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Effect of long-term cultivation on morphological and biological characteristics of human periodontal ligament stem cells

Michal Straka [1,#], Ida Kupcová [2,#], Ivan Varga [3], Andreas Nicodemou [4], Zuzana Varchulová Nováková [4], Ľuboš Danišovič [4,5]

1 Department of Dentistry, Faculty of Medicine, Slovak Medical University, Bratislava, Slovak republic
2 Department of the Oromaxillofacial Surgery, Faculty of Medicine, Comenius University, Bratislava, Slovak Republic.
3 Institute of Histology and Embryology, Faculty of Medicine, Comenius University, Bratislava, Slovak Republic
4 Institute of Medical Biology and Genetics, Faculty of Medicine, Comenius University, Bratislava, Slovak Republic
5 REGENMED Ltd., Bratislava, Slovak Republic.

[#]*These authors contributed to the work equally and should be regarded as co-first authors*

Correspondence to: Dr. Ľuboš Danišovič, PhD., Institute of Medical Biology and Genetics, Faculty of Medicine, Comenius University, Sasinkova 4, 811 08 Bratislava, Slovak republic; TEL.: +421259357215, E-MAIL: lubos.danisovic@fmed.uniba.sk

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Key words: **human periodontal ligament stem cells; long-term cultivation; morphological changes; immunophenotype; cell cycle analysis; telomerase activity**

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Abstract

OBJECTIVES: Recently, it was demonstrated that human periodontal ligament stem cells have great potential for tissue engineering and regenerative medicine not limited to oro-maxillofacial region. They are easily accessible and they may be expanded under *in vitro* conditions. In this study we assessed the effect of long-term cultivation on the selected biological and morphological properties of human periodontal ligament stem cells.

METHODS: Periodontal tissues were obtained from normal impacted third molars of healthy donors (n=5; aged 18–27 years), after obtaining informed consent. The explant technique was used to initialize cell culture and further expansion *in vitro* was carried out in complete culture medium (D-MEM + 10% foetal bovine serum + gentamicin) with passaging in 80% of confluence using trypsine up to 25th passage. Cells were continually analyzed for morphology changes by inverted and transmission electron microscope. The analysis of selected biological characteristics (expression of surface antigens and selected genes involved in cell regulation and apoptosis, cell cycle analysis and cell senescence) were performed, as well.

RESULTS AND CONCLUSIONS: Obtained results showed that long-term cultivation lead in to considerable changes in morphology and affect the proliferation and cell cycle of human periodontal ligament stem cells. On the other hand, it did not affect their immunophenotype as well as function of cell cycle, apoptosis regulators and telomerase activity also in high passages. However, further studies considering stem cells bio-safety have to be carried out prior their clinical application.

ORIGINAL ARTICLE

Abbreviations

periodontal ligaments – PLs;
human periodontal ligament stem cells – hPLSCs;
mesenchymal stem cells – MSCs;
human adipose tissue-derived stem cells – hATSCs;
Dulbecco´s minimum essential medium – D-MEM;
foetal bovine serum – FBS;
phosphate buffered saline – PBS;
Transmission electron microscope – TEM

INTRODUCTION

The periodontal ligaments (PLs) belong to specialized connective tissue composed predominantly of collagen fibers with small amount of ground substance and cells. PLs are localized in highly vascularized space between the cementum (surface layer of the tooth root) and the inner wall of the alveolar bone socket. They play pivotal role in sustaining and helping to constrain teeth within the jaw maintaining tooth nutrition and homoeostasis (Mantesso & Sharpe, 2009).

From embryological point of view PLs originates from neural crest and contain very heterogeneous population of cells, including fibroblasts, osteoblasts and cementoblasts (Chai *et al.* 2000). Moreover, several investigations have been carried out to provide evidence about presence of multipotent stem cells within PLs, which are involved in processes of maintaining homeostasis and promoting regeneration of periodontal tissue (Park *et al.* 2011; Wang *et al.* 2011). It was also shown that periodontal ligament stem cells (PLSCs) have similar characteristics as stem cells obtained from different sources (e.g. bone marrow, adipose tissue, placenta and cord blood) including long-term self-renewal, plasticity, increased proliferation activity and ability of active migration into damaged tissue (Laitinen *et al.* 2016; Zachar *et al.* 2011). Therefore, they gradually became object of interest in respect to regenerative medicine, not limited only to oro-maxillofacial region (Yam *et al.* 2015; Mayo *et al.* 2014; Huang *et al.* 2009).

It have been demonstrated that PLSCs may be relative easily obtained from PLs by explant outgrowth or enzymatic digestion followed by subsequent expansion *in vitro*. Two media have been mostly used for human PLSCs (hPLSCs) cultivation without negative effect on their biological characteristics – α-minimum essential medium and Dulbecco´s minimum essential medium (D-MEM) containing L-glutamine and L-ascorbic-acid-2-phosphate (Zhu and Liang 2015). Young *et al.* (2013) showed that hPLSCs maintain their properties within 8 passages. However, several authors, including our group, provided information about alterations in the morphology, cytogenetic stability or proliferation activity in different mesenchymal stem cells (MSCs). Moreover, there are also several evidences about negative effect on regulation of cell cycle, DNA repair and apoptosis or expression of immune genes (Jayaraman *et al.* 2016; Varga *et al.* 2015; Froelich *et al.* 2013).

In the present study, we isolated hPLSCs and expanded them *in vitro* up to 25th passage. We investigated changes of the morphological and selected biological properties, including expression of surface antigens and selected genes involved in cell regulation and apoptosis, cell cycle analysis and cell senescence.

MATERIAL AND METHODS

Normal human impacted third molars (n=5) were collected from healthy patients (aged 18-27 years) after obtaining informed consent in accordance with Helsinki Declaration. Freshly extracted teeth were stored in serum-free D-MEM (Sigma-Aldrich, USA) with gentamicin (Lek, Slovenia) and were immediately transported to the laboratory for processing. First, PLs were removed from teeth and were carefully rinsed with phosphate buffered saline (PBS), followed by cutting PLs into small fragments (approximately 2×2 mm) and placed in a Petri dish (6 mm, TPP, Swiss) with minimal amount of complete culture medium consisted of D-MEM, 10% fetal bovine serum (FBS, PPA, Austria) and gentamicin at final concentration of 80 μg/ml. After 2 hours of incubation at 37 °C in 5% CO2, 3 ml of complete culture medium was carefully added, to not release attached tissues from the Petri dish. Culture medium was refreshed every 3 days until sub confluence. After hPLSCs reached sub confluency, they were passaged using 0.25% trypsine (Sigma-Aldrich, USA) up to 25th passage, always at the cell density of 5000 cells/cm2.

To asses the cell surface antigens of hPLSCs the flow cytometry was used. Cells were detached by 0.25% trypsin digestion and then resuspended in blocking buffer consisting of PBS with 0.5% bovine serum albumin (PAA, Austria). The MSC Phenotyping kit (Miltenyi Biotec, Germany) containing antibodies against human CD14, CD20, CD34, CD45, CD73, CD90 and CD105 were used in all experiments. Cell suspensions were processed according to protocol provided by manufacturer. The samples were analyzed by MACSQuant® Analyzer (Miltenyi Biotec, Germany).

The morphology of vital hPLSCs was continually analyzed during cultivation using an inverted microscope Zeiss AxioVert A1 FL LED (Carl Zeiss, Germany). The ultra-structure of hPLSCs was analyzed by transmission electron microscopy (TEM). Cells appointed for TEM were fixed in 2.5% glutaraldehyde

Table 1. Primers and probes used in Quantitative real-time polymerase chain reaction.

Target Gene	Acession Number of TaqMan®Gene Expression Assay
TP53	Hs01034249_m1
Bcl-2	Hs00608023_m1
CDK1	Hs00938777_m1
GAPDH	Hs03929097_g1

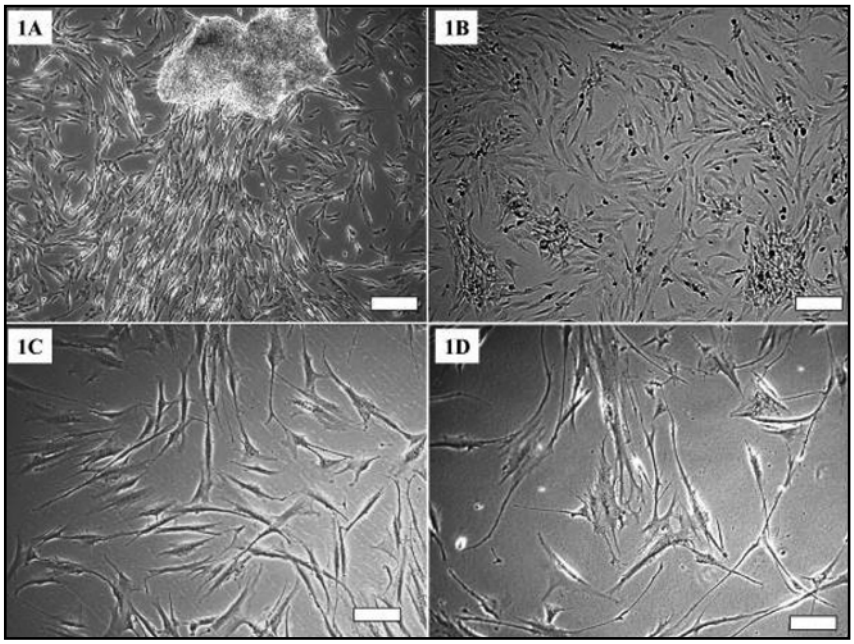

Fig. 1. Representative micrographs presenting the morphology of hPLSCs during long-term cultivation. (A) Outgrowth of hPLSCs from fragment of periodontal ligament (scale bar = 100 µm). (B) Proliferation of hPLSCs in P5 (scale bar = 100 µm). (C) Changes of hPLSCs morphology in P15 (scale bar = 20 µm). (D) The morphology of hPLSCs during termination of experiment, in P25 (scale bar = 20 µm).

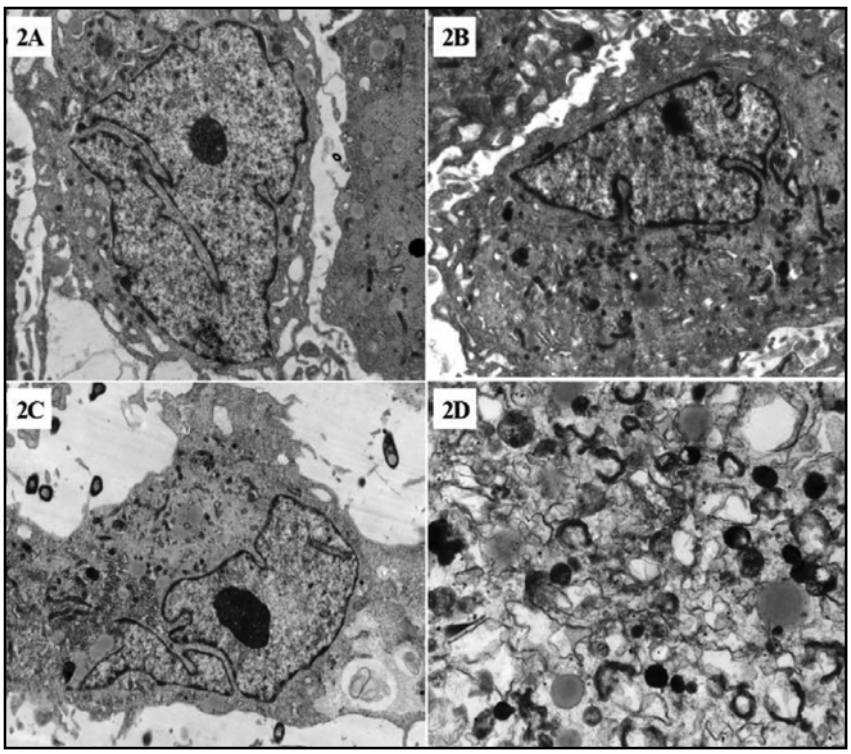

Fig. 2. Ultrastructural analysis by transmission electron microscope. (A) hPLSCs in P5. Cells contain large and pale nucleus (with predominance of euchromatine) with noticeable nucleolus and relatively small amount of cytoplasm (the nucleus-cytoplasm ratio is 4:1). Cell nuclei have deep invaginations for the increase of the surface between the nucleus and cytoplasm. The cytoplasm contains relatively less organelles. Orig. Magn. 11,000x. (B) hPLSCs in P15. The nucleus-cytoplasm ratio decreases. Based on the morphology of the nucleus (predominance of euchromatine and large nucleolus) and presence of deep invaginations of the nuclear envelope the process of transcription is in high level. The cytoplasm contains numerous cell organelles important for proteinsynthesis, but also dark (electron-dense) residual bodies. Orig. Magn. 7,100x. (C) hPLSCs in P25. The nucleus is still very active and contains extremely large compact type of nucleolus (typical for stem cells but also malignant cells). The cytoplasm is filled by lipid droplets and residual bodies. Orig. Magn 5,600x. (D) Detail of cytoplasm of hPLSCs in P25. The whole cytoplasm is filled by lipid droplets, dark residual bodies, lipid bodies ("worm like bodies") and myelin figures ("zebra bodies"). Orig. Magn. 11,000x

(Sigma-Aldrich, USA), pH 7.2, at 4 °C for 2 hours. After fixation, cells were carefully rinsed by PBS and post-fixed with 1% osmium tetroxide (Serva, Germany) for 2 hours, then rinsed in distilled water and dehydrated in a graduated series of ethanol (Centralchem, Slovakia). Subsequently, the samples were embedded in Durcupan (Sigma Aldrich, USA) and cut into semi-thin sections. The obtained sections were stained by toluidine blue (Sigma Aldrich, USA) for 10 minutes, and minced into ultra-thin sections. Then, they were mounted on 200 mesh copper grids, double stained using uranyl acetate and lead citrate (Sigma-Aldrich, USA) and examined using a TEM FEI Morgagni 268D (FEI, USA).

In vitro expanded hPLSCs were analyzed using Muse® Cell Cycle Assay Kit (Merck Millipore, USA) in every 5th passage. All procedures were performed according to the protocol provided by manufacturer. Briefly, cells were detached with 0.25% trypsine and centrifuged (1200 rpm, 6 min.). Obtained cells were washed with PBS and then fixed with cold ethanol. Prior to analysis, hPLSCs were treated with Muse Cell Cycle Reagent (Merck Millipore, USA) for 30 minutes. Analyses were

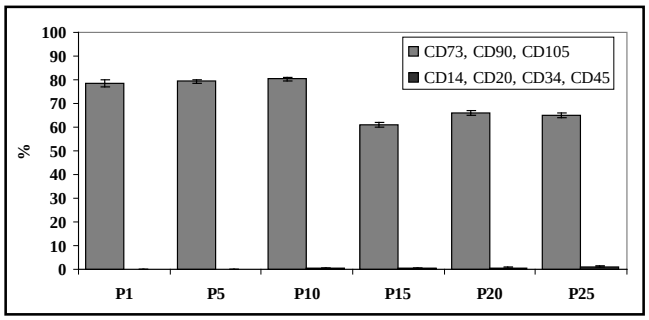

Fig. 3. Flow cytometry revealed the expression of MSCs markers (CD73, CD90 and CD105) in hPLSCs during long-term cultivation. They lack expression o hematopoietic and endothelial cell (CD14, CD20, CD34 and CD45).

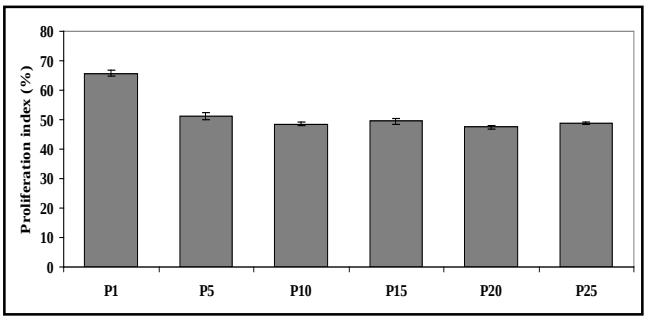

Fig. 4. Proliferation index in hPLSCs during long-term cultivation.

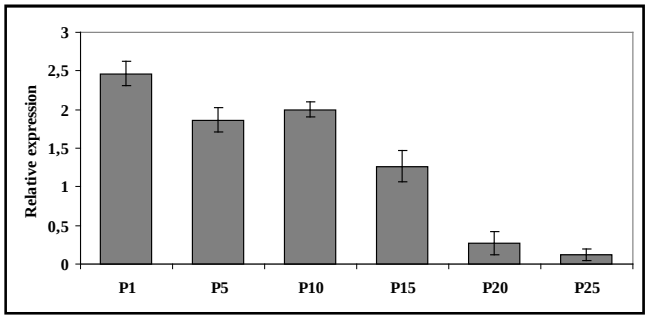

Fig. 5. Telomerase activity in hPLSCs during long-term cultivation.

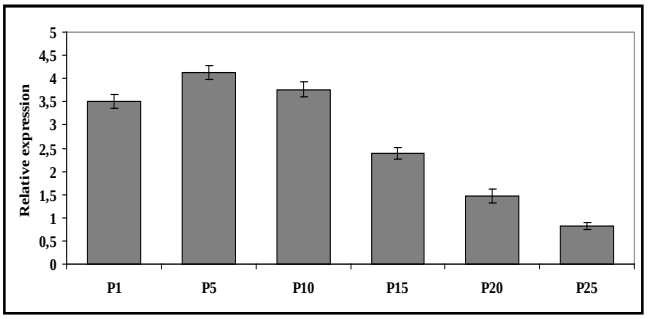

Fig. 6. The expression of CKD1 in hPLSCs during long-term cultivation.

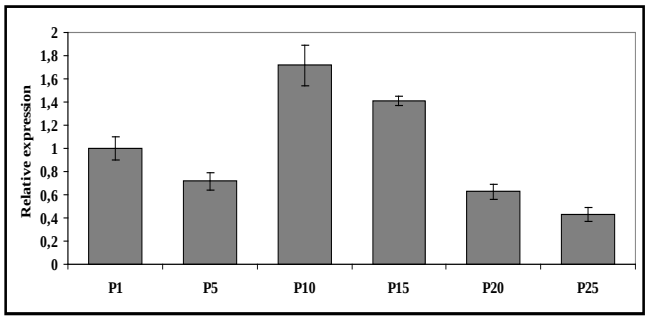

Fig. 7. The expression of Bcl-2 in hPLSCs during long-term cultivation.

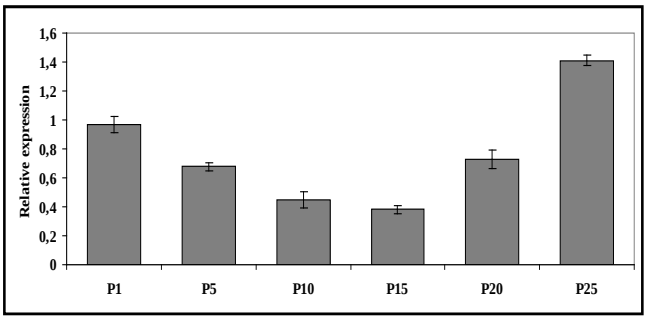

Fig. 8. The expression of TP53 in hPLSCs during long-term cultivation.

performed by Muse Cell Analyzer (Merck Millipore, USA). The proliferation index (PI) was calculated using the formula: PI (%) = (S + G2/M)/(G0/G1 + S + G2/M) × 100% (Waladali *et al.* 2009).

Detection of telomerase activity of hPLSCs was continually analyzed by using TRAPeze® RT Telomerase Detection Kit (Merck Millipore, USA) according to the manufacturer's recommendations. The cells were resuspended in 200 µL CHAPS Lysis Buffer (Merck Millipore, USA) and incubated on ice for 30 minutes. Subsequently, cells were centrifuged at 12000 rpm for 20 min. at 4 °C. 160 µL of the supernatant was transferred into a tube and the protein concentration was determined. Finally, the "Master Mix" was prepared by mixing all reagents with sample. The PCR reactions were performed on Eco Real-Time PCR System (Illumina, USA).

Total RNA was extracted from hADSCs in every passage using GeneJET RNA Purification Kit (Thermo Scientific, USA) according to the manufacturer's protocol. First-strand cDNA was prepared from total RNA with Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific, USA). The reaction was carried out according to protocol recommended by manufacturer. The thermal cycling conditions were composed of 25 °C for 10 min., 50 °C for 15 min. and 85 °C for 5 min. Obtained cDNA was used as a template for quantitative real-time PCR to determine the expression level of the selected genes associated with senescence, regulation of cell cycle and apoptosis, including CDK1, TP53 and Bcl-2. Glyceraldehyde-3-phosphate dehy-

drogenase (GAPDH) was used as housekeeping gene. All primers used in this study are listed in *Table 1*. The PCR reactions were performed on Eco Real-Time PCR System. The reaction was performed in 5 μl mixture consisted of 2.5 μl Maxima Probe/ROX qPCR Master Mix (2x) (Thermo Scientific), 0.25 μl of each primer – TaqMan®Gene Expression Assay (Applied Biosystems), 0.5 μl of cDNA and the rest of the reaction volume was adjusted with water. The thermal cycling conditions were composed of 50 °C for 2 min. followed by an initial denaturation step at 95 °C for 10 min., 45 cycles at 95 °C for 15 s., 60 °C for 1 min. Expression of all analyzed genes was normalized to GAPDH.

RESULTS

Cells started to grow from PLs at approximately day 4 to 8. They had heterogeneous morphology, from trigonal to spindle-shaped (fibroblast-like) morphology (*Fig. 1A*). After passaging hPLSCs proliferated in colonies (*Fig. 1B*) and during subsequent passages they gradually changed their morphology; the cells had irregular shape with prolonged processes (*Figs. 1C and 1D*).

Transmission electron microscopic ultrastructural analysis showed that the cell nuclei have the same morphology in all passages. The nuclei contain predominantly euchromatine and well developed compact type nucleolus (*Fig. 2A–2C*). The nuclear envelope creates deep invaginations, probably for increase of the surface between the nucleus and cytoplasm. The ultrastructure of nuclei suggests extremely high level of transcription and is typical for stem cells (but also for malignant cells). At the first passages the nucleus – cytoplasm ratio was 4:1, what is typical for non-differentiated cells (e.g., stem cells). In higher passages this ratio decrease at level 2:1 or 1:1. The cytoplasm in first passages contains fewer organelles. In the highest passages accumulation of lipid droplets, residual bodies, storage vesicles, myelin-like figures (so-called zebra bodies) and lipid bodies (with worm-like shape) create the largest part of cytoplasm (*Fig. 2B*).

As shown in Figure 3, immunophenotypic analysis of hPLSCs was positive for CD73, CD90 and CD105 and did not express CD14, CD20, CD34 and CD45. The expression of CD73, CD90 and CD105 slightly decreased in late passages (P15–P25).

The analysis of cell cycle phase's distribution and subsequent calculation of proliferation index showed that majority of hPLSCs were characterized by high proliferation activity in P1. In subsequent passages (P5 –P25), the proliferation index was slightly decreased, and showed similar values until the end of the experiment in P25 (*Fig. 4*).

TRAPeze assay demonstrated that hPLSCs gradually lost the telomerase activity in passage increasing manner (*Fig. 5*).

The analysis of CDK1 expression in hPLSCs (*Fig. 6*) showed increased expression between P1 and P10 and minimum levels in the later passages (P20-P25). The expression of Bcl-2 reached highest levels in P10 and P15, while during the termination experiment the expression reached low values (*Fig. 7*). The expression of TP53 is presented in *Figure 8*. Its expression gradually decreased from initiation the cultures to P15, but increased in higher passages P20 and P25.

DISCUSSION

Recently, PLs represent promising and easy accessible source of adult stem cells. As other adult stem cells, hPLSCs possess unique characteristics such as long-term self-renewing, plasticity and active migration to diseased tissues. Moreover, they have immunomodulatory potential. Several studies support their potential for tissue engineering and regenerative medicine (Amrollahi *et al.* 2016; Chen *et al.* 2016; Ji *et al.* 2013). The most important prerequisite of their translation to clinical practice is obtaining their therapeutically efficient number by expansion under proper *in vitro* conditions. The time of expansion seem to be important because several studies reported changes of biological properties of stem cells during prolonged cultivation (Nava *et al.* 2015; Froelich *et al.* 2013). Our study was therefore aimed at comprehensive analysis of selected biological and morphological properties of hPLSCs in long-term cultivation system.

The explant technique was used to initialize culture of hPLSCs in combination with D-MEM and FBS. Similar approach was used in many previous experiments to obtain hPLSCs with high proliferation activity and properties typical for other MSCs (Chen *et al.* 2013). The culture medium, which is often ignored, may affect the biological properties of hPLSCs. In our study, we used D-MEM, which belong to most used to maintain stem cell phenotypes (Zhu and Liang 2015). Our cells start to migrate from fragments about day 6 and they have to be sub-passaged during next 7–12 days. Indeed, that proliferation activity was maintained up to the end of the experiment. To verify their MSCs phenotype as well as to record any changes through prolonged cultivation, the cells were characterized by flow cytometry. They were positive for markers of mesenchymal cells CD73, CD90 and CD105; and did not express CD14, CD20, CD34 and CD45 typical for haematopoietic and endothelial cells (Dominici *et al.* 2006). We did not record any significant alterations in the expression of mentioned markers up to 15th passage. During later passages the expression of CD73, CD90 and CD105 decreased. Similar findings were obtained in experiments dealing with other types of MSCs, for instance bone marrow MSCs lost their typical immunophenotype in 12th passage (Pogozhykh *et al.* 2015) and in case of tonsil-derived MSCs it was in 15th passage (Yu *et al.* 2014). This may be associated with increased concentration of contaminating fibroblasts. It can be overlapped by utilization special synthetic media or

specific growth factors such as BMP-4 and FGF-2 (Zhu and Liang 2015; Liu *et al.* 2013).

The first evidence of cell transformation and senescence is changing of morphology. In our study, the first changes were recorded in 15th passage. Cells became bigger and had prolonged cytoplasmic projections. The most visible morphological alterations were visible during last passages; the cells had irregular morphology. Also observations performed on TEM revealed changes of hPLSCs as the number of passages increases. The most important finding was accumulation of numerous dark electron-dense bodies, lipid droplets and lamellar structures in cytoplasm (resemble myelin figures). Subgroup of the more compact lamellar dense bodies may represent the residual bodies, which arise after the breakdown of cellular organelles, notably mitochondria termed as multivesicular bodies or endosomes. In our case, they represent late stage of endosome maturation with partial electron dense areas prior to final breakdown by lysosomes (Huotari and Helenius, 2011).

Our data obtained from telomerase activity analysis showed, that long-term cultivation led in its incremental reduction. This was in agreement with results of other authors dealing with MSCs from different tissues (Fu *et al.* 2015; Chen *et al.* 2014). However, there is still option of alternative lengthening of telomeres which was detected in some human tumours of mesenchymal origin. Alternative lengthening of telomeres is maintained by homologous recombination-mediated DNA replication mechanism (Lee *et al.* 2015). Nava *et al.* (2015) analyzed alternative lengthening of telomeres in hATSCs in long-term cultivation system, but their results did not proved its presence.

The expression analysis of genes involved in regulation cell cycle and apoptosis (e.g. CDK1, TP53 and Bcl-2) is crucial because they play important roles in the pathogenesis of many cancers (Halasova *et al.* 2010; Halasova *et al.* 2013). It was revealed the influence of long-term cultivation system. For instance, the expression of TP53 had decreasing tendency up P15, but in higher passages (from P20) when the proliferation activity was lowered the expression of TP53 started to grow. That was probably associated with DNA damage accumulation and initiation of apoptosis. It was also underlined by fact that values of CDK1 expression had a decreasing trend. Similar findings were published by Jiang *et al.* (2012), who analyzed the effect of cyclin A on the cell cycle distribution and regulation of proliferation of hATSCs. It is thought that cyclin A in association with CDK1 is required for entry into M phase (Bendris *et al.* 2015). Moreover, low values of Bcl-2 in late passages indicated that hPLSCs, may undergo the process off apoptosis as prevention against malignant transformation (Tsujimoto & Shimizu, 2000). Mentioned above, probably explained fact that MSCs which underwent prolonged cultivation *in vitro* did not result in tumor formation after transplantation into nude mice (Zaman *et al.* 2014).

In summary, our data indicate that long term *in vitro* expansion of hPLSCs results not only in significant changes of morphology but also affect the proliferation kinetics and cell cycle. On the opposite, there seem to be low tumorigenic potential mainly because of maintenance the normal function of regulators associated with cell cycle and apoptosis regulation. Moreover, there is lack of telomerase activity which is typical for cancer cells. However, further studies considering the bio-safety of MSCs have to be performed prior their translation to regenerative medicine.

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