***In vitro* polydeoxyribonucleotide effects on human pre-adipocytes**

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Abstract. *Objectives:* Adipose tissue is the most abundant and accessible source of adult stem cells. Human processed lipoaspirate contains pre-adipocytes that possess one of the characteristic pathways of multipotent adult stem cells and are able to differentiate *in vitro* into mesenchymal and also neurogenic lineages. Because stem cells have great potential for use in tissue repair and regeneration, it would be significant to be able to obtain large amounts of these cells *in vitro*. As demonstrated previously, purine nucleosides and nucleotides mixtures can act as mitogens for several cell types. The aim of this study was to evaluate the effects of polydeoxyribonucleotides (PDRN), at appropriate concentrations, on human pre-adipocytes grown in a controlled medium, also using different passages, so as to investigate the relationship between the effect of this compound and cellular senescence, which is the phenomenon when normal diploid cells lose the ability to divide further. *Materials and methods:* Human pre-adipocytes were obtained by liposuction. Cells from different culture passages (P6 and P16) were treated with PDRN at different experimental times. Cell number was evaluated for each sample by direct counting after trypan blue treatment. DNA assay and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test were also carried out in all cases. *Results and Conclusions:* PDRN seemed to promote proliferation of human pre-adipocytes at both passages, but cell population growth increased in pre-adipocyte at P16, after 9 days as compared to control. Our data suggest that PDRN could act as a pre-adipocyte growth stimulator.

INTRODUCTION

Several researchers have shed new light on the importance of the action of extracellular nucleotides and nucleosides to increase cell proliferation and activity. Purine nucleosides and nucleotides in particular, bind to specific receptors triggering different signal transduction

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pathways. They act as mitogens for fibroblasts, endothelial cells and other cell types (Rathbone *et al.* 1992; Muratore *et al.* 1997; Thellung *et al.* 1999) and work synergistically with different growth factors (epidermal growth factor, platelet-derived growth factor, fibroblast growth factor) (Dingji *et al.* 1990; Chavan *et al.* 1994). Furthermore, polydeoxyribonucleotides (PDRN) are commonly used by plastic surgeons in pre-surgical cutaneous treatments, as they stimulate fibroblast metabolism, promoting both an increase in the number of fibroblasts and dermal matrix component production (Bigliardi 1982). Recently, the effect of PDRN has been analysed in a number of tissues, such as human corneal epithelium (Lazzarotto *et al.* 2004) and both rat and human bone (Bowler *et al.* 2001; Guizzardi *et al.* 2007), showing enhancement of tissue regeneration in all cases. In addition, PDRN leads to improvement in angiogenesis in burn wound treatment (Bitto *et al.* 2006) and activates the salvage pathway of nucleic acids to provide available nucleosides and nucleotides (Guizzardi *et al.* 2003). PDRNs are also able to influence immunological responses (Edgington 1992; Gailit & Clark 1994) and production of different cytokines and growth factors (Musk *et al.* 1989; Edgington 1992). They have offered a protective and regenerative effect on ultraviolet-irradiated mouse cell cultures (Henning *et al.* 1996) and also on human ultraviolet B-exposed dermal fibroblasts (Belletti *et al.* 2007).

Human stem cells represent an important starting point for carrying out medical and biological clinical studies, as they have a large potential for use in tissue repair and regeneration. Adipose tissue contains mesenchymal-like multipotent stem cells, pre-adipocytes, that can be maintained for long periods of time with low levels of senescence (Zu *et al.* 2002), and these possess multilineage potential *in vitro* (Gimble & Guilak 2003; Raposio *et al.* 2007) in the presence of lineage-specific induction factors. Our team is able to take a large volume of tissue from conventional liposuction procedures of healthy donors (standard procedures, low cell morbidity and minimal patient discomfort). Pre-adipocytes possess many characteristics that are present in bone marrow-derived mesenchymal stem cells, such as cell surface marker expression of CD105, CD90, CD71 and CD44 (De Ugarte *et al.* 2003). Similarly, they do not express the haematopoietic and endothelial markers, CD14, CD45 and CD31 (Gronthos *et al.* 2001; Astori *et al.* 2007). It is of great importance to be able to increase multipotent pre-adipocyte number *in vitro* to obtain a greater proportion of these cells. Thus, this review is a first simple step to verify and investigate the behaviour and characteristics of human pre-adipocytes at different passages *in vitro*, when treated with PDRN. Our choice to use pre-adipocytes is of interest as these cells are considered to be a new source for adult multipotent stem cell populations (Strem *et al.* 2005) and their final characterization is still in progress.

MATERIALS AND METHODS

Materials

Polydeoxyribonucleotide is product of Officina Bio-Farmaceutica Mastelli s.r.l. (Sanremo, Italy). The commercial preparation (Placentex Integro) contains 5625 mg of PDRN in a 3-mL ampoule sterilized at 121 °C for 20 min. PDRN is a pure substance with a titre superior to 95%, constituted of different lengths of PDRN (from 50 to 2000 base pairs) obtained from sperm of salmon trout (*Oncorhynchus mykiss*) for human alimentation, through an original purification and sterilization process. The other chemical reagents, unless otherwise specified, were from Sigma-Aldrich (St. Louis, MO, USA). All cell culture products, unless otherwise specified, were from Euroclone, Life Sciences Division (Pero, Italy).

Processed lipoaspirate cell processing and cell culture

Human adipose tissue was obtained from healthy donors during liposuction procedures under local anaesthesia. All procedures were approved by the Human Subject Protection Committee (Protocol HSPC 98-08-011-01) of Italy. Lipoaspirate samples were processed as previously described by Zuk *et al.* (2001) to obtain pre-adipocytes. Briefly, samples were repeatedly washed in Dulbecco's phosphate-buffered saline (PBS), to remove blood cells and anaesthetic. The extracellular matrix was digested with 2 mg/mL type I collagenase solution for 1 h at 37 °C, then was centrifuged to obtain the pellet which contains the stromal vascular components, including adipocyte progenitor cells, in addition to haematopoietic lineage cells. The pellet was then incubated with 160 mM ammonium chloride for 10 min, to lyse contaminating erythrocytes. The resulting cells were re-suspended in cell culture control medium [Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 1 : 1, 10% foetal bovine serum (FBS), 2 mM glutamine, penicillin (100 UI/mL), streptomycin (50 µg/mL), amphotericin B (2.5 µg/mL)], filtered through a 100-µm nylon mesh to remove cellular debris, and was incubated overnight at 37 °C in an atmosphere of 5% CO₂. After 24 h, cells were extensively washed in PBS and were maintained in control medium. Once arriving 80% confluence (after about 10 days culture), the cells were usually detached using trypsin 0.05%–sodium EDTA 0.02% solution and then were seeded in sterile flasks at a density of 2×10^4 per cm². The culture medium was generally replaced with fresh medium three times a week. Cells were observed microscopically, daily (Nikon Eclipse TS100; Nikon Corp., Tokyo, Japan).

Vitality evaluation

Vitality of the pre-adipocytes was analysed when the cells were at the 6th and 16th passages *in vitro*. Then, they were treated for 24, 48 and 72 h with 100 µg/mL and 80 µg/mL PDRN. The number of cells was evaluated for each sample by detaching with trypsin 0.05%–sodium EDTA 0.02% solution and counting using a Neubauer chamber and trypan blue solution. Pre-adipocytes were then plated in 96 multiwells (Corning Co., Corning, NY, USA), at a density of 5000 per well, in DMEM/Ham's F-12 (1 : 1) supplemented with 10% FBS. Forty-eight hours after seeding, cells were treated with PDRN (100–80 µg/mL) for the whole experimental time: 24, 48 and 72 h. At the end, cell viability was evaluated in both treated and untreated cultures by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test as described by Mosmann (1983). Briefly, pre-adipocytes were incubated for 3 h at 37 °C with 500 µg/mL MTT in serum-free medium. In viable cells, mitochondria reduce MTT to formazan that accumulates as blue crystals. These were dissolved by adding a solution of 1 N hydrogen chloride–isopropanol (1 : 24 v/v). Then, the plates were left at room temperature for 10 min, after which the absorbance of the product was measured spectrophotometrically at 570 nm.

MTT proliferation assay

Pre-adipocytes at the 6th and 16th passages *in vitro* were plated in 6 multiwells (Corning Co.) at a density of 10 000 per well, in DMEM/Ham's F-12 (1 : 1) supplemented with 10% FBS. Forty-eight hours after the seeding, cells were treated with PDRN (80 µg/mL) for 3, 6, 9, 12 and 15 days. During these steps, growth medium was changed every 2 days. After each experimental culture condition, cell proliferation was evaluated in treated and untreated cultures by MTT test, as described above.

DNA assay

Pre-adipocytes at the 6th and 16th passages *in vitro* were plated in 12 multiwells (Corning Co.), at a density of 5000 per well, in DMEM/Ham's F-12 (1 : 1) supplemented with 10% FBS. Twenty-four hours after seeding, cells were treated with PDRN (80 µg/mL) for 3, 6, 9, 12 and

15 days. In this period, growth medium was changed every 2 days. After each experimental culture condition, cell proliferation was evaluated in treated and untreated cultures by DNA assay. Cell proliferation index was observed as cellular DNA content, measured by the method of Rao & Otto (1992). At pre-determined time points, the experimental medium was removed; after washing with warm PBS, 1 mL of lysis solution [urea 10 M, 0.01% sodium dodecyl sulfate in saline sodium citrate buffer (SSC) 0.154 M NaCl, 0.015 M Na₃ citrate, pH 7] was added to each well. Dissolved cell suspensions were incubated at 37 °C in a shaking bath for 2 h, then 1 mL of Hoechst 33258 dye 1 µg/µL in SSC buffer was added in the dark. Absorbance was measured with an LS5 PerkinElmer spectrofluorometer (Norwalk, CT, USA) at excitation and emission wavelengths of 355 nm and 460 nm, respectively. Cell proliferation was estimated by referring fluorescence units to a linear standard curve for DNA fluorescence versus cell number.

Senescence-associated β -galactosidase assay

Pre-adipocytes at the 6th and 16th passages *in vitro* underwent β -galactosidase (β -gal) staining. In this assay, β -gal activity tallies with senescent cells at pH 6.0 (Dimri *et al.* 1995; Gary & Kindell 2005). When cells were subconfluent (1×10^6 cells per flasks), monolayers were repeatedly washed with PBS, fixed in 2% formaldehyde/0.2% glutaraldehyde for 3–5 min at room temperature and washed twice in PBS. Then, cells were incubated at 37 °C (without CO₂) with the senescence-associated β -gal staining solution: 1 mg of 5-bromo-4-chloro-3-indolyl β -D-galactoside (X-Gal) per millilitre in dimethylformamide; 40 mM citric acid/sodium phosphate (pH 6.0); 5 mM potassium ferrocyanide; 5 mM potassium ferricyanide; 150 mM sodium chloride; 2 mM magnesium chloride. The X-Gal solution had to be added just before use. After 2–4 h of incubation, it was possible to observe the blue-green staining, that was maximal after 12–16 h.

5-Bromo-2'-deoxyuridine staining

Pre-adipocytes at the 6th and 16th passages *in vitro* were seeded in sterile flasks at a density of 6×10^3 per cm² in DMEM/Ham's F-12 (1 : 1) with 10% FBS. Cell population growth and antibody staining for detection of 5-bromo-2'-deoxyuridine (BrdU) incorporation has been shown previously by Dolbeare *et al.* (1983). Briefly, when pre-adipocytes reached exponential growth, BrdU at a final concentration of 10 µM was added to the culture medium for 45 min at 37 °C in 5% CO₂. Then, cells were detached with trypsin 0.05%–sodium EDTA 0.02% solution, and were centrifuged and washed once with cold PBS. Pellets were gently re-suspended in 200–300 µL of PBS and cells were fixed by dropping them slowly into ice cold 70% ethanol; finally, they incubated at 4 °C for at least 30 min. Next, they were washed once with cold PBS and incubated for 30 min at room temperature with 2 N HCl/Triton X-100 to denature the DNA. After DNA denaturation, cells were permeabilized with 0.5% Tween-20 in PBS, and were incubated with anti-BrdU, pure diluted 1 : 10 (Becton Dickinson, NJ, USA) in Tween-20 0.5% in PBS plus 1% bovine serum albumin for 1 h at room temperature in the dark. Cells were washed with PBS and then incubated with secondary antibody (Alexa Fluor 488 F(ab') fragment of goat antimouse IgG (H + L), Invitrogen, Carlsbad, CA, USA) diluted 1 : 500 in Tween-20 0.5% in PBS, for 1 h at room temperature in the dark. Finally, cells were washed once with cold PBS, re-suspended in propidium iodide and RNasi solution (Becton Dickinson) and stored at 4 °C overnight in the dark. Samples were acquired by FACSCalibur flow cytometer (Becton Dickinson) and data were analyzed by Cell Quest software (Becton Dickinson).

Carboxyl fluorescein succinimidyl ester staining

Carboxyl fluorescein succinimidyl ester (CFSE) staining was performed as follows: 1 day before seeding, cells were washed three times with PBS and were incubated in the dark for 10 min at

37 °C in 5% CO₂ with 5 µM CFSE solution in PBS, then washed three times with PBS, added with 10% FBS (Urbani *et al.* 2006). Pre-adipocytes at the 6th and 16th passages *in vitro* were seeded in sterile flasks at a density of 4000 per cm² in DMEM/Ham's F-12 (1 : 1) with 10% FBS. Pre-adipocytes at different times after CFSE staining (1, 2, 5 and 9 days) were harvested using trypsin 0.05%–sodium EDTA 0.02% solution, centrifuged, and washed in PBS. Samples were acquired using a FACSCalibur flow cytometer (Becton Dickinson). FACS data analysis was performed using Cell Quest software (Becton Dickinson) and ModFit (Mac3.1 SP2, Verity Software House Inc., Topsham, ME, USA).

Immunocytochemistry

Pre-adipocytes, treated and untreated with PDRN (100 µg/mL) for 24, 48 and 72 h, were cultured using Laboratory-Tek™ II Chamber Slide™ System (Nunc A/S, Roskilde, Denmark) and were fixed with absolute methanol at –20 °C for 4 min. Monoclonal antihuman Ki-67 (BGX-Ki67 clone, 1 : 30, BioGenex, San Ramon, CA, USA), a cell proliferation marker, was used. Super Sensitive™ Link-Label Detection Systems Concentrated Format (BioGenex), avidin-biotin complex technique (Hsu *et al.* 1981; Van der Loos *et al.* 1989) and 3,3'-diaminobenzidine tetrahydrochloride (DAB chromogen, BioGenex) were used as the chromogen. Counterstaining with haematoxylin was performed to identify the cells.

RESULTS

Level of senescence of the pre-adipocytes was verified at the 6th and 16th passages *in vitro* by the senescence-associated β-gal assay (Fig. 1). The results obtained were: 4 senescent pre-adipocytes per 133 cells (3%) per mm² at the 6th *in vitro* passage and 7 senescent pre-adipocytes per 133 cells (5.2%) per mm² at the 16th *in vitro* passage. Our data represent means of three separate experiments in duplicate. The BrdU assay showed that only 1% (6th passage) and 2% (16th passage) of cells were in S phase, the greater amount of being in G₀ phase (Fig. 2). Pre-adipocyte proliferation was assessed at different time points, comparing the 6th and 16th passages. At 24 h after CFSE staining, both samples displayed only the parental peak. After 5 and 9 days, we confirmed modest proliferation activity; pre-adipocytes at the 16th passage showed virtually five generations but cells at the 6th passage possessed virtually six generations without parental peak (Fig. 3). The effect of PDRN treatment on pre-adipocytes at the 6th and 16th passages was demonstrated by our following experiments. Index of viability showed no toxic effects of PDRN for each dose (80 and 100 µg/mL) and experimental time (24, 48 and 72 h) (Table 1). The proliferation MTT test of human pre-adipocytes is shown in Figs 4 and 5. In these graphics, the values represent averages of three different experiments in quadruplicate and show that PDRN treatment had no significant effect on pre-adipocyte population growth at the 6th passage. On the other hand, PDRN treatment on pre-adipocytes at the 16th passage seemed to indicate an enhancement of growth rate compared to the untreated controls. We could also affirm that cell proliferation under the effect of PDRN showed a time-dependent response. After 15 days of treatment, proliferation of pre-adipocytes, both at the 6th and 16th passages, seemed to reach a saturation point, displaying a lower proliferative effect of PDRN. Moreover, we evaluated the cells using trypan blue staining in the same experimental conditions as the proliferation MTT test, as represented in Figs 6 and 7. We also employed DNA assay with 80 µg/mL PDRN on pre-adipocytes at the 6th and 16th passages for 3, 6, 9, 12 and 15 days to confirm results obtained previously, and values are shown in Figs 8 and 9. Cell proliferation was estimated

Figure 1. Senescence-associated β -galactosidase assay in pre-adipocytes at the 6th (a) and 16th (b) passages *in vitro*. The blue-green staining represents senescent cells (original magnification $\times 100$).

Table 1. Effect of the treatment with PDRN 100 and 80 $\mu\text{g/mL}$ for 24, 48 and 72 h on the viability of human pre-adipocytes to passages 6 and 16, as evaluated by MTT assay

Treatment	MTT P6			MTT P16		
	Control	PDRN (100 $\mu\text{g/mL}$)	PDRN (80 $\mu\text{g/mL}$)	Control	PDRN (100 $\mu\text{g/mL}$)	PDRN (80 $\mu\text{g/mL}$)
24 h	100	100 $\pm$ 0.1	100 $\pm$ 0.1	100	100 $\pm$ 0.5	100 $\pm$ 2
48 h	100	100 $\pm$ 0	100 $\pm$ 0.6	100	96 $\pm$ 1	100 $\pm$ 4
72 h	100	100 $\pm$ 0.5	100 $\pm$ 1	100	92 $\pm$ 1	98 $\pm$ 1

Values are expressed as percentage of the untreated controls, and represent the mean $\pm$ SEM of three separate experiments in quadruplicate.

Figure 2. BrdU staining in human pre-adipocytes at the 6th (P6) and 16th (P16) passages *in vitro*. The axis of graphics illustrate propidium iodide (PI) versus secondary antibody Alexa Fluor 488 F(ab') fragment of goat antimouse IgG (H + L); 1% pre-adipocytes at P6 and 2% pre-adipocytes at P16 are in S phase.

by referring fluorescence units to a linear standard curve for DNA fluorescence versus cell number of three different experiments in duplicate. After 3 days, the difference in growth rate between PDRN-treated and untreated cells was not relevant, and only on the 6th day did it reach significance between the two groups ($P < 0.05$), mainly at the 16th passage, as shown in Fig. 8. By immunocytochemistry, Ki-67 nuclear expression was prevalently displayed in the treated pre-adipocytes, in comparison with the untreated ones (Fig. 10). Bearing these results in mind, we counted numbers of mitoses in PDRN-treated pre-adipocytes and in controls by staining with haematoxylin and eosin. The results revealed that mitosis of pre-adipocytes treated with 80 $\mu\text{g/mL}$ PDRN was greater than that observed in control pre-adipocytes (data not shown).

Figure 3. CFSE staining in human pre-adipocytes at the 6th (P6) and 16th (P16) passages at different times (1, 2, 5 and 9 days).

Figure 4. Effect of PDRN on the growth of cultured human pre-adipocytes at the 16th passage *in vitro*. Cells were cultured in the experimental conditions in the absence and in the presence of 80 µg/mL PDRN for 3, 6, 9, 12 and 15 days. Values are expressed as an arbitrary unit (the absorbance was measured spectrophotometrically at 570 nm), and represent the mean $\pm$ SEM of three separate experiments in quadruplicate. * $P < 0.05$, compared to control by analysis of variance followed by Dunnett's test.

Figure 5. Effect of PDRN on the growth of cultured human pre-adipocytes at the 6th passage *in vitro*. Cells were cultured in the experimental conditions in the absence and in the presence of 80 µg/mL PDRN for 3, 6, 9, 12 and 15 days. Values are expressed as an arbitrary unit (the absorbance was measured spectrophotometrically at 570 nm) and represent the mean $\pm$ SEM of three separate experiments in quadruplicate.

Figure 6. Effect of PDRN on the growth of cultured human pre-adipocytes at the 16th passage *in vitro*. Cells were cultured in the experimental conditions in the absence and in the presence of 80 µg/mL PDRN for 3, 6, 9, 12 and 15 days. Cell counting was measured by Trypan blue staining. Values are expressed as a cell number and represent the mean $\pm$ SEM of three separate experiments in quadruplicate.

Figure 7. Effect of PDRN on the growth of cultured human pre-adipocytes at the 6th passage *in vitro*. Cells were cultured in the experimental conditions in the absence and in the presence of 80 µg/mL PDRN for 3, 6, 9, 12 and 15 days. Cell counting was measured by Trypan blue staining. Values are expressed as a cell number and represent the mean $\pm$ SEM of three separate experiments in quadruplicate.

DISCUSSION

As shown in previous pieces of research (Rathbone *et al.* 1992; Muratore *et al.* 1997, 2003; Guizzardi *et al.* 2003), PDRN can stimulate the growth of osteoblasts, fibroblasts and other kind of cells *in vitro*. PDRN, as described before, is a sterile, aprotogenic pure substance constituted of more than 95% (generally 99%) deoxyribonucleotide polymers of different lengths (from 50 to 2000 base pairs). It was observed by Guizzardi *et al.* (2003), Rubegni *et al.* (2001) and Sini *et al.*

Figure 8. Effect of PDRN on the growth of cultured human pre-adipocytes at the 16th passage *in vitro*. Cells were cultured in the experimental conditions, in the absence and in the presence of 80 μ g/mL PDRN for 3, 6, 9, 12 and 15 days. Cell proliferation was estimated by referring fluorescence units to a linear standard curve for DNA fluorescence versus cell number, and represent the mean $\pm$ SEM of two separate experiments in duplicate. * $P < 0.05$, compared to control by analysis of variance followed by Dunnett's test.

Figure 9. Effect of PDRN on the growth of cultured human pre-adipocytes at the 6th passage *in vitro*. Cells were cultured in the experimental conditions, in the absence and in the presence of 80 μ g/mL PDRN for 3, 6, 9, 12 and 15 days. Cell proliferation was estimated by referring fluorescence units to a linear standard curve for DNA fluorescence versus cell number, and represent the mean $\pm$ SEM of two separate experiments in duplicate. * $P < 0.05$, compared to control by analysis of variance followed by Dunnett's test.

(1999) that nucleotides and nucleosides may operate on cell proliferation in two different ways: they can stimulate nucleic acid synthesis through the salvage pathways (Pickard & Kinsella 1996; Nakamura *et al.* 2000) and they can bind and activate purinergic receptors (Rathbone *et al.* 1992; Thellung *et al.* 1999; Nakamura *et al.* 2000), in particular, P1 and P2 receptors, which bind nucleosides and nucleotides, respectively. In this report, we have discussed the PDRN effect on pre-adipocyte cell cultures. These types of cell are abundant and easily found

Figure 10. Immunostaining with Ki-67 antibody: (a) in untreated pre-adipocytes (original magnification $\times 600$) and (b) in pre-adipocytes treated with PDRN (100 $\mu\text{g/mL}$) (original magnification $\times 600$), as shown by the arrows.

after suction-assisted lipectomy (liposuction) as discarded tissue from surgical interventions. As mentioned above, pre-adipocytes are multipotent cells, differentiating *in vitro* along numerous lineages, such as adipocytes, chondrocytes, osteoblasts, myocytes, neuronal cells, pancreatic and hepatic lineages (Ashjian *et al.* 2003; Shi & Cheng 2004; Seo *et al.* 2005; Timper *et al.* 2006). For that reason, pre-adipocytes could be used in the future for cell therapy and tissue engineering (Katz *et al.* 1999); they could be employed in regeneration of damaged tissues and in treatment of several disorders (diabetes mellitus, musculoskeletal, nervous and hepatic diseases) in combination with appropriate biomaterials (Shaffler & Buchler 2007). We have analysed pre-adipocytes at different passages *in vitro* (P6 and P16) as we wanted to understand the effect of PDRN on early and mature cells. Cellular senescence is a phenomenon which develops when normal diploid cells lose the ability to divide. In general, mesenchymal stem cells, like pre-adipocytes, have low levels of senescence (Zuk *et al.* 2001; Mizuno & Hyakusoku 2003). By the β -galactosidase test, we observed an increase in positive cell numbers from 6th to 16th passages. Measurement of cell proliferation using BrdU was carefully examined by multicolour flow cytometry and microscopic analysis (Forster *et al.* 1989; Brockhoff *et al.* 2001; Leif *et al.* 2004; Rothausler & Baumgarth 2006). BrdU staining has been applied in different cell lines used for immunocytochemistry and immunofluorescence (Coltrera & Gown 1991), it becomes incorporated into nuclear DNA of dividing cells (Gratzner 1982). We verified that there was no a significant difference between amounts of cells in G_0 and S phases between pre-adipocytes in

the two passages. In our opinion, S phase was restricted because our cells originated from adult adipose tissue and were not stabilized cell lines. A further test to determine proliferation of pre-adipocytes at the 6th and 16th passages was CSFE staining. The CSFE method has previously been applied for labelling human pre-adipocytes (Hemmrich *et al.* 2006) leucocytes and lymphocytes (De Clerck *et al.* 1994; Urbani *et al.* 2006). CSFE has the ability to covalently label long-lived intracellular molecules with the fluorescent dye, carboxyfluorescein. Following each cell division, the equal distribution of this fluorescent molecule to progeny cells results in halving of the fluorescence to each daughter cell (Quah *et al.* 2007). Our more significant discovery was that pre-adipocytes at the 6th passage showed six generations after 9 days of treatment and at the same time, we verified the loss of a parental peak. Otherwise, pre-adipocytes at the 16th passage had five generations only, and we found parental peak yet. According to these considerations, we could affirm that pre-adipocytes at the 6th passage possess a higher capability of proliferation. Therefore, we thought to use these different passages for our studies. At the beginning, we observed that the effect of PDRN (80–100 µg/mL) on our cells was not toxic during the exposure time (24, 48 and 72 h). According to these considerations, we decided to use only one PDRN concentration (80 µg/mL) in subsequent analysis and at the same time, to extend treatment up to 15 days. However, on the contrary to what we thought at the beginning of this work, pre-adipocytes at the 16th passage seem to grow more than pre-adipocytes at the 6th passage. In the MTT test, this enhancement is clearer to the 12th day, while in the DNA assay the point of saturation of pre-adipocyte proliferation seems to be 9 days. Similar results were obtained by corresponding cell counting. The answer to this strange behaviour is perhaps connected to a major adaptation of cells at the 16th passage in these culture conditions and external stimulation. The fibroblast-like morphology of treated and untreated cells at all passages is similar; finally, by immunocytochemical analysis, we have found a prevalent presence of Ki-67 in PDRN-treated pre-adipocytes, indicative of cell proliferation. At the same time, we observed an increase in mitoses in PDRN-treated pre-adipocytes. According to these considerations, we could deduce that both provided evidence for enhancement in proliferation in treated cells. The Ki-67 is a 360-kDa protein which is expressed by proliferating cells in all phases of the active cell cycle (G_1 , S, G_2 and M phases). In G_1 phase, the Ki-67 antigen is predominantly localized in the perinucleolar region, but in the later phases of the cell cycle it is also detected throughout the nuclear interior, being predominantly localized in the nuclear matrix. In mitosis, the Ki-67 antigen is present in all chromosomes and appears in a reticulate structure surrounding metaphase chromosomes (Bullwinkel *et al.* 2006). Thus, presence of the Ki-67 antigen is strictly associated with the cell cycle and is confined to the nucleus, suggesting an important role of this structure in the maintenance and/or regulation of the cell division cycle. From analysis of our data, we can conclude that PDRN also can stimulate proliferation of pre-adipocytes. The effect of PDRN does not seem to be so remarkable; perhaps it is due to the origin of our particular cells, that is, adult adipose tissue. In our opinion, this research is a good starting point for further investigation of differentiated pre-adipocytes in various lineages, such as hepatic and pancreatic. In future, we propose to study pre-adipocyte cells at passages above the 16th *in vitro*, to evaluate and to confirm our hypothesis.

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