Title: ABCG2 is a selectable marker for enhanced multilineage differentiation potential in periodontal ligament stem cells

Áron Szepesi[1, 2], Zsolt Matula[1], Anna Szigeti[1], György Várady[1], Gyula Szabó[5], Ferenc Uher[4], Balázs Sarkadi[1, 3, 4] and Katalin Német[2]

[1] *Research Centre for Natural Sciences, Hungarian Academy of Sciences, Budapest, Hungary* (Mailing address: 1525 Budapest, Pf. 17.)

[2] *Creative Cell Ltd, Budapest, Hungary* (Mailing address: 1119 Budapest, Budapest, Puskás Tivadar utca 13.)

[3] *Molecular Biophysics Research Group of the Hungarian Academy of Sciences, Semmelweis University, Budapest, Hungary* (Mailing address: 1094 Budapest, Tűzoltó u. 37-47.)

[4] *National Blood Service, Budapest, Hungary* (Mailing address: 1518 Budapest, Pf. 44.)

[5] *Department of Dentistry, Oral and Maxillofacial Surgery, University of Pécs Medical School, Pécs, Hungary* (Mailing address: 7621 Pécs, Dischka Győző utca 5.)

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Corresponding author:

Katalin Német, PhD

Creative Cell Ltd.

Budapest, Puskás Tivadar utca 13.

1119 Hungary

Phone/Fax number: +36 1 372 4332

E-mail: knemet@gmail.com

Contact information:

Áron Szepesi: aron.szepesi@biomembrane.hu

Zsolt Matula: matula.zsolt@gmail.com

Anna Szigeti: anna.szigeti@biomembrane.hu

György Várady: varady@biomembrane.hu

Gyula Szabó: gyula.szabo@aok.pte.hu

Ferenc Uher: uher.ferenc@gmail.com

Balázs Sarkadi: sarkadi@biomembrane.hu

Katalin Német: knemet@gmail.com

Abbreviations

ABCG2 - ATP-binding cassette sub-family G member 2

AD-MSC - adipose tissue-derived mesenchymal stem cell

ALP - alkaline phosphatase

BM-MSC - bone marrow-derived mesenchymal stem cell

CEMP1 - cementum protein 1

CFU-F - colony forming unit-fibroblast

DCV - DyeCycle Violet

HUES - human embryonic stem cell

MSC - mesenchymal stem cell

OCN - osteocalcin

OPN - osteopontin

OSX - osterix

PDL - periodontal ligament

PDLSC - periodontal ligament stem cell

PPARγ - peroxisome proliferator-activated receptor gamma

SP - side population

SSEA4 - stage-specific embryonic antigen-4

Abstract

Periodontal ligament stem cells (PDLSC) 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 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 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 (SP) 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 regeneration of the attachment apparatus that is destroyed as a result of periodontitis. Periodontal regeneration requires new connective tissue attachment to the root surface, a process that involves the regeneration of periodontal fibers and the insertion of these 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 periodontal ligament 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 (PDLSC). These progenitor cells are capable of differentiating both into osteoblasts, cementoblasts or fibroblasts, and produce the extracellular matrix of the periodontal ligament [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, 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 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 cells [14-16]. It has been shown that the periodontal ligament also contains an ABCG2-expressing side population [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 PDLSC showing SP features, although in this case the dye extrusion was ABCB1-dependent.

Based on these studies, the selection of human PDLSC 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, USA) at 37°C for 1 hour. Digestion was stopped by adding identical volume of MSC expansion medium (D-MEM/F-12, 10% v/v fetal bovine serum (FBS), 1 ng/ml FGF-2). The digested cell suspension was centrifuged at 800 g for 10 minutes, washed with PBS, and resuspended in MSC expansion medium. Initially, cells were plated at a density of $2x10^5/cm^2$. Following selection for plastic adherence, PDLSCs were subcultured once a week at a density of $4x10^3/cm^2$.

Human embryonic stem cell line 9 (HUES9) was a kind gift of Douglas Melton, Harvard University, USA. 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, HLA-G), the cells were subsequentially 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 FACSCalibur flow cytometer (BD Biosciences), data were analyzed with CellQuest Pro software (BD Biosciences). For cell sorting $1x10^6$ cells were labeled with the anti ABCG2 antibody as described above. 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 minutes 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).

STRO-1 immunohistochemistry

Cells were fixed with 4% w/v paraformaldehyde, blocked against nonspecific staining with 1% w/v BSA and 4% v/v nonimmune serum, and incubated with primary mouse anti-STRO-1 monoclonal antibody or mouse IgM isotype control for 1 hour at room temperature. AlexaFluor 546 secondary antibody were applied for fluorescent detection, and nuclei were counterstained with 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/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 (CFU-F)

To assess the colony-forming efficiency of PDLSC samples, cells were seeded at a density of 400 cells/10 cm 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) colonies. 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 alkaline phosphatase activity. Following cell lysis with 0.1% w/v Triton X-100 solution diluted in ALP buffer (50 mM TRIS, 150 mM NaCl, 10 mM MgCl2, 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 hour 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 (Perkin-Elmer, Waltham, MA, USA). 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, USA), according to the manufacturer's instructions.

For chondrogenic differentiation $2x10^5$ PDLS cells were plated in 5 µl culture medium in a 24 well cell culture dish for 1 hour, 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 µm 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, than fixed and stained with Oil Red O. To quantify the lipid accumulation, the stain was dissolved with 100% isopropanol, 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 (PPARγ) 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). The list of primers and assays shown in Supplemental Table 1.

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 ± SD. Student's *t-test* was used for the comparison of the sorted cell populations.

Results

Characterization of PDLSC isolates

The cells isolated from periodontal ligament as described in the Methods section are most probably close relatives of the mesenchymal stem cells (MSCs), with variable differentiation potential in different tissues. In order 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 Supplemental Table 2, 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, 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 types (CD14, CD34, CD45, CD117, CD133, CD31, CD144 and CD309) were practically absent, or expressed at a low level (HLA-DR).

The potential mesenchymal stem cell marker STRO-1 was also measured on the surface of cells by flow cytometry, and was found in less than 1% in all PDLSC isolates (Supplemental Table 2). Immunohistochemical staining of STRO-1 was also performed, in order to confirm the flow cytometry measurements (Supplemental Figure 2A, 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 neglible (ΔCT values were 6.68-10.01), as compared to the embryonic stem cell line HUES9 (ΔCT values 0.68-2.93) – see Supplemental Figure 1.

The ABCG2 (MXR/BCRP) multidrug transporter has been indicated to provide a marker for tissue-derived stem cells, corresponding to the side-population (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 e.g. 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 Methods).

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 1-17% of the cells, while the 5D3 method indicated a higher fraction of the ABCG2 positive cells, approximately between 2-64% positivity, depending on the actual source of isolation. The mRNA expression levels for the ABCG2 transporter, measured by qPCR, 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 passage at least) with moderate decrease of ABCG2 expression in the PDLSC cultures.

Culturing 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 0.3-1.5 days. In this regard, we found no correlation of the cell growth with the marker expressions or with the levels of ABCG2 expression as estimated by flow cytometry (Supplemental Figure 3A and 3B).

Mesenchymal stem cells have colony forming ability on plastic surfaces. All of the PDLSC isolates contained a subpopulation that was capable of generating colonies from single cells (Supplemental Figure 4A, B). However the CFU-F frequency varied between 14-32% and no correlation was found between the ABCG2 expression level and the CFU-F frequency (Supplemental Figure 4C). 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 both 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 (Figure 2A, B), and the Calcium Liquicolor test (Supplemental Figure 5). 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 (Figure 2C).

The glycosaminoglycan formation was visualized with hematoxylin and eosin staining after 14 days of *chondrogenic* differentiation (Supplemental Figure 6A). No significant correlation between ABCG2 and SOX9 expression (a transcription factor involved in chondrogenesis; Supplemental Figure 6B) was noticeable in the case of the PDLSC isolates (Supplemental Figure 6C).

We have also studied the *adipogenic* differentiation of the the PDLSC isolates, by culturing these cells in adipogenic medium for 14 days and performed a quantification by oil red O staining (Supplemental Figure 7A, B), and by an RT-qPCR measurement of the PPARγ gene expression level (Supplemental Figure 7C). We found that the amount of the accumulated lipids and the changes in the expression level of the PPARγ gene showed a weak correlation with the ABCG2 expression in the various PDLSC isolates (Supplemental Figure 7D).

Isolation and characterization of ABCG2-expressing PDLSCs

In order to further investigate the correlation between ABCG2 expression and the osteogenic ability of PDLS cells we sorted three PDLSC sample (namely F48, F38 and F28) to $ABCG2^{high}$ and $ABCG2^{low}$ populations (Figure 3A), by using an anti-ABCG2 antibody (see Methods). 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 (Supplemental Figure 8A). The mRNA expression level of ABCG2 was in accordance with the flow cytometer measurements (Supplemental Figure 8B). The ABCG2 expression of sorted cells was followed for a long period (15 passage at least) and moderate decline was noticeable.

In these sorted samples we have examined the proliferation ability, osteogenic and adipogenic differentiation potential of the cells. When measuring cell growth, we found that the sorted cell populations showed no differences in their proliferation activities (Supplemental Figure 9).

For *osteogenic* induction, the unsorted (parental), $ABCG2^{high}$ and $ABCG2^{low}$ cell populations were cultured in an osteogenic medium for 7 days. Alkaline phosphatase (ALP) activity, greatly increasing during osteogenic differentiation, was significantly higher in the $ABCG2^{high}$ cells, as compared to the parental or the $ABCG2^{low}$ cell populations (Supplemental

Figure 10). The gene expression of ALP also strengthens these observations (Figure 3D). As a key parameter of osteogenic differentiation, calcium deposition measured - after 14 days osteogenic induction - with alizarin red S staining, was much greater in the ABCG2^{high} cell populations than in the parental cells or the PDLSC that expressed low levels of ABCG2 (Fig. 3B, C). Thus the mineralization of the sorted ABCG2^{high} PDLS cells was significantly accelerated.

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 osteocalcin (OCN, Fig. 4A, and the transcription factor osterix (OSX, Fig. 4B), involved in bone matrix maturation, were also higher in the ABCG2^{high} cells after 7 days induction. In addition, the mRNA expression level of the cementum protein 1 (CEMP1) gene, a marker of cementogenesis, was also higher in the ABCG2^{high} cells (Figure 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 (Figure 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 (Figure 3F).

Under *adipogenic* induction of the PDLSCs, the gene expression level of PPARγ (Figure 3I) and the lipid accumulation (quantitated in the sorted cells by oil red O staining – Figure 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.

In order 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 μM, 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 cell (Supplemental Figure 11A). 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 (Supplemental Figure 11B), and a loss of ABCG2 from the cell surface after 7 days in osteogenic medium (see Supplemental Figure 8C for a representative sample).

Discussion

Human periodontal ligament stem cells (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 mesenchymal stem cells (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 (side population, SP) excluding the fluorescent dye Hoechst33342 [17]. This Hoechst, or the analog Vibrant DyeCycle Violet [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 PDLS cells. 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.

In order 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 line 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 to 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 glicosaminoglycans was detectable.

The elevation of PPARγ 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. Furthermore, 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 low populations may have eliminated these differences (see Figure 3G, H, 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 to 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 determining 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 periodontal ligament cell transplantation has been performed [34].

A limitation in clinical applications is the available cell number for transplantation. The self-renewing ability of PDLSCs and the fact they can be kept in culture for long periods of time without phenotypical changes, allow potential clinical applications. Furthermore, these cells can be isolated from cryopreserved periodontal ligaments, thus providing a ready source of MSCs [35]. 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, 36]. 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 if 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 down-regulated 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 mesenchymal stem cell isolates derived from bone marrow (BM-MSC) and adipose tissue (AD-MSC), 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.

Mesenchymal stem cells in the craniofacial region are derived from neural crest, in contrast to other skeletal MSCs (like BM-MSC and AD-MSC) which develop from the mesoderm [37, 38]. High ABCG2 expression has already been described in other neural crest derived MSC populations, isolated from dental pulp [29], and corneal stroma [39]. 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 periodontal ligament for clinical applications in the future.

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Author Disclosure Statement

No competing financial interests exist.

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