ORIGINAL RESEARCH

Highly osteogenic PDL stem cell clones specifically express elevated levels of ICAM1, ITGB1 and TERT

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Abstract Cells derived from the periodontal ligament (PDL) have previously been reported to have stem cell-like characteristics (PDL stem cells; PDLSCs) and play an important part in bone engineering, including that of alveolar bone. However, these populations have been heterogeneous, and thus far no specific marker has yet been established from adult human stem cells derived from PDL tissue. We have previously isolated highly purified single cell-derived PDLSC clones and delineated their phenotypic and functional characteristics. In this report, we further obtained three homogeneous and distinct PDLSC clones demonstrating low, moderate and high mineralized matrix forming ability-namely PC12, PC4 and PC3, respectively, and the expression of mesenchymal stem cell pathway-specific genes in these clones was investigated. PCR array revealed that the expression of intercellular adhesion molecule 1 (ICAM1), integrin beta 1 (ITGB1) and telomerase reverse transcriptase (TERT) was associated with highly osteogenic PDLSC clones, as determined by the expression of key osteoblastic markers and their ability to form alizarin red S positive mineralized matrix in vitro. The present results suggest that these three mesenchymal stem cell-associated markers could potentially be used to isolate PDLSCs with high osteogenic capability for engineering new bone.

Introduction

Bone engineering has been proposed to treat bone defects in oral and maxillofacial region using a combination of scaffold, growth factors and stem cells, which possess a high potential to differentiate into cells of osteoblastic lineage, and thus help regenerate new bone (Langer and Vacanti 1993). Seo et al. (2004) identified cells with the characteristic features of stem cells (e.g., self-renewal, clonogenicity and multi-tissue differentiation potential) resided in human periodontal ligament (PDL) tissue, a specialized soft connective tissue which anchors the teeth to the jaw bone and helps the tooth withstand natural chewing forces (Bartold et al. 2000; Shimono et al. 2003). The PDL stem cells (PDLSCs) have been shown to differentiate into osteoblasts and thus forming mineralized bone matrix both in vitro and in vivo (Chen et al. 2006; Gay et al. 2007; Gronthos et al. 2006; Kemoun et al. 2007; Seo et al. 2004). Such putative PDLSCs represent a subpopulation of cells that are also likely to contain a heterogeneous population of periodontal as well as other cells (Kramer et al. 2004). We have recently reported the isolation,

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characterization and osteogenic function of a highly purified and homogeneous PDLSC clone which appears to exhibit, at least in vitro, the definitive features of an adult human stem cell (Singhatanadgit et al. 2009). These PDLSC clones showed different capability to form mineralized matrix in vitro and thus far no information is available regarding the marker used to isolate stem cells with high osteogenic potential under suitable environment. The present study was therefore aimed to identify mesenchymal stem cell (MSC) genes associated with highly osteogenic PDLSC clones using a PCR array technique, which is able to simultaneously examine the expression of 84 MSC signaling pathway-specific genes with high accuracy and specificity.

Materials and methods

Isolation of primary human PDL cells

Periodontally healthy teeth were collected from patients undergoing routine extraction at the Dental Unit, Thammasat University Hospital. The participants signed informed consent to the use of these tissues, in accordance with the protocol approved by the Thammasat University Ethics Committee. The PDL tissue was gently separated from the surface of the middle portion of the root and digested in 3 mg/mL of collagenase type I and 4 mg/mL of dispase (both from Sigma, Gillingham, UK) for 1 h at 37 °C. Single-cell suspensions were obtained by passing the cells through a 70 μm strainer (Falcon, Becton–Dickinson, Cowley, UK) and cultured in α-Modified Eagle's Medium (α-MEM) (Gibco Life Technologies Ltd, Paisley, UK) containing 15% fetal calf serum (FCS) (PAA Laboratories, Yeovil, UK) (15% FCS-MEM) supplemented with 200 U/mL penicillin, 200 μg/mL streptomycin, 2 mM L-glutamine (all from Gibco) at 37 °C in a humidified atmosphere of 5% CO_2 in air.

Cloning of PDL cells

One thousand cells were seeded into 10 cm diameter culture dishes (NUNC, Fisher Scientific UK Ltd, Loughborough, UK) and incubated as above. Individual clones were allowed to develop for 14–21 days until they reached approximately 200–500 cells per colony and then 'ring-cloning' carried out by placing

8 mm diameter cylinder polystyrene rings (Sigma) around the colonies. The cells were detached with 0.05% (w/v) trypsin and 0.05 mM (w/v) EDTA (trypsin–EDTA), transferred to 24-well plates and re-cultured as above.

Colony forming unit-fibroblast (CFU-F) assay

To test CFU-F capability of the PDL clones, single-cell suspensions (1×10^3 cells) were seeded into 10 cm diameter culture dishes (Fisher Scientific UK Ltd, Loughborough, UK) and incubated for 14 days, then stained with 0.1% (w/v) toluidine blue in 1% paraformaldehyde for 5 min. Aggregates of approximately ≥50 cells were scored as colonies.

Adipogenic differentiation of PDLSC clones

Cells were plated (2×10^4 cells/cm^2) in 24-well plates for 48 h. The samples were then cultured for 5 weeks in 10% FCS-MEM with adipogenic supplements containing 0.5 mM hydrocortisone, 0.5 mM isobutylmethylxanthine and 60 mM indomethacin (adipogenic media; AM) and subjected to oil red O staining for the presence of lipid droplets. Briefly, cells were fixed with 10% formalin for 30 min, incubated with 60% propan-2-ol for 5 min and then stained with 0.2% oil-red-O in propan-2-ol for 5 min. For quantification of staining, the absorbed dye was extracted with 100% isopropanol and the absorbance (A490 nm) was measured. In some experiments, the samples were counterstained with Harris Hematoxylin and photographed under a light microscope.

Osteogenic differentiation of PDLSC clones

Cells were plated (2×10^4 cells/cm^2) in 24-well plates and incubated for 48 h at which time osteogenic medium (OM) was added (10% FCS-MEM and osteogenic supplements containing 10 nM dexamethasone, 0.1 mM L-ascorbic acid 2-phosphate and 10 mM β-glycerophosphate), and the cultures re-incubated for 21 days. Alizarin red S staining for calcium-containing deposits and mineralized matrix were then carried out as follows. Cell monolayers were fixed with cold methanol for 30 min at 4 °C and washed with distilled water. The samples were incubated for 10 min at room temperature with 1% alizarin red S (Sigma) (pH 4.2), rinsed twice with methanol and

photographed using light microscopy. Incorporated alizarin red S was extracted from the cell matrix with 100 mmol/L cetylpyridinium chloride (Sigma) for 20 min at room temperature, and the absorbance (A530 nm) was measured.

PCR array of mesenchymal stem cell and osteogenesis pathways

Total RNA was extracted from the cells using RNeasy® Mini Kit (Qiagen, West Sussex, UK), in accordance with manufacturer's instructions. To assess the quality of the extracted RNA and as an internal RT-PCR standard, the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAP-DH) was also amplified.

For reverse-transcription, 1 μg of total RNA was used with 5 ng of oligo-dT in 40 μL of water. After 5 min at 65 °C, the first stand of cDNA was synthesized in a total volume of 50 μL, containing 50 U of cloned Moloney murine leukemia virus (M-MuLV) reverse transcriptase, $1\times$ M-MuLV buffer, 40 μM of each dNTP and 40 U of RNase block. After incubation at 37 °C for 60 min, the enzyme was inactivated by incubation for 5 min at 90 °C. The resulting cDNA reaction (from non-induced and OM-induced samples) was mixed with ready-to-use RT^2 SYBR green qPCR master mix and transfer into 96-well RT^2 Profiler™ PCR array plate for human mesenchymal stem cell and osteogenesis signaling pathway, respectively, (SA-Biosciences). These PCR array plates contain appropriate primers for genes associated with the desired specific signaling pathway and primers for house keeping genes. A two-step real-time PCR reaction was performed starting at 95 °C (10 min) for one cycle and followed by 95 °C (15 s) and 60 °C (1 min) for 40 cycles. Normalized threshold cycle data from real-time instrument was calculated and interpreted using the PCR array data analysis web tool (SABiosciences). A subset of the genes whose expression revealed significant changes (≥ 2 fold) was subjected to quantitative real-time PCR (Q-PCR) individually and the results from the PCR array were confirmed.

Quantitative real-time PCR (Q-PCR)

Q-PCR was carried out to further confirm the expression of ICAM1, ITGB1 and TERT in PDLSC clones. Total RNA was isolated and first strand cDNA

synthesized from 1 μg RNA, as described above. The first strand cDNA were used for amplifications performed with specific primers for ICAM1, ITGB1 and TERT genes (Applied Biosystems, Cheshire, UK). Primer sequences were designed with the Primer Express® program from Applied Biosystems. For Q-PCR analysis in an ABI Prism® 7300 Sequence Detector, the TaqMan® Gene Expression Assays with the Assays-on-Demand™ Gene Expression products were used. TaqMan PCR reaction mixtures were set up as suggested by the manufacturer. Briefly, a 5 μL aliquot of cDNA was used in a final volume of 25 μL reaction mixture containing 12.5 μL of $2\times$ TaqMan® Universal PCR Master Mix (P/N 4304437), 1.25 μL of $20\times$ Assays-on-Demand™ Gene Expression Assay mix and 6.25 μL of nuclease-free water (all from Applied Biosystems). Thermal cycler conditions consisted of AmpErase® UNG activation at 50 °C for 2 min, AmpliTaq Gold® DNA polymerase activation at 95 °C for 10 min and 40 PCR cycles, each of which was 95 °C for 15 s and 60 °C for 60 s. All PCR reactions were performed in triplicate and each of the signals was normalized to the GAPDH signal in the same reaction.

Flow cytometry (FCM)

FCM was carried out to confirm the cell surface expression of ICAM1 and ITGB1 proteins in PDLSC clones. Exponentially growing cells were obtained by incubating the cells in standard culture media for 48 h and the samples were then prepared for FCM as described previously (Kuru et al. 2001). In brief, the cells were detached using a scraper and 20 mM EDTA without trypsin, in order to prevent the loss of cell surface-associated proteins resulting from proteolytic activity of this enzyme, and then fixed with 1% paraformaldehyde. They were washed with PBS containing 2% FCS (PBS-FCS) after each step described below. Following centrifugation, 50,000 cells were treated for 30 min at room temperature with PE-mouse anti-human ICAM1 (1:5) and PE-mouse anti-human ITGB1 (1:5) (all BD Pharmingen, CA, USA), diluted in PBS-FCS. PE-mouse IgG1, κ Isotype Control (BD Pharmingen) was used as the negative control. The specific reactivity of the antibodies with their corresponding antigens had been tested and confirmed by the supplier. After centrifugation, the cells were resuspended in 400 μL of PBS-FCS and the fluorescence

intensity of 5,000 individual cells was measured using a flow cytometer (FACScan; Becton–Dickinson, Cowley, UK). The values are presented as arbitrary units of fluorescence intensity, which depend on the electronic input and output settings of the cytometer and were kept constant in all experiments. Analysis of data was performed using the CELLQuest software program (Becton–Dickinson). The geometric means of the fluorescence values of the cell population are shown as the average fluorescence intensity (AFI) of 5,000 individual cells. For each antibody, the arbitrary AFI obtained is proportional to the level of expression of the specific antigen. Thus, differences in AFI between different PDLSC clones after reaction with a specific antibody reflect the relative levels of expression of that particular antigen by each of the different PDLSC clones. However, AFI values obtained using different antibodies cannot be compared with each other as they do not indicate the relative level of the different antigens, only differences in reactivity of the antibodies with their respective antigens.

Results

Highly purified PDL cell clones and their CFU-F ability

A total number of 12 PDL cell clones (namely PC1-PC12, respectively) were obtained from individual single cell-derived colonies of primary human PDL cells (Fig. 1a). Although all the PDL cell clones were able to form CFU-F, each of these clones showed different CFU-F ability. The results in Fig. 1b show that PC1, PC3, PC5, PC8, PC 9 and PC11 formed approximately more than 100 CFU-F per 1,000 cells seeded initially, whereas the other clones (PC2, PC4, PC6, PC7, PC10 and PC12) had low ability to form CFU-F (less than 100 CFU-F per 1,000 cells) (Fig. 1b).

Adipogenic differentiation of PDL clones

The PDL cell clones were further tested for the ability to differentiate along with the adipogenic lineage by culturing them in AM and the presence of intracellular lipid droplets examined by oil red O staining. A representative result of the presence of lipid droplets
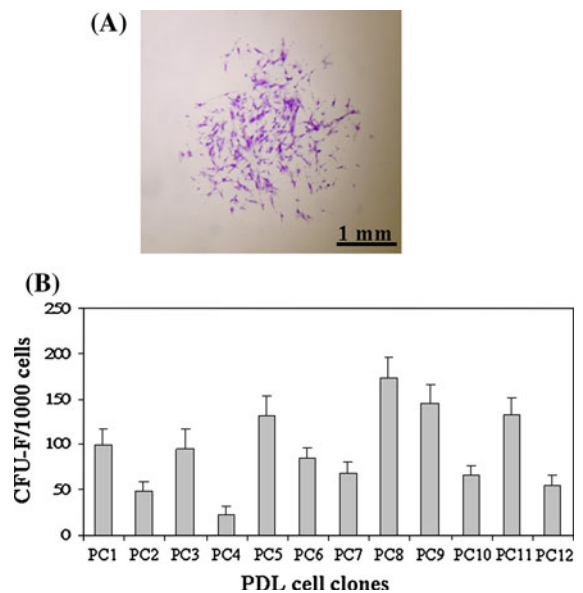

Fig. 1 Colony-forming unit fibroblast (CFU-F) potential of PDL cell clones. Single cell suspensions were cultured at a low density, as described in the "Materials and methods". The colony-forming efficiency was assessed on day 14, with aggregates of approximately ≥50 cells being scored as colonies. **a** shows a representative micrograph of a single cell-derived colony stained with 0.1% toluidine blue. **b** reveals a summary of CFU-F potential of each of the PDL clones. The results are presented as the mean ± SD of the number of CFU-F per 1,000 initially seeded PDL clone cells obtained from three separate experiments, as described in the "Materials and methods"

obtained from PC3 culture is shown in Fig. 2a. Analysis of three separate experiments in Fig. 2b established that only PC2, PC3, PC4, PC8 and PC12 were able to be induced to undergo adipogenic differentiation and thus forming oil red O-positive lipid droplets while the other clones were negative for oil red O staining.

Osteoblast differentiation and mineralized matrix-forming ability of PDL cell clones

The PDL cell clones were further tested for the ability to differentiate along with the osteogenic lineage by culturing them in OM and the presence of mineralized matrix examined by alizarin red S staining. In Fig. 3a, a representative result obtained from PC3 cultures demonstrated the presence of alizarin red S-positive mineralization, as indicated by bright red deposits, in

Fig. 2 Adipogenic differentiation of PDL cell clones. In **a**, the figures show representative oil red O staining of PC3 cultured in standard culture medium (control) and in adipogenic medium, as described in "Materials and methods". In adipogenic medium, increased accumulation of intracellular lipid was shown by stained droplets (*arrows*) when compared with the sample cultured in the control medium (**a**). Oil red O staining in all stimulated PDL cell clones was extracted and quantified as described in "Materials and methods", and the results are presented as the mean $A_{490} \pm$ SD of triplicate experiments (**b**)
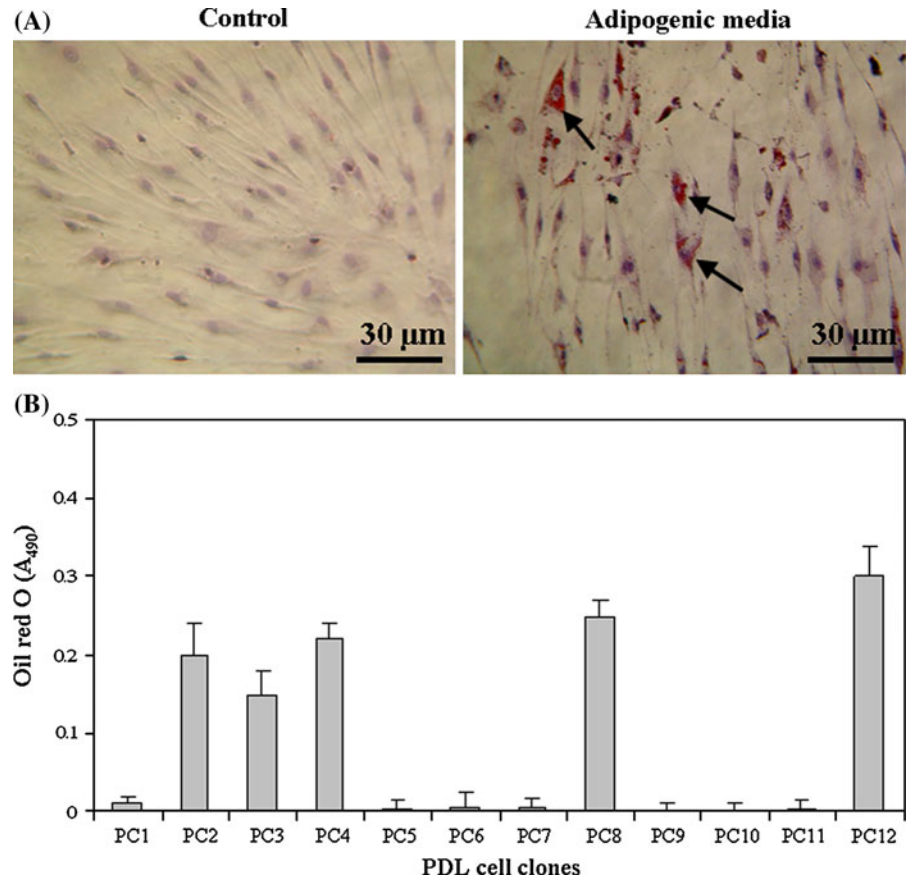
the OM culture compared with cells cultured in the standard media (control). Analysis of three separate experiments showed that unlike their adipogenic differentiation ability, all clones were able to differentiate into osteoblasts, forming alizarin red S-positive mineralized matrix under OM cultures although their osteogenic property differed markedly (Fig. 3b). Together with the results mentioned above, PC2, PC3, PC4, PC8 and PC12 possessed stem cell-like characteristics, i.e., CFU-F ability (Fig. 1) and multilineage differentiation (Figs. 2 and 3), suggesting that these PDL cell clones appeared to be PDLSCs.

Based on the analysis of alizarin red S-positive mineralization, PC12, PC4 and PC3 were selected to be representative PDLSC clones that possessed low, moderate and high mineralized matrix forming ability, respectively. These PDLSC clones were thus used to examine the expression of genes involving in stem cell and osteogenic signaling pathways using PCR array technique.

Expression of mesenchymal stem cell-related genes and osteoblast-related genes in PDLSC clones by PCR array

PCR array was used to examine the expression of genes associated with mesenchymal stem cell pathway in PDLSC clones (PC12, PC4 and PC3, demonstrating low, moderate and high mineralized matrix forming ability, respectively) cultured in standard medium and that related to osteoblasts in these clones grown in OM for 3 weeks. The affected genes with more than twofold changes in PC4 and PC3 compared with those in PC12 are shown in Tables 1 and 2 whereas the other tested genes not listed in the Tables were unaffected (<2-fold changes). The results in Table 1 show the expression of genes associated with mesenchymal stem cell signaling which reveals that in control medium (without OM), the expression of intercellular adhesion molecule 1 (ICAM1), integrin beta 1 (ITGB1) (fibronectin receptor or CD29) and telomerase reverse
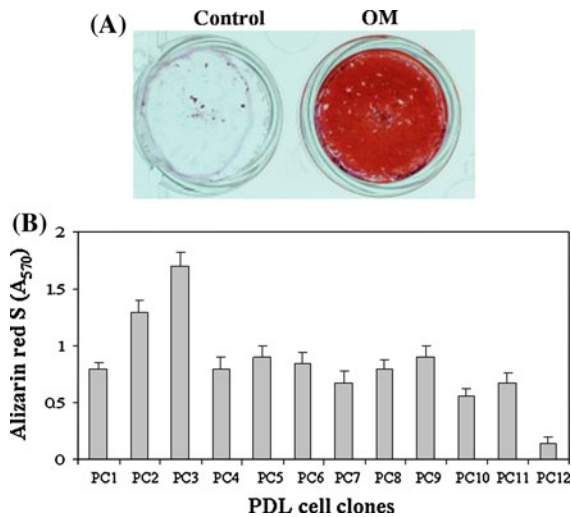
Fig. 3 Osteogenic differentiation of PDL cell clones. In **a**, the figures show representative alizarin red S staining of PC3 cultured in standard culture medium (control) and in OM, as described in "Materials and methods". The presence of alizarin red S-positive mineralized matrix was shown by stained deposits (in OM culture) compared with cells cultured in the control medium. Alizarin red S staining in all stimulated PDL cell clones was extracted and quantified as described in "Materials and methods", and the results are presented as the mean $A_{570} \pm$ SD of triplicate experiments (**b**)

transcriptase (TERT) was approximately twofold and threefold higher in PC4 and PC3, respectively, than that of the control clone PC12. The elevated expression of these three genes was confirmed by Q-PCR (data not shown). In contrast, PC4 and PC3 expressed approximately 50–70% lower mRNA levels of histone deacetylase 1 (HDAC1), Notch homolog 1 (NOTCH1) and Wingless-type MMTV integration site family, member 3A (WNT3A) than the control clone PC12.

The results suggest possible association of these stem cell signaling genes and the ability of PDLSC clones to form mineralized matrix.

The results in Table 2 show the expression of osteogenesis-associated genes in PC12, PC4 and PC3 cultured in OM for 3 week. The PCR array indicated that there was a significant increase (more than twofolds) in the expression of alkaline phosphatase (ALP), osteocalcin (OCN), biglycan (BGN), collagen type 1A2 (COL1A2) and runt-related transcription factor 2 (RUNX2) in PC4 and PC3 when compared with the control clone PC12, with an gradually increasing expression being observed in PC4 and PC3, respectively. The gradually increased expression of these key osteogenesis-associated markers, i.e., low expression in PC12 versus high expression in PC3, supported their mineralization capability, previously shown in Fig. 3b.

Expression of cell surface ICAM1 and ITGB1 antigens in PDLSC clones PC12, PC4 and PC3

To test whether the pattern of cell surface expression of ICAM1 and ITGB1 in the three PDLSC clones PC12, PC4 and PC3 would be comparable to the expression pattern of their corresponding mRNA transcripts, the surface expression of ICAM1 and ITGB1 antigens was examined using FCM, as described in "Materials and methods". The representative histograms in Fig. 4 show that all PDLSC clones tested were found to be positive for ICAM1 and ITGB1 antibodies, as demonstrated by the shift of the histograms of the fluorescence of the tested antibody (clear histograms) to the right of the fluorescence of the isotype-matched antibody used

Table 1 The expression of affected stem cell marker-related genes in PC12, PC4 and PC3 when cultured in standard culture medium

GeneBank	Symbol	Description	Fold change		
			PC12	PC4	PC3
NM_004964	HDAC1	Histone deacetylase 1	1.0	0.5	0.4
NM_000201	ICAM1	Intercellular adhesion molecule 1 (CD54), human rhinovirus receptor	1.0	2.1	3.2
NM_002211	ITGB1	Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29)	1.0	2.2	2.9
NM_017617	NOTCH1	Notch homolog 1, translocation-associated (Drosophila)	1.0	0.5	0.3
NM_198253	TERT	Telomerase reverse transcriptase	1.0	2.2	2.9
NM_033131	WNT3A	Wingless-type MMTV integration site family, member 3A	1.0	0.4	0.5

Table 2 The expression of affected osteoblastic cell marker-related genes in PC12, PC4 and PC3 when cultured in OM

GeneBank	Symbol	Description	Fold change		
			PC12	PC4	PC3
NM_000478	ALP	Alkaline phosphatase, liver/bone/kidney	1.0	2.6	4.8
NM_199173	BGLAP	Bone gamma-carboxyglutamate (gla) protein (osteocalcin)	1.0	2.3	5.3
NM_001711	BGN	Biglycan	1.0	2.1	2.8
NM_000089	COL1A2	Collagen, type I, alpha 2	1.0	3.5	3.4
NM_004348	RUNX2	Runt-related transcription factor 2	1.0	2.3	5.1

as controls (shaded histograms). Analysis of three separate experiments further established that in standard culture medium (without OM), the expression of cell surface ICAM1 was approximately threefold and sixfold higher in PC4 and PC3, respectively, than that of the clone PC12 (Fig. 4). As with ICAM1, cell surface ITGB1 expression was approximately sixfold and ninefold higher in PC4 and PC3, respectively, than in the clone PC12 (Fig. 4).

Discussion

In the present study, PDL cell clones were isolated by cloning ring technique based on their ability to form single cell-derived colonies (Digirolamo et al. 1999, Pittenger et al. 1999, Sekiya et al. 2002). A total number of 12 clones, namely PC1-PC12, were cloned from primary PDL-derived cells, which are believed to comprise progenitor cells of PDL-producing fibroblasts, of cementoblats (cells that form cementum, a mineralized matrix layer covering the tooth root) and of bone forming osteoblasts (Ivanovski et al. 2006, Kramer et al. 2004, Lekic et al. 2001, McCulloch and Bordin 1991, Melcher 1976, Murakami et al. 2003, Seo et al. 2004). Although all 12 clones had an ability to form CFU-F, only PDL clones PC2, PC3, PC4, PC8 and PC12 underwent adipogenic and osteoblastic differentiation, suggesting stem cell-like characteristics of these clones.

In order to investigate the markers expressed by stem/progenitor cells that have a high potential to differentiate into the osteoblastic lineage, we first examined the expression of stem cell-related markers in these highly purified putative stem cell clones PC12, PC4 and PC3 (representing low, moderate and high mineralized matrix forming ability, respectively) cultured in non-stimulated condition. We utilized a new technology PCR array, which provides highly accurate expression of a large number of pathway-specific genes of interest to examine the expression of stem cell and osteogenic signaling genes. The result of the present study suggested that ICAM1, ITGB1
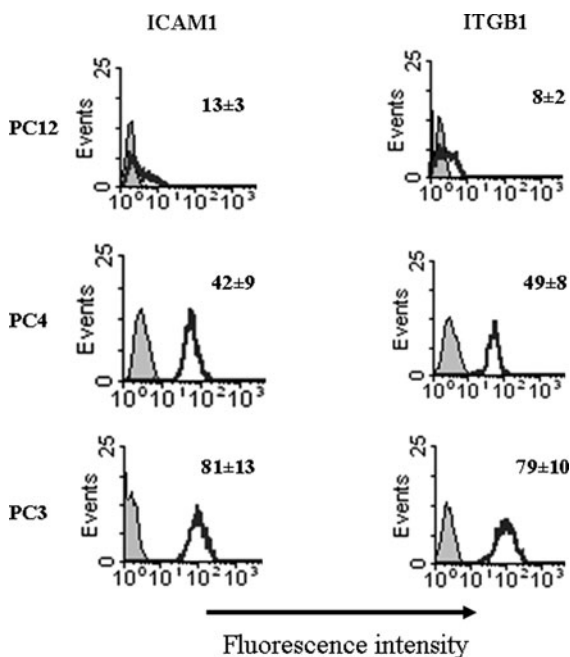

Fig. 4 FCM analysis of cell surface expression of ICAM1 and ITGB1 antigens in PDLSC clones PC12, PC4 and PC3. The relative levels of cell surface expression analyzed using ICAM1 and ITGB1 antibodies are shown by the shift of the clear (*solid line*) histogram to the right compared with the fluorescence of the isotype-matched antibody used as controls (*shaded histograms*). The numbers shown are the mean AFI ± SD of PDLSC clones of three separate experiments

and TERT were highly expressed in a certain PDL clone specifically associated with cells of osteoblasic lineage.

ICAM1 is an important cell surface adhesion molecule found in many cell types including in stem/progenitor cells and osteoblasts (Fu et al. 2009, Tanaka et al. 1995). Several lines of evidence support that ICAM1-mediated direct cell contact has a major impact on a wide range of biological responses including cell differentiation (Gortz et al. 2004, Long et al. 1995, Olsen et al. 1988, Saho et al. 2003, Tanaka et al. 1995). It has been shown that ICAM1 plays an important role in direct cell-to-cell contact-mediated signals via mitogen-activated protein kinase (MAPK) pathway including p38, extracellular signal-regulated kinase (ERK) 1/2 and c-Jun N-terminal kinase (JNK) 1/2/3 (Cernuda-Morollon and Ridley 2006, Turowski et al. 2005), which could subsequently stimulate osteoblast differentiation of stem/progenitor cells. Fu et al. (2009) further suggested that ICAM1 and p38 MAPK also significantly influence the migration of stem cells to their target sites, such as PDL tissue and alveolar bone. These previous evidence, together with our present results, suggest that ICAM1 could also play a significant part in signals initiated by direct cell-to-cell and cell-to-matrix contact, thus subsequently enhancing osteoblast differentiaon of stem cells. However, further studies are required to test this possibilty. Like ICAM1, ITGB1 serves as a cell surface adhesion protein that facilitates direct cell-to-ECM contact and cell-to-cell binding, regulating growth and function of many cell types including osteoprogenitor cells (Calderwood 2004, Liddington and Ginsberg 2002) and controlling early differentiation phase of stem cells via modulation of Wnt1 and BMP4 expression (Czyz and Wobus 2001). Telomerase reverse transcriptase (TERT) is one of key nuclear proteins which control DNA metabolism and thus cell proliferation (Bodnar et al. 1998). TERT involves in processing single stranded RNA into double stranded DNA by telomerase, which adds DNA sequence TTAGGG repeats at the telomere region of the DNA strand (Bodnar et al. 1998). Defective TERT could thus result in significant DNA degradation and hence reduced cell proliferation and undergoing cell senescence (Zakian 1995). However, unlike ICAM1 and ITGB1, which are cell surface molecules, TERT is found in nucleus, and it is therefore practically difficult to use this protein as a selective marker to isolate stem cells by using currently available techniques.

As mentioned above, unlike TERT, ICAM1 and ITGB1 are known to be cell surface-associated proteins, and we have therefore examined their cell surface expression to determine the possibility to use these proteins as selective markers of PDL-derived stem cells possessing high osteoblastic activity. FCM analysis of ICAM1 and ITGB1 showed similar pattern of their cell surface expression and their corresponding mRNA transcript expression among the three PDLSC clones PC12, PC4 and PC3, thus confirming the PCR array results and establishing that ICAM1 and ITGB1 could potentially be used as putative markers of highly osteogenic stem cells isolated from PDL tissue. However, we are currently investigating whether these two markers are specifically expressed in osteogenic stem cells derived from other human tissues known to be sources of mesenchymal stem cells.

It has been reported that adult human bone marrow-derived CFU-F are capable of differentiating into functional osteoblasts and that osteoprogenitors are present in the STRO-1$^+$ population (Gronthos et al. 1994). In addition, STRO-1 expressing stem cells have been shown to possess a marked proliferative capacity and CFU-F potential (Simmons and Torok-Storb 1991). However, little is known about markers associated with PDLSCs that highly differentiate into cells of osteoblastic lineage. The present study has shown, for the first time, that ICAM1, ITGB1 and TERT expression could be considered as putative markers for mesenchymal stem cells with a characteristic of high osteoblast differentiation capability.

It is noteworthy that the expression of key osteoblast marker-related genes ALP, OCN, BGN, COL1A2 and RUNX2 in PC12, PC4 and PC3 cultured in OM (Table 2) corresponded to their ability to form mineralized matrix assessed by alizarin red S staining (Fig. 3). However, the expression of other known osteoblast-associated genes in these clones did not correspond to their mineralization forming ability, suggesting that ALP, OCN, BGN, COL1A2 and RUNX2 could be good specific markers of osteoblast differentiation and mineralization of PDLSCs. Moreover, it is important to point out that although the expression of these osteogenic-related genes was not examined in undifferentiated mesenchymal cells in the present study, it has previously been shown that

the expression of these genes is highly up-regulated in more differentiated progenitor cells compared with those in undifferentiated cells (Ducy et al. 1997, Gay et al. 2007, Gronthos et al. 2006, Parisuthiman et al. 2005, Seo et al. 2004, Singhatanadgit et al. 2009). RUNX2 is a key transcription factor involving in osteoblast differentiation and bone formation (Ducy et al. 1997, Komori et al. 1997, Shirakabe et al. 2001). RUNX2 controls the expression of many bone matrix proteins including ALP, COL1A2, osteopontin (OP), bone sialoprotein (BSP) and OCN (Barnes et al. 2003, Ducy et al. 1997). ALP is an important early marker of osteoblast differentiation (Kim et al. 2004, ten Dijke et al. 2003) which controls the precipitation of hydroxyapatite crystals in the early phase of mineralization in bone formation process (Anderson 2003, Manolagas 2000). Moreover, Chaudhary et al. (2004) have reported that the ALP activity is closely associated with the formation of mineralized matrix while OCN, BGN and COL1A2 are major proteins commonly found in extracellular bone matrix and are believed to play an important role in bone formation (Chen et al. 2003, 2004, Kuroki et al. 1994, Parisuthiman et al. 2005, Pischon et al. 2004, Regazzoni et al. 2001, Sierra et al. 2003, Takeuchi et al. 1996, Takuwa et al. 1991, Viereck et al. 2002, Young et al. 2002).

In conclusion, the present results have shown, for the first time, that two cell surface molecules ICAM1 and ITGB1 are favorably expressed in highly osteogenic PDL-derived stem cell clones, suggesting their potential use as putative markers of functionally-active bone-forming stem cells derived from human PDL tissue.

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