug transporter has been indicated to provide a marker for tissue-derived stem cells, corresponding to the SP fraction. The SP cells show a low-level Hoechst or DCV dye accumulation, due to the dye transport activity of ABCG2, which can be inhibited by blocking ABCG2 function, for example, by verapamil, FTC, or Ko143. The ABCG2 protein, although in variable proportions, has also been shown to be present in human embryonic stem cells and induced pluripotent stem cells [21,22]. Therefore we examined the functional presence of the ABCG2 protein in PDLSCs by measuring DCV dye "side-populations," and the cell surface ABCG2 expression by using a monoclonal antibody (5D3), recognizing this protein on the cell surface. Since the 5D3 mAb is a conformation-sensitive antibody [23], we assured maximum labeling in these experiments (see "Materials and Methods" section).

As shown in Figure 1, PDLSC isolates showed variable expression levels for the ABCG2 transporter. By using the DCV extrusion (SP detection) method, we found that the SP fraction varied between about 1% and 17% of the cells, while the 5D3 method indicated a higher fraction of the ABCG2-positive cells, approximately between 2% and 64% positivity, depending on the actual source of isolation. The mRNA expression levels for the ABCG2 transporter, measured by reverse transcription quantitative PCR (RT-PCR), also showed variable levels, although consistent with the protein expression pattern for each isolate. As a summary, based on all assays, the F38 and F28 isolates had a significantly higher ABCG2 mRNA and protein expression than the other isolates. All these marker and mRNA expression patterns were well reproducible for an extended time period (15 passages at least) with moderate decrease of ABCG2 expression in the PDLSC cultures.

Culture and differentiation of the PDLSC isolates

When measuring the proliferation activity of the different PDLSC isolates, these were variable in each preparation, showing doubling times between 0.3 and 1.5 days. In this

FIG. 1. Periodontal ligament stem cell (PDLSC) isolates express the ATP-binding cassette subfamily G member 2 (ABCG2) transporter at different levels. **(A)** Expression was detected with antibody labeling: *dotted lines* mark isotype controls and *continuous lines* the 5D3-antibody-labeled cell populations. **(B)** Detection of the side-population (SP) with DyeCycle Violet (DCV) extrusion method: *rectangular gate* marks the DCV excluding SP cells inhibitable with Ko143 or verapamil. **(C)** The ABCG2 mRNA expression determined by RT-qPCR.

regard, we found no correlation of the cell growth with the marker expression or with the levels of ABCG2 expression as estimated by flow cytometry (Supplementary Fig. S3A, B).

MSCs have colony-forming ability on plastic surfaces. All of the PDLSC isolates contained a subpopulation that was capable of generating colonies from single cells (Supplementary Fig. S4A, B). However the CFU-F frequency varied between 14% and 32% and no correlation was found between the ABCG2 expression level and the CFU-F frequency (Supplementary Fig. S4C). The densities of the colonies correlated with the proliferation ability of the cells, which was independent from the level of ABCG2 expression.

The differentiation potential of the PDLSCs was followed by standard induction protocols, measuring osteogenic, chondrogenic, and lipogenic differentiation.

For bone differentiation, after 14 days of osteogenic induction we quantified the calcium deposition in the cells with Alizarin red S staining (Fig. 2A, B), and the Calcium LiquiColor test (Supplementary Fig. S5). We found that the PDLSC isolates showing higher ABCG2 expression (F38 and F28) achieved significantly higher calcium accumulation than the cell isolates with low-level ABCG2 expression. Thus, the cellular ABCG2 expression showed a positive correlation with the overall osteogenic differentiation potential of the PDLSC isolates (Fig. 2C).

The glycosaminoglycan formation was visualized with hematoxylin and eosin staining after 14 days of chondrogenic differentiation (Supplementary Fig. S6A). No significant correlation between ABCG2 and SOX9 expression (a transcription factor involved in chondrogenesis; Supple-

mentary Fig. S6B) was noticeable in the case of the PDLSC isolates (Supplementary Fig. S6C).

We have also studied the adipogenic differentiation of the PDLSC isolates, by culturing these cells in adipogenic medium for 14 days and performed a quantification by oil red O staining (Supplementary Fig. S7A, B), and by an RT-qPCR measurement of the PPAR γ gene expression level (Supplementary Fig. S7C). We found that the amount of the accumulated lipids and the changes in the expression level of the PPAR γ gene showed a weak correlation with the ABCG2 expression in the various PDLSC isolates (Supplementary Fig. S7D).

Isolation and characterization of ABCG2-expressing PDLSCs

To further investigate the correlation between ABCG2 expression and the osteogenic ability of PDLSCs, we sorted three PDLSC sample (namely, F48, F38, and F28) to ABCG2^{high} and ABCG2^{low} populations (Fig. 3A), by using an anti-ABCG2 antibody (see “Materials and Methods” section). After cell sorting, by measuring dye extrusion, we found that the high-ABCG2-expressing cells indeed corresponded to a higher level of the SP population (Supplementary Fig. S8A). The mRNA expression level of ABCG2 was in accordance with the flow cytometer measurements (Supplementary Fig. S8B). The ABCG2 expression of sorted cells was followed for a long period (15 passages at least) and moderate decline was noticeable.

In these sorted samples we have examined the proliferation ability, osteogenic and adipogenic differentiation potential of

FIG. 2. Alizarin red S staining of differentiated PDL cells induced for 14 days in osteogenic medium (A), and the colorimetric quantitation of Alizarin red S stain (B). The calcium accumulation of the differentiated cells shows positive correlation with ABCG2 expression (C).

FIG. 3. The trilineage differentiation capacity of sorted PDLSC samples. The ABCG2 expression of sorted PDLSCs measured by flow cytometer: dotted lines mark isotype controls and continuous lines the 5D3-antibody-labeled cell populations (A). The demonstration of osteogenic differentiation potential of sorted PDLSCs with Alizarin red S staining after 14 days of induction into osteogenic lineage (B), and the quantitation of the stain (C). Elevation of alkaline phosphatase mRNA expression of cell populations in the early stage of bone differentiation (D). The glycosaminoglycan pattern of PDLSCs differentiated into chondrogenic direction (E), and SOX9 expression of the sorted cells (F). Oil red O staining of cells induced for 14 days into adipogenic lineage (G), the quantitation of the stain (H), and the changes of peroxisome-proliferator-activated receptor gamma (PPAR γ) mRNA expression after 7 days in adipogenic medium (I). Student's *t*-test was performed between ABCG2^{high} versus ABCG2^{low} and ABCG2^{high} versus parental cell populations; *P* values of <0.05 were considered significant (*).

the cells. When measuring cell growth, we found that the sorted cell populations showed no differences in their proliferation activities (Supplementary Fig. S9).

For osteogenic induction, the unsorted (parental), ABCG2^{high}, and ABCG2^{low} cell populations were cultured in an osteogenic medium for 7 days. ALP activity, greatly increasing during osteogenic differentiation, was significantly higher in the ABCG2^{high} cells, as compared with the parental or the ABCG2^{low} cell populations (Supplementary Fig. S10). The gene expression of ALP also strengthens these observations (Fig. 3D). As a key parameter of osteogenic differentiation, calcium deposition measured—after 14 days of osteogenic induction—with Alizarin red S staining was much greater in the ABCG2^{high} cell populations than in the parental cells or the PDLSCs that expressed low levels of ABCG2 (Fig. 3B, C). Thus, the mineralization of the sorted ABCG2^{high} PDLSCs was significantly accelerated.

FIG. 4. Bone differentiation marker expression of sorted PDLSCs in the early stage of osteogenic induction—presented on a representative sample. The mRNA level of osteocalcin (A), osterix (B), and the cementogenesis marker cementum protein 1 (C). Student's *t*-test was performed between ABCG2^{high} versus ABCG2^{low} and ABCG2^{high} versus parental cell populations; *P* values of <0.05 were considered significant (*).

When measuring the gene expression for several markers of the osteogenic differentiation, we found a similar positive correlation with the ABCG2 expression level. As documented in Figure 4, the mRNA level of OCN (Fig. 4A), and the transcription factor OSX (Fig. 4B), involved in bone matrix maturation, was also higher in the ABCG2^{high} cells after 7 days of induction. In addition, the mRNA expression level of the CEMP1 gene, a marker of cementogenesis, was also higher in the ABCG2^{high} cells (Fig. 4C).

Chondrogenic differentiation was performed on F38-parental cells and the isolated ABCG2^{high} and ABCG2^{low} cells. The density of newly formed glycosaminoglycans was significantly higher in the ABCG2^{high} cell population after 14 days of differentiation (Fig. 3E). We also found an elevated mRNA expression level for the chondrogenic transcription factor SOX9, in correlation with the hematoxylin and eosin staining in the cartilage pellet (Fig. 3F).

Under adipogenic induction of the PDLSCs, the gene expression level of PPAR γ (Fig. 3I) and the lipid accumulation (quantitated in the sorted cells by oil red O staining; Fig. 3G, H) after 14 days of differentiation also showed a positive correlation with the level of ABCG2 expression. Thus, a general effect of higher ABCG2 levels on the differentiation potential of PDLSCs was observed.

To examine the role of ABCG2 function in PDLSC differentiation, we used a specific, selective inhibitor of the ABCG2 function, Ko143. We kept a PDLSC sample (F38) in osteogenic medium for 14 days, complemented with the ABCG2 inhibitor Ko143 in a final concentration of 1 μ M, shown to have a full ABCG2 inhibitory activity. When measuring the calcium accumulation in the cells, we found no difference between the Ko143-treated and the control cells (Supplementary Fig. S11A). These data indicate no direct functional role of the ABCG2 protein in the osteogenic differentiation. Moreover, when we measured the changes in ABCG2 expression during osteogenic differentiation, we found a significant reduction in the mRNA level (Supplementary Fig. S11B), and a loss of ABCG2 from the cell surface after 7 days in osteogenic medium (see Supplementary Fig. S11C for a representative sample).

Discussion

Human PDLSCs are easily accessible, rapidly growing cells, characterized by the mesenchymal cell markers, and providing promising candidates for cell therapy applications. In this study we have used human PDLSCs obtained from tissues after necessary surgeries, and characterized the isolated cells for general expression markers. We found that all PDLSC preparations corresponded to the minimal criteria [24] defining MSCs. The conventional cell surface markers of MSCs were present on the surface of isolated cells, while the stromal cell marker STRO-1 was present only in a low percentage. The cells possessed clonogenic ability and a proper differentiation capacity in the osteogenic, chondrogenic, and adipogenic direction, indicating a close relationship of these cells with MSCs.

PDLSCs were reported to contain a smaller cell population (SP) excluding the fluorescent dye Hoechst 33342 [17]. This Hoechst or the analog vibrant DCV [25] extrusion is mediated in most cases by the ABCG2 multidrug transporter. The SP cells were found to possess stem-cell-like

features in various tissues [26,27], including the dental follicle of rat [28], or human dental pulp [29]. Therefore in the present experiments we have studied the potential correlation of the ABCG2 protein expression profiles and differentiation potential of PDLSCs.

In our experiments we have used two different technologies to estimate the SP cells and the ABCG2 protein expression in the PDLSCs. These were flow cytometry measurements, either using a cell-surface-reacting, ABCG2-specific monoclonal antibody (5D3), or the vibrant dye (DCV) exclusion method. Moreover, DCV extrusion was blocked either by verapamil or the specific inhibitor of ABCG2, Ko143. Since the two inhibitors gave similar results, we suggest that in PDLSCs for most of the dye exclusion feature the activity of the ABCG2 protein was responsible. In these experiments the most sensitive estimation of the ABCG2-positive population was obtained by the specific-monoclonal-antibody-based flow cytometry detection.

In the various PDLSC isolates we found a variable level of ABCG2 expression but we observed that the PDLSCs showing higher ABCG2 expression achieved significantly higher calcium accumulation than the cell isolates with low-level ABCG2 expression. Thus, in general, the cellular ABCG2 expression showed a significant, positive correlation with the osteogenic differentiation potential of PDLSCs.

To further investigate the role of ABCG2 expression in the differentiation ability of human periodontal-tissue-derived cells, and to avoid interdonor variance in age and genetic background, we isolated ABCG2^{high} and ABCG2^{low} cell populations from three PDLSC lines by using an antibody-based cell sorting. In these experiments we found that ABCG2^{high} cells, after an osteogenic induction, showed a significant upregulation of all examined osteoblastic/cementoblastic markers, as compared with the ABCG2^{low} and the parental PDLSC populations.

The increased differentiation ability was not limited to the osteogenesis. Although the chondrogenic and adipogenic differentiation potential of isolates showed no (or only a weak) correlation with ABCG2 expression, following the antibody-based selection of cells, in the case of chondrogenesis the ABCG2^{high} population showed an elevated SOX9 expression and a higher density of glycosaminoglycans was detectable.

The elevation of PPAR γ expression and lipid accumulation was also typical in ABCG2^{high} samples after 14 days of induction in adipogenic differentiation medium. It has to be noted that the lipogenic potential of PDLSCs is limited, and the number and size of lipid droplets are variable in different isolates. Further, the age and genetic background of donors can also influence the differentiation ability of the isolates. These features may explain why a significant correlation was not found in the isolates, while the sorting of the samples into ABCG2^{high} and ABCG2^{low} populations may have eliminated these differences (Fig. 3G–I).

Our results are in accordance with the observations on human dental pulp stem cells, in which an enhanced *in vitro* osteogenic, adipogenic, neurogenic, and endothelial differentiation ability was detected in the ABCG2-expressing SP cells, as compared with the main population [29]. These data indicate that SP cells in the dental region may possess multilineage potential.

Our simple, antibody-based selection method of cells enriched in ABCG2 may be a proper basis for studies that

determine the potentially increased *in vivo* osteogenic/cementogenic potential ABCG2^{high} PDLSCs. The bone/cementum-forming ability of PDLSCs has been already confirmed in an ectopic mouse model [30], rat periodontal fenestration defect model by using allogenic PDLSCs [31], in a combined sheep-mouse xenogenic model [32], and in a canine model [33]. In humans the therapeutic application of PDLSCs has been suggested to be safe, as a successful pilot study has been performed with three human patients suffering from periodontitis. In these patients autologous PDL cell transplantation has been performed [3].

A limitation in clinical applications is the available cell number for transplantation. The self-renewing ability of PDLSCs and the fact that they can be kept in culture for long periods of time without phenotypical changes allow potential clinical applications. Further, these cells can be isolated from cryopreserved PDLs, thus providing a ready source of MSCs [34]. As a most promising subpopulation of PDLSCs for potential therapy, with an enhanced multilineage differentiation capacity, was suggested the population of CD146⁺/STRO-1⁺ cells, comprising about 2%–3% of the PDLSCs [6,35]. In contrast, ABCG2 expression, as found in this study, occurs in a much greater PDLSC population, allowing the selection of a higher number of cells for efficient therapeutic applications.

A key question was in these experiments whether ABCG2 provides only a cellular marker for an increased differentiation capacity of PDLSCs, or the function of this protein (potentially exporting endo- and xenobiotics from the cells) is also an important factor. Our present experiments argue against such a functional role as ABCG2 expression was significantly downregulated in the early stages of the differentiation process. Moreover, the selective inhibition of the ABCG2 function by a high-affinity inhibitor, Ko143, had no measurable effect on the osteogenic differentiation of PDLSCs.

In parallel experiments, not detailed here, we also investigated the presence of ABCG2 transporter in MSC isolates derived from bone marrow (BM-MSCs) and adipose tissue (AD-MSCs), and negligible ABCG2 expression was detectable at mRNA as well as at protein level. However, the osteogenic differentiation capacity of these cells was commensurable with the bone-forming activity of PDLSCs (data not shown). Based on these observations we suggest that ABCG2, although serves as a marker for differentiation in PDLSCs, has no functional role in the process of bone differentiation.

MSCs in the craniofacial region are derived from neural crest, in contrast to other skeletal MSCs (like BM-MSCs and AD-MSCs) that develop from the mesoderm [36,37]. High ABCG2 expression has already been described in other neural-crest-derived MSC populations, isolated from dental pulp [29] and corneal stroma [38]. Therefore it is possible that ABCG2 expression represents a progenitor cell marker in the neural-crest-derived craniofacial region, although further investigations are necessary to strengthen this hypothesis.

As a conclusion, we found that ABCG2 expression in PDLSCs shows close correlation with their osteogenic and, in general, their differentiation capacity. Moreover, a non-invasive sorting of ABCG2-expressing PDLSCs may be a suitable tool to enrich cells with increased osteogenic and/or cementogenic capacity from the PDL for clinical applications in the future.

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Author Disclosure Statement

No competing financial interests exist.

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