

The effect of strontium chloride on human periodontal ligament stem cells

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Summary

The complete repair of periodontal structures remains an exciting challenge that prompts researchers to develop new treatments to restore the periodontium. Recent research has suggested strontium ion to be an attractive candidate to improve osteogenic activity.

In this study, we have isolated a clonal finite cell line derived from human periodontal ligament (PDL) in order to assess whether and in which way different doses of SrCl₂ (from 0.5 to 500 µg/ml) can influence both the proliferation and the mineralization process, for future application in oral diseases.

PDL cells were cloned by dilution plating technique and characterized by FACS. Cell proliferation analysis and mineralization were performed by [³H]-thymidine incorporation and spectrofluorometric assay.

Results have evidenced that the higher SrCl₂ concentrations tested, from 25 to 500 µg/ml, have increased the proliferation activity after only 24 h of treatment. Interestingly, the same higher concentrations have decreased the mineralization, which was conversely increased by the lower ones, from 0.5 to 10 µg/ml.

Our findings suggest the possible use of SrCl₂ in appropriate delivery systems that release, at different time points, the specific dose, depending on the biological response that we want to induce on periodontal ligament stem cells, providing a more efficient periodontal regeneration.

KEY WORDS: periodontal ligament stem cells; periodontal regeneration; strontium; cell therapy.

Introduction

The periodontal ligament (PDL) is a soft connective tissue embedded between the cementum, covering the root of the tooth, and the inner wall of the alveolar bone socket (1). The PDL provides anchorage and support to the functional tooth, dispersing the mechanical forces associated with mastication (2).

Periodontitis, a chronic inflammatory disease, is a common and widespread condition that causes the destruction of the tooth-supporting tissues including the PDL, the alveolar bone and the root cementum. This pathology affects more than 60% of adults with a 10% of aggressive forms, which are able to produce a quick progression of the tissue damage (1); in fact, if left untreated, periodontitis will result in progressive periodontal attachment and bone loss that may lead to premature tooth loss, representing a harmful implication on the individual's quality of life and a substantial public health burden worldwide (3). It is therefore evident that the regeneration of a healthy periodontium, that has been destroyed by periodontal disease, is the major goal of periodontal therapy.

Current conventional techniques for the treatment of periodontal disease, which aim to decrease microbial levels and modify the local environment to reduce inflammation, have shown a limited potential for complete periodontal regeneration. The topical direction to enhance periodontal tissue reconstruction and its biomechanical integration is represented by the use of mesenchymal stem cells for the development of scaffolding matrices in combination with specific bioactive molecules that can drive and promote the complete process of regeneration.

Mesenchymal stem cells have been isolated from teeth including dental pulp, apical papilla, and dental follicle (4). Of particular interest are the cell populations derived from PDL, as the presence of multiple cell types (cementoblasts, osteoblasts, fibroblasts) within PDL suggests that this tissue contains progenitors, which maintain tissue homeostasis and regeneration of the periodontal tissues (5). Earlier evidence has shown that PDL contains cell populations which have the characteristics of postnatal stem cells, are self-renewable and possess the ability to give rise to a range of different phenotypes (cementoblasts, adipocytes and chondrocytes) (1, 6, 7). Moreover, in various animal models, implanted PDL-derived mesenchymal stem cells (PDLSCs) have shown the capacity to generate a cementum/PDL-like structure similar to the native periodontal complex (1). Based on these findings, PDLSCs are now considered a more appropriate cell type for developing novel tissue strategies for regenerating dental tissue.

Strontium, Sr, an alkaline earth metal, is an important trace element in the human body. It has interesting properties and is chemically similar to calcium, thus, it can substitute calcium in the bone matrix relatively easily. It has been found to induce osteoblast activity by stimulating bone formation and reducing bone resorption (8). Moreover, in several studies, it has been reported to stimulate osteogenic differentiation and proliferation in mesenchymal stem cells and osteoblasts (9, 10).

However, to our best knowledge, there are few studies regarding the effects of strontium on the proliferation and mineralization processes of mesenchymal stem cells isolated from human PDL (11, 12). In particular, there are no articles which simultaneously analyze both of the above mentioned processes in human PDLSCs treated with the same SrCl₂ concentrations. For this reason, the aim of our study was to evaluate and define the *in vitro* effects of a wide range of SrCl₂ concentrations, from 0.5 to 500 µg/ml, in order to bring additional and more specific evidences to support the possible use of SrCl₂ in combination with PDLSCs, helping to improve the outcome of cellular therapy for periodontal regeneration.

Materials and methods

Isolation of primary human PDL cells and cultures

A normal impacted third molar was extracted from a patient (male, age 23) after orthodontic treatment, and processed within 3 hours from the extraction. The procedure was approved by the Local Ethics Committee AOU Careggi (Rif. n. 140-12) and was performed after obtaining signed informed consent from a patient, in accordance with the ethical standard stated in the Declaration of Helsinki. Tissue from the PDL was gently excised with a scalpel from the middle third of the freshly extracted tooth. The PDL tissue was then enzymatically digested with 3 mg/ml collagenase type I (Sigma-Aldrich, Saint Luis, MO, USA) for 1 hour at 37°C. Single-cell suspensions were obtained with a 70 µm cell strainer (Merck Millipore, Darmstadt, Germany). To separate cells expected to be stem cells, we allowed the suspension to attach and proliferate, until confluence, to 100 mm dishes (Falcon, Madison, SD, USA) in growth medium (GM) composed by Ham's F12 Coon's medium supplemented with 10% fetal bovine serum (FBS), (Sigma-Aldrich, Saint Luis, MO, USA), 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in 5% carbon dioxide in humidified atmosphere.

Immunomagnetic isolation of CD44+ PDL cells

The isolation of putative PDLSCs was made through immunomagnetic isolation by MicroBeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Single-cell suspension of 10⁶ PDLSCs was incubated with 20 µl of CD44 MicroBeads for 15 minutes on ice. The cells were then washed with phosphate buffered saline containing 1% bovine serum albumin and centrifuged at 300 x g for 10 minutes. Beads positive cells were isolated with a magnetic particle concentrator according to the manufacturer's instruction, and left proliferating in 100 mm-dishes in GM. The medium was refreshed twice a week and the cells were used for subculturing or cryopreservation upon reaching 70 - 80% confluence. Isolated cells were expanded and used at passage 2 for cell cloning.

Cloning of CD44+ PDL cells

CD44+ PDL cells at the second passage were used for cell cloning. Cells in an active phase of growth were cloned by the dilution plating technique. Cells were detached with trypsin 1:250 (BD, Franklin Lakes, NJ, USA) 0.4 mg/ml in Dulbecco's Phosphate Buffered Saline (DPBS) without Ca²⁺ and Mg²⁺, with EDTA 0.2 mg/ml and with glucose 1 mg/ml. The cells were then resuspended in Coon's medium with 20% FCS. The cell suspension was diluted to a concentration of 10 cells/ml in the following cloning medium: Coon's medium with 20% FCS supplemented with 25% conditioned medium prepared from human fetal fibroblast culture. The cell suspension was maintained in agitation, and 0.1 ml/well was rapidly distributed in a 96-well half area tissue culture plate. Each well was carefully observed during the following days and the wells containing only one cell were scored. The cloning culture was incubated at 37°C in humidified air with 5% CO₂. When the colonies reached 500-1000 cells, they were detached, collected, transferred in 24 well plates and subsequently expanded in 60 mm and 100 mm dishes.

Flow cytometry analysis

Stemness surface markers of PDLSCs finite clonal line were evaluated by flow cytometry with a CyFlowSpace cytometer (Sysmex Partec GmbH, Görlitz, Germania), equipped with FlowMax software. The antibodies used were directed against the following antigens (the tags are given in parentheses): CD44 (PE/Cy7), CD105 (FITC), CD90 (APC), CD45 (PerCP), CD34 (PE) (all Abcam, Cambridge, UK), CD133 (PE), CD271 (FITC) (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and CD117 (FITC) (Bioss Antibodies). Each antibody was diluted according to manufacturer's instruction. Briefly, 1 x 10⁵ cells were labeled with antibodies in PBS with 1% bovine serum albumin (BSA) for 20 min RT in the dark, then washed once and promptly analyzed.

Soft agar assay for neoplastic transformation

Neoplastic transformed cells form colonies that grow progressively in soft agar. A 35 mm dish was coated with 1% agar prepared in culture medium and maintained in liquid state at 45°C. The dish was immediately cooled. Cells in growth phase were detached, suspended in medium, diluted to double the required final concentration, and maintained at 37°C. Agar (0.68%) was prepared in medium and maintained at 45°C. The cell suspension was mixed with an equal volume of 0.68% agar, distributed into the agar coated dish to obtain a final concentration of 2.5 x 10³ cells/dish, and immediately cooled. The cells were cultured at 37°C in humidified air with 5% CO₂ for 3-4 weeks until the formation and growth of the colonies. The colonies that formed in each dish were observed and counted in phase contrast microscopy. Suspended clusters greater than 0.2 mm diameter are considered as colonies.

Colony forming unit assay

To test colony forming unit (CFU) capability of the PDLSCs, single-cell suspension of 10² cells was seeded into 100-mm diameter culture dishes and cultured for 2 weeks in GM. After fixation for 20 minutes with 4% p-formaldehyde, and extensive washes with ultrapure water, cells were stained with 0.1% (w/v) toluidine blue to assess colony formation efficiency. Fifty or more cells clustered together were considered a colony.

Multiple differentiation potential of PDLSCs

Multipotency evaluation was assessed inducing PDLSCs toward both the osteogenic and adipogenic phenotypes, as described below.

Osteogenic differentiation: PDLSCs were plated on tissue culture dishes at a cell density of 1×10^4 cells/cm² in GM and grown to 70 - 80% confluence. Afterwards, the medium was changed to osteogenic medium (OM): Ham's F12 Coon's modification medium supplemented with 10% FBS, 100 IU/ml penicillin, 100 µg/ml streptomycin, 10 nM dexamethasone, 0.2 mM sodium L-ascorbyl-2-phosphate, and 10 mM β-glycerol phosphate. In order to visualize calcium mineral deposits in epifluorescence microscopy, fluorophore calcein 1 µg/ml was added to the OM during the entire study period. The medium was refreshed twice a week. The expression of the osteoblastic phenotype was evaluated until 21 days from induction by monitoring ALP activity and mineralization by cytochemical staining. For ALP staining, the cells were washed with DPBS (twice), stained with a specific dye mixture: 5 mg naphthol-AS-MX phosphate sodium salt, previously dissolved in 1 ml dimethyl sulfoxide, 40 mg Fast Red Violet LB, dissolved in 49 ml Tris-HCl Buffer 280 mM pH 9.0 for 30 minutes at 37°C. The cells were then washed with DPBS (twice), fixed in 4% p-formaldehyde for 15 minutes, and washed with ultrapure water (three times). ALP+ cells, stained in red, and nuclei, counterstained in blue with Mayer's acid hemalum, were observed in brightfield microscopy. For mineralization staining, the cells were washed with DPBS (two times), fixed in 4% p-formaldehyde for 15 minutes, and washed with ultrapure water (three times). Calcium mineral deposits, stained with calcein, and nuclei, counterstained with 10⁻⁵M propidium iodide, were visualized in epifluorescence microscopy.

Adipogenic differentiation: PDLSCs were cultured with a specific adipogenic medium (AM): Ham's F12 Coon's modification medium supplemented with 10% FBS, 100 IU/ml penicillin, 100 µg/ml streptomycin and 1 µM dexamethasone, 1 µM bovine insulin, 0.5 mM isobutylmethylxanthine (IBMX). The medium was refreshed twice a week. The expression of the adipogenic phenotype was evaluated on cells cultured in AM or GM for 21 days by Oil Red O staining. Cultures were fixed directly with fresh Oil Red O in alcoholic solution for only 3 minutes, to avoid the rupture of the lipid droplets, and immediately observed in brightfield microscopy for photo acquisition.

Immunocytochemistry

PDLSCs were seeded into 24-well plates (1×10^4 cells/well) and cultured for 24 hours in GM. Then, cells were fixed for 10 minutes with 4% p-formaldehyde and permeabilized for 10 minutes with 0.2% triton X 100 at RT. Afterwards, cells were treated for another 30 minutes at 37°C with RNase in 2% bovine serum albumin (BSA) in order to degrade RNA and block non-specific sites. Then samples were incubated with primary antibody for CD44 (Abcam, Cambridge, UK) in PBS at 4°C, overnight. After extensive washes with PBS, goat anti-mouse IgG (H+L) SuperClonal secondary antibody, Alexa Fluor 488 conjugate (Thermo Scientific, Waltham, MA, USA) was incubated for 1 hour at room temperature in the dark. Subsequently, nuclei were counterstained with 10⁻⁵M propidium iodide. Samples were then washed with PBS for observation in laser scanning confocal microscopy (LSCM), using a LSM 510 Meta microscope (ZEISS, Jena, Germany).

Treatment with SrCl₂ concentrations

Different concentrations of SrCl₂·6H₂O (Mr 266.6, Sigma-Aldrich, Saint Luis, MO, USA) were used in the experiments in order to evaluate the effects of the bioactive ion on PDLSCs proliferation and osteogenic differentiation. All the experiments were performed using SrCl₂ concentrations from 0.5 to 500 µg/ml added to the media.

Cell proliferation analysis

Incorporation of [³H]-thymidine (PerkinElmer, Waltham, MA, USA) was performed using semi-confluent PDLSCs in 24 well plates, starved for 48 hours in Ham's F12 Coon's modification medium supplemented with 0.1% FBS and stimulated with or without different concentrations of SrCl₂ for 24 hours. After 7 hours of incubation with the addition of 1 µCi of [³H]-thymidine / 500 µl / well, cells were harvested for analysis. [³H]-thymidine incorporation was determined using the LS6500 scintillation counter (Beckman Coulter, Miami, FL, USA) and radioactivity was expressed as disintegration per minute (DMP) / well.

Calcium content assay

Confluent PDLSCs in 24 well plates were induced to mineralize with several concentrations of SrCl₂ in the presence of fluorophore calcein (1 µg/ml), at different experimental times (T₀, 7, 14, 21, 28 days). The multiwell plate was fixed with 4% p-formaldehyde for 10 minutes. After washes in DPBS, each well was incubated overnight with 1 ml of 50 mM NaEDTA at RT. The contents of each well were then transferred into a cuvette and the relative fluorescence measured by spectrofluorometer LS55 (PerkinElmer, Waltham, MA, USA) at 494 λ (excitation) and 517 λ (emission), and expressed in ng calcium deposits/ng DNA, using a standard curve of hydroxyapatite 25 ng/ml – 2.5 µg/ml solubilized in NaEDTA.

Statistical analysis

Statistical analysis was performed using the Kruskal-Wallis Test with calculation of least significant difference, comparing the values obtained in presence of different SrCl₂ concentrations with respect to the control without stimuli. A p-value of less than 0.05 was considered significant.

Results

Isolation and characterization of PDLSCs

The plastic-adherent cell population derived from human periodontal ligament, called PDL5, was separated by immunomagnetic isolation using CD44 MicroBeads, to identify putative stem cells. Selected cells were expanded in 100-mm culture dishes. CD44+ PDLSCs were cloned at passage 2. Nine clones were obtained and, among them, clone PDL5-F3 was randomly selected for subsequent characterization.

The PDL5-F3 cell line showed an elongated and fusiform, fibroblast-like shape, with a number of flattened cytoplasmic extensions (Figure 1 a). Immunocytochemical staining of CD44, observed in LSCM, has allowed the expression of the specific stemness marker on the PDL5-F3 cell surface to be assessed. As expected, it is clearly evidenced how the CD44 protein is presented over the membrane in the 100% of PDL5-F3 cells (Figure 1 b).

The ability of these cells to form adherent clonogenic cell clusters of fibroblast-like cells, with a typical fibroblastic spin-

dle shape, was shown by the formation of 27 ± 5 single colonies, generated from 10^2 single cells cultured at low density for 2 weeks, with a cloning efficiency of $27 \pm 5\%$ (Figure 1 c). On the contrary, the PDL5-F3 line did not show growth in soft agar after 4 weeks in culture, demonstrating no malignant transformation in cells (Figure 1 d).

In order to better characterize the PDL5-F3 line, cells were analyzed by flow cytometry. The phenotype analysis has revealed that PDL5-F3 cells expressed surface markers CD44, CD90, CD105, commonly used to identify mesenchymal stem cells, with a very close percentage to 100. In contrast,

the hematopoietic lineage marker CD45 was negative (Figure 2). Moreover, to further confirm that the cell line, used in the experiments, is not malignant, we assessed the principal tumor cell surface markers. The negative results, obtained for CD133, CD117, CD34 and CD271, together with the soft agar assay previously shown, were an additional proof that our cell line was not malignant (Figure 2).

Multipotentiality of PDLSCs

The multipotentiality of PDLSC cloned was verified by the induction toward the osteogenic and adipogenic phenotypes,

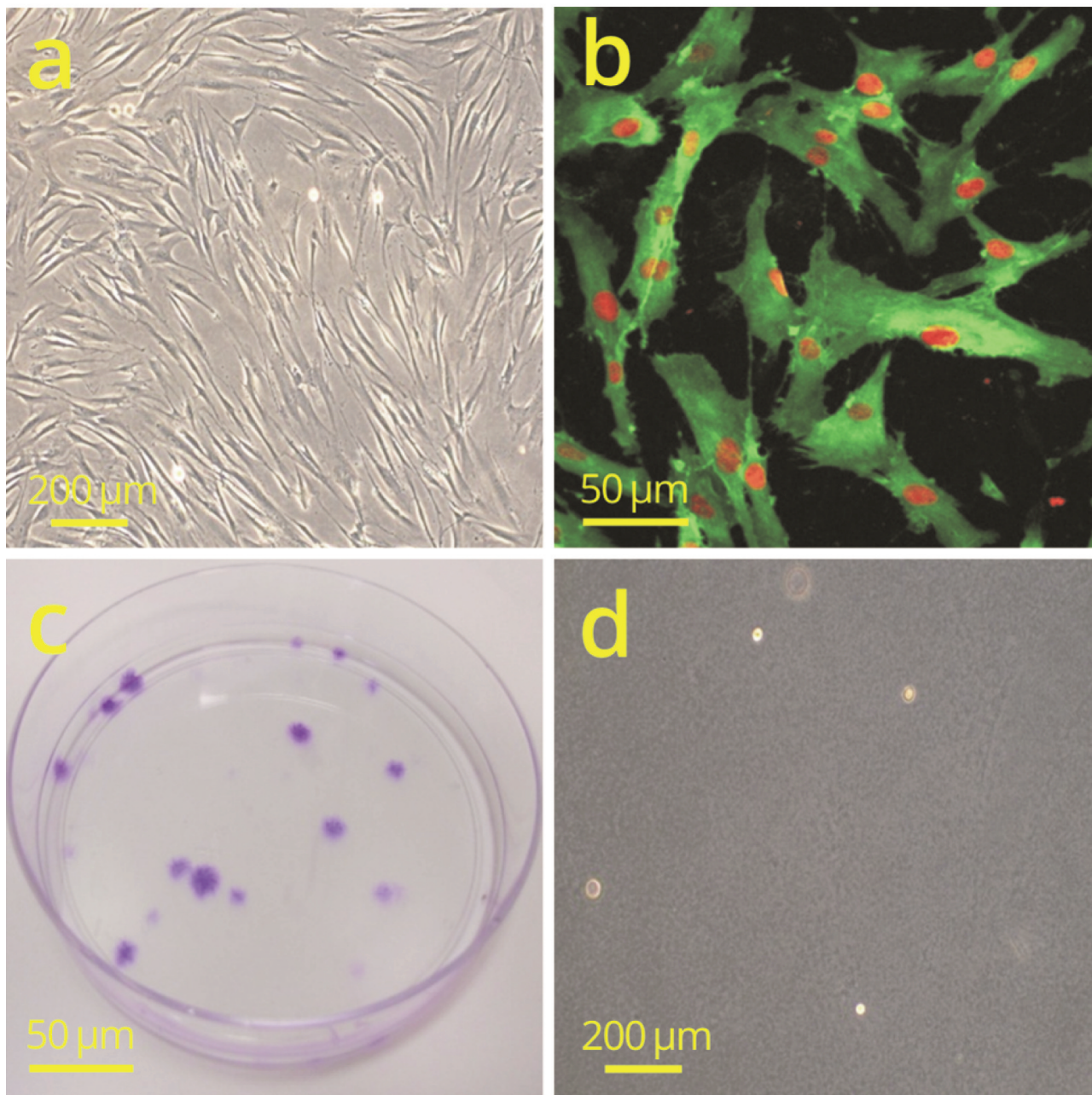


Figure 1 - Representative image from phase contrast microscopy of the PDL5-F3 clonal cell line grown on plastic (a); observation in LSC microscopy of CD44 marker (conventional green color) on PDL5-F3 and nuclei counterstained with propidium iodide (conventional red color), objective 20x (b); representative photo of CFU generated by PDL5-F3 cells (c); representative image from phase contrast microscopy of the PDL5-F3 cells grown in soft agar, objective 10x (d).

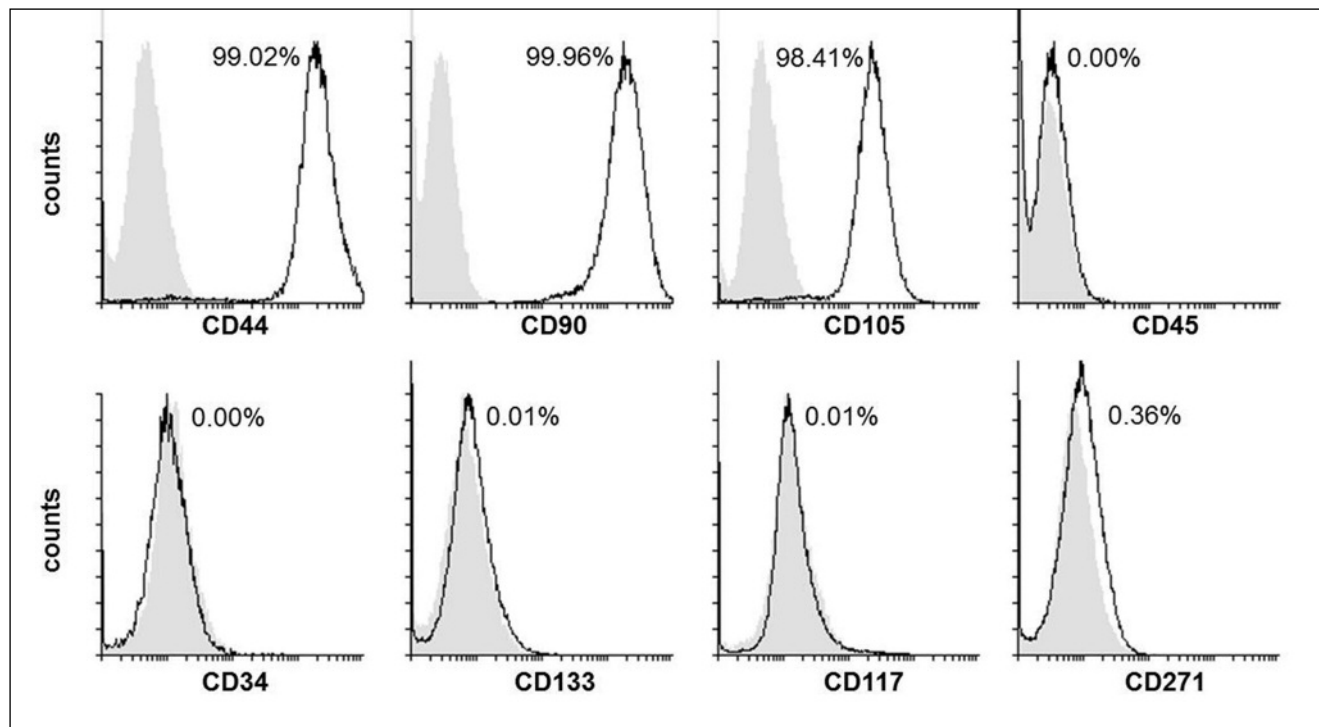


Figure 2 - Phenotype characterization of PDL5-F3 cells by flow cytometry. The overly plots show the percentage of PDL5-F3 cells expressing the surface markers CD45, CD105, CD90, CD44, CD34, CD133, CD117 and CD271 (open histograms); grey histograms: autofluorescence of unstained cells. One experiment representative of three independent ones.

using appropriate medium described in the ‘Materials and methods’ section.

Osteogenic induction of the PDL5-F3 line was assessed with OM up to 21 days and observed during the entire period of study, monitoring the ALP expression and the production of mineralized calcium deposits.

Cytochemical staining, performed at different time points (T_0 , 4, 7, 14 days), has revealed an increase in ALP activity with respect to the control incubated in GM, (T_0), as shown by brightfield microscopic images (Figure 3 a-d), highlighting the activation of the early biochemical marker of the differentiation process after only 4 days of induction. Subsequently, the ALP activity of PDL5-F3 cells underwent a decrease after 14 days of induction. Control cells grown in GM for the same time did not show any ALP+ cells.

In order to evaluate the effective deposition of an osteoid matrix by PDL5-F3 clonal cell line, cells were analyzed at different time points of osteogenic induction (T_0 , 7, 14, 21 days). The osteoblast activity of the cell line was evidenced by an increase of mineralized calcium nodules deposition, adding the fluorophore calcein in OM, yielding a great deposition 3 weeks after induction. Epifluorescence microscopic observations have shown calcein uptake starting from 14 days of induction (Figure 3e-h). In contrast, control cells grown in GM for the same time did not show any deposition of calcium deposits.

Adipogenic differentiation was performed culturing PDL5-F3 cells in AM for 21 days, and confirmed by Oil Red O staining of the multiple intracellular lipid-filled droplets accumulation and microscopic observations in brightfield control (Figure 4).

The same behavior, using OM or AM, was confirmed in the CD44+ PDL population, before cell cloning (data not shown).

Proliferation process of PDL5-F3 cells in presence of different concentrations of SrCl₂

We have evaluated the effects of different concentrations of SrCl₂ (0.5 -1 -5 -10 -25 -50 -100 -250 -500 µg/ml) on the proliferation process of human PDL5-F3 cells.

Figure 5 shows a significant increase in the incorporation of [³H]-thymidine when PDL5-F3 cells were treated for 24 hours with SrCl₂ at concentrations from 25 to 500 µg/ml, with respect to the control group in Coon’s modification medium with 0.1% FBS (with $p \leq 0.05$). In particular, this increase in proliferation rate was highest in PDL5-F3 cells stimulated with 100 µg/ml SrCl₂ (+37.7%) and lowest when PDL5-F3 cells were treated with 25 µg/ml SrCl₂ (+14.9%). On the contrary, lower concentrations of SrCl₂, from 0.5 to 10 µg/ml, did not have any effect on the proliferation process, given that we did not detect any increment of [³H]-thymidine incorporation with respect to the control 0.1% FBS.

Cell cultures, analyzed during proliferation in the presence of various concentrations of SrCl₂ for 24 hours and assessed by MTT assay, have shown a viability higher than 96% (data not shown) (Figure 5).

Mineralization process of PDL5-F3 in presence of different concentrations of SrCl₂

In order to evaluate the effect of SrCl₂ on the mineralization process of PDL5-F3 cells, osteogenic differentiation media with concentrations of SrCl₂ from 0.5 to 500 µg/ml were used and refreshed twice a week, for the entire study period (28 days).

Not surprisingly, the calcium content increased in a time-dependent manner in PDL5-F3 cultivated in OM, without SrCl₂, used as positive control (Figure 6).

Figure 6 shows that concentrations of SrCl₂ lower than 25

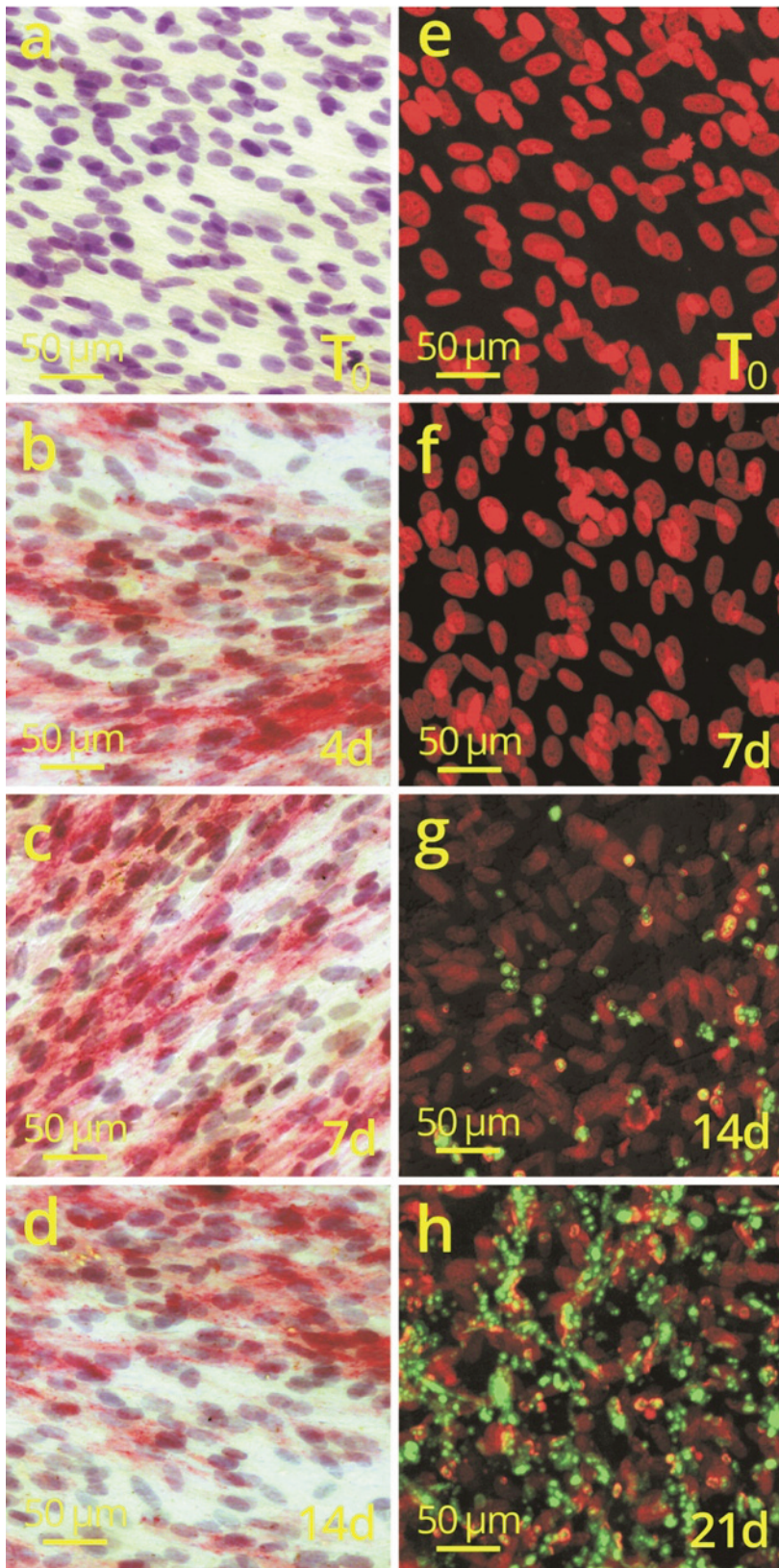


Figure 3 - Osteogenic phenotype evaluation of the PDL5-F3 cell line. a, b, c, d are representative images of Fast Red Violet BB staining at time 0 and after 4, 7 and 14 days of osteogenic induction; ALP+ cells are in red and nuclei counterstained with Mayer's acid hemalum in blue. Images acquired in brightfield microscopy. Objective 40x. e, f, g, h are representative images of calcein staining at time 0 and after 7, 14 and 21 days of osteogenic induction; mineralized calcium deposits are in fluorescent green and nuclei counterstained with propidium iodide in conventional red color. Images acquired in epifluorescence microscopy. Objective 40x.

µg/ml have caused significant increases in the production of mineralized calcium nodules already observed after 14 days of osteogenic induction and reached a maximum at 28 days,

compared to the not induced control. In particular, 28 days after induction, the minimum percentage increase of calcium deposits is valued at 10 µg/ml SrCl₂ (+71.6%) and the maxi-

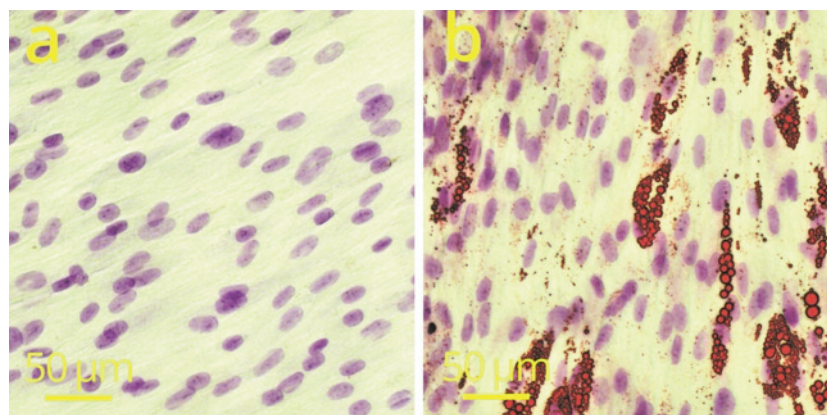


Figure 4 - Adipogenic phenotype evaluation of the PDL5-F3 cell line. Representative images of Oil Red O staining at time 0 (a) and after 21 days (b) of adipogenic induction; the intracellular droplets of lipids are in red, and nuclei counterstained with Mayer's acid hemalum in blue. Images acquired in brightfield microscopy. Objective 40x.

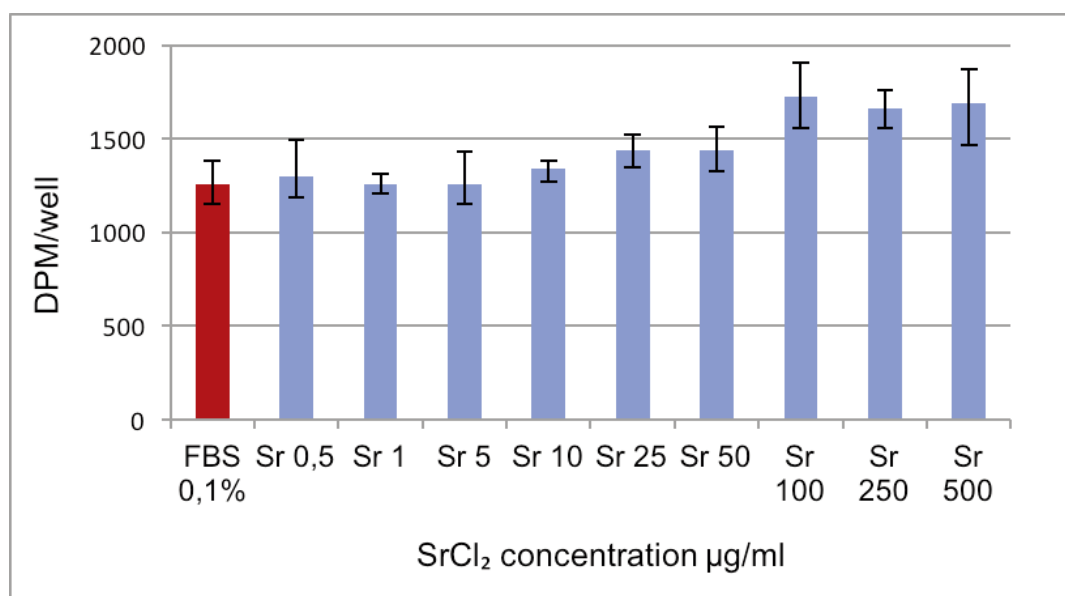


Figure 5 - ³H-thymidine incorporation in PDL5-F3 cell line assessed after 24h in presence of different concentrations of SrCl₂ (from 0.5 to 500 µg/ml). The values are the median of six independent experiments. Significance was obtained by Kruskal-Wallis Test, comparing the value of ³H-thymidine incorporation in presence of different SrCl₂ concentrations with respect to control without stimuli (FBS 0.1%). Radioactivity was expressed as disintegration per minute (DPM)/well. *p≤0.05 vs control. Whiskers represent a non-parametric 95% confidence interval.

mum at 0.5 µg/ml SrCl₂ (+153.5%). Concentrations of 25 and 50 µg/ml did not produce any significant increment of calcium deposition with respect to the osteogenic positive control during the entire study period. Otherwise, SrCl₂ concentrations of 100, 250 and 500 µg/ml affected mineralization after 28 days of osteoinduction, leading to a decrease in calcium deposits respectively of 29.8, 35 and 28%, compared to the osteogenic control.

Quantitative data reporting mineralization and Ca²⁺ deposition of PDLSCs, after 28 days of osteoinduction in presence of SrCl₂ treatments, were supported by epifluorescence microscopy observations (Figure 7). As shown in Figure 7, Ca²⁺ mineral deposits were increased, respect to the control group, in samples treated with SrCl₂ concentrations from 0.5 to 10 µg/ml; not differences were microscopically detected in samples treated with SrCl₂ 25 and 50 µg/ml, whereas in samples treated with concentrations from 100 to 500 µg/ml, consistent decrements in Ca²⁺ nodules were observed.

Discussion

Numerous studies have demonstrated that the human PDL contains a population of multipotent postnatal stem cells that can be expanded *ex vivo*, providing a unique reservoir of stem cells to be used for periodontal therapy (1, 13). Indeed, they have shown that PDL has a good potential of promoting regeneration of cementum and PDL-like tissues, as reported in *in vivo* animal models and in human case reports (1, 14-16). According to these findings, we have focused our study on supporting the efficacy of utilizing autologous PDL cells in the treatment of human periodontitis.

In our study, we successfully isolated PDLSCs from a human third molar, extracted for orthodontic purpose. Positive selection with CD44, transmembrane glycoprotein and marker of mesenchymal stemness, has permitted us to obtain and isolate putative stem cells that were highly enriched for this particular subset, which were used in our experiments (17, 18).

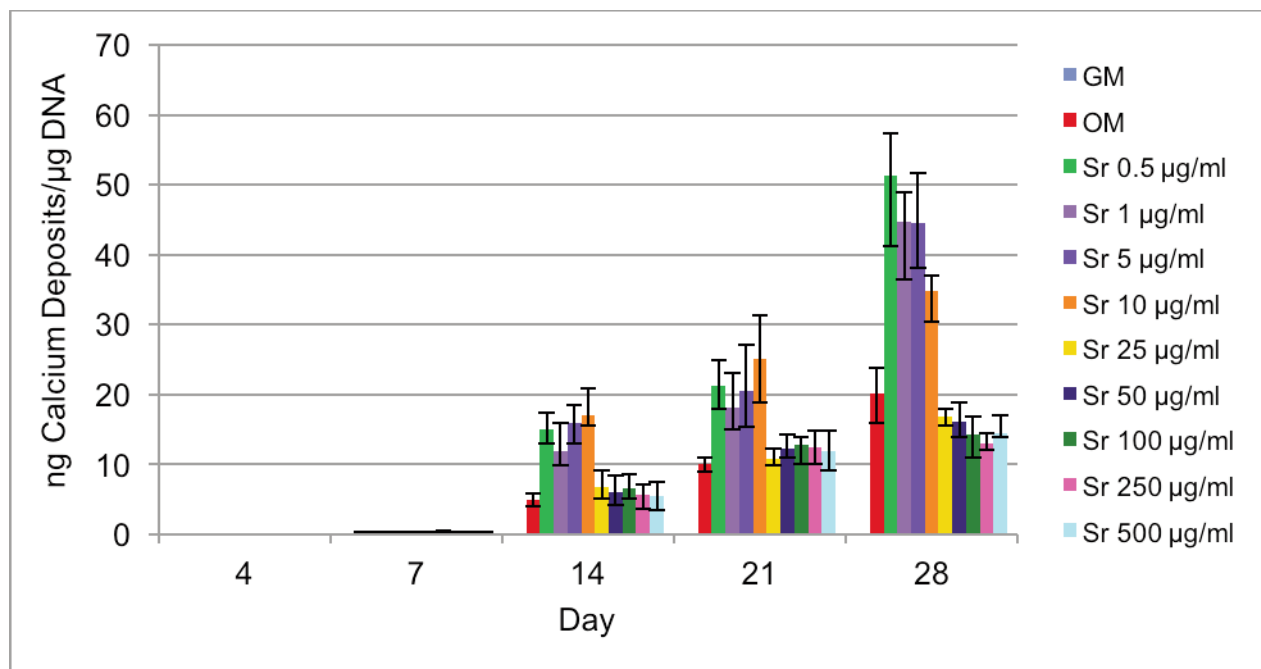


Figure 6 - Quantitative analysis of mineralized calcium deposits in PDL5-F3 induced in presence of different concentrations of SrCl_2 , from 0.5 to 500 $\mu\text{g/ml}$, up to 35 days. The values are the median of six independent experiments. The significance was evaluated by Kruskal-Wallis Test, comparing the value of calcium deposits in presence of different Sr^{2+} concentrations at different experimental points with respect to control group without stimuli. * $p \leq 0.05$ vs OM control at the same time; § $p \leq 0.01$ vs OM control at the same time. Whiskers represent a non-parametric 95% confidence interval.

Moreover, we have purposely chosen CD44 membrane marker for selecting PDLSCs because of this protein is largely known to be crucial for the proliferation and mineralization of PDL cells and may play multiple important roles in the biological function of periodontal regeneration (19, 20). Afterwards, we have established a CD44+ PDL clonal cell line, since this has permitted us to analyze a more homogeneous population and obtain experimental reproducibility, with the final aim to clarify the PDL-regenerative mechanism (21, 22).

The FACS analysis has assessed not only the mesenchymal origin but also the non-malignancy of our clone. In particular, the negative results obtained analyzing the main surface markers expressed within the majority of solid tumors (CD133, CD117, CD34 and CD271) (23-27), together with the cell inability to form colonies in soft agar (28), confirmed that no malignant transformation occurred during cloning of PDL cells.

In order to assess its potential and capability to differentiate into different lineages, we induced the clonal CD44+ PDL line toward adipogenic and osteogenic phenotypes. Both of the phenotypes were verified, confirming the efficacy of the selected clone for further experiments.

Advanced research has involved the combination of PDLSCs with growth factors such as BMP-2 (29), PDGF, IGF-1 (30-32) but also FGF-2 and VEGF-A (33) with the final aim to support the PDL tissue regeneration process.

In this study, we have focused our attention on strontium, an alkaline earth metal, since it promotes bone formation and decreases bone reabsorption. It has been reported that strontium preferentially stimulates cell proliferation of mesenchymal and osteoblastic cells (10, 34) and, because of the osteoblast-like characteristics of the PDL cells, we may con-

sider it an elective factor to be used in PDL regenerative therapy.

Literature reports the effects of strontium in PDL regeneration, demonstrating its ability to promote proliferation and osteogenic differentiation of PDLSCs (11, 12, 35, 36), but in these studies they have tested few and different concentrations of strontium and examined only a single process at once.

Therefore, our purpose was to assess whether and in which way the same concentrations of SrCl_2 (from 0.5 to 500 $\mu\text{g/ml}$) may influence both of the proliferation activity and the mineralization process of PDLSCs. It is desirable indeed, in order to assume the effective use of strontium in the PDL regeneration, to have a full knowledge of the effects that this bioactive ion may induce on PDLSCs, identifying the most active concentrations for proliferation and differentiation.

The obtained results showed that only the higher tested concentrations of strontium (from 25 $\mu\text{g/ml}$ to 500 $\mu\text{g/ml}$) can significantly affect the proliferation activity of PDLSCs, demonstrating a fast proliferative response during the first 24 hours of treatments with SrCl_2 . On the contrary, when we assayed the mineralization activity of PDLSCs in the presence of the entire range of strontium concentrations, we found that the ion induced a biphasic effect on this process. Specifically, with respect to the control group without treatments, low strontium concentrations, from 0.5 to 10 $\mu\text{g/ml}$, had stimulatory effects on calcium nodule deposition, whereas intermediate concentrations, from 25 to 50 $\mu\text{g/ml}$, induced no significant effects on mineralization. Higher concentrations, from 100 to 500 $\mu\text{g/ml}$, inhibit the deposition of mineralized nodules at 28 days after induction, probably due to the fact that these concentrations of strontium may disturb mineralization, interfering with the hydroxyapatite formation and physico-

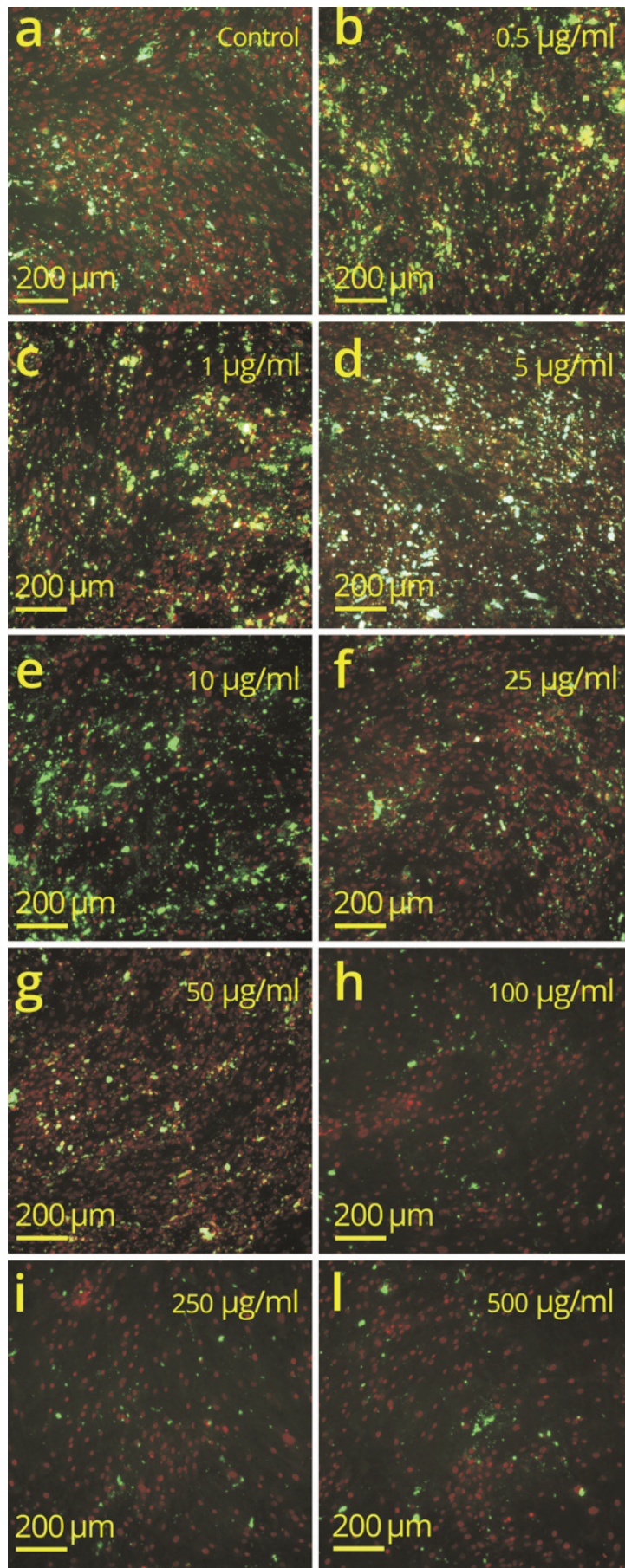


Figure 7 - Representative images of mineralized calcium deposits after 28 days of osteoinduction in absence of Sr-Cl₂ (OM) or in presence of different concentrations of the ion: control (a); 0.5 µg/ml (b); 1 µg/ml (c); 5 µg/ml (d); 10 µg/ml (e); 25 µg/ml (f); 50 µg/ml (g); 100 µg/ml (h); 250 µg/ml (i); 500 µg/ml (l). Mineralized calcium deposits, stained with calcein, are in fluorescent green and nuclei, counterstained with propidium iodide, in conventional red color. Images acquired in epifluorescence microscopy. Objective 10x.

chemical crystal properties (37, 38).

Our finding agree with previous data obtained by our research group, according to which strontium can distinctly induce proliferation or mineralization activity on human adipose tissue-derived mesenchymal stem cells, according to the used high or low concentrations (10).

Altogether, our results have provided evidence that the wide range of tested SrCl₂ concentrations can promote on PDLSCs either the one or the other process at different time points, underlining the complexity of the mechanism of actions of the ion, which, to date, is still unknown and subject to further investigation.

Indeed, it has been demonstrated *in vitro* in osteoblasts that strontium promotes extracellular-signal regulated kinase (ERK) phosphorylation via the calcium sensing receptor (CaSR), which in turn improves cell replications (39). This interaction with CaSR also resulted in activation of inositol triphosphate production and mitogen-activated protein kinase signaling and induction of cyclooxygenase-2 expression, which brought to an increase of prostaglandin-E₂ production (40-42). Furthermore, other mechanisms are also thought to be involved in regulation or mediation of mesenchymal and osteoblast cell replication via protein kinase C/D and p38 signaling pathways (43, 44). Recently, it was shown that the Wnt signaling pathway was involved in strontium-induced proliferation and differentiation of the murine osteoblasts (45). Moreover, it was found that strontium could enhance the osteogenic differentiation of mesenchymal stem cells, with upregulated extracellular matrix gene expression and activated Wnt/ β catenin pathway (9).

Despite the remaining questions regarding mechanism of action, our study demonstrates the importance of strontium on PDL proliferation/osteogenesis and confirms it as a promising bioactive factor in PDL regeneration. We have shown evidence of the proliferative and mineralization effects in strontium-exposed PDLSCs, but concentrations to obtain the specific event are opposite. Both observed phenomena offer desirable conditions for guided periodontal regeneration and might help to improve the outcome of periodontal regeneration therapy. In fact, incorporation of strontium into a delivery system that can release, with a control release profile over time, the appropriate concentration, chosen according to the specific biological response of PDLSCs that we want, might represent a possibility for developing innovative strategies capable not only of improving proliferation but also of increasing new bone formation. Thus, it is plausible that the use of strontium may become an elective treatment in many future options for a large variety of dental applications. Additional clinical studies are required to fully characterize the possible beneficial effects of strontium *in vivo*.

Conflict of interests

The Authors declare that there is no conflict of interest regarding the publication of this paper.

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