Effect of Human Platelet Lysate as Cultivation Nutrient Supplement on the Expansion of Human Natal Dental Pulp Stem Cells In Vitro

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Research Article

Keywords: Stem cell cultivation, human platelet lysate, mesenchymal stem cells, human natal stem cells, fetal bovine serum, culture medium nutrient supplement, regenerative medicine

Posted Date: April 6th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1419877/v1

Abstract

Background

Despite scientific and ethical issues, fetal bovine serum (FBS) remains the standard nutrient supplement in the mesenchymal stem cell cultivation medium. Given that cell amplification plays an important role in human stem cell therapies, there is an increasing interest in finding suitable human alternatives to FBS for *in vitro* cell propagation. One such alternative is human platelet lysate (hPL).

Methods

Our study aimed at evaluating the influence of 2% hPL in growth medium for *in vitro* expansion of human natal dental pulp stem cells (hNDP-SCs). We determined the effect this medium had on proliferation rate, viability, phenotype profile, expression of several markers, relative telomere length change, and differentiation potential of four lineages of hNDP-SCs. As a control, hNDP-SCs were simultaneously cultivated in 2% FBS.

Results

hNDP-SCs cultivated in hPL showed a statistically significantly higher proliferation rate in initial passages. We did not observe a statistically significant effect on mesenchymal stem cells marker (CD29, CD44, CD73, CD90) or stromal-associated marker (CD13, CD166) expression. The cell viability, relative telomere length, and multipotency remained unaffected in hNDP-SCs cultivated in hPL-medium.

Conclusions

In conclusion, hPL produced under controlled and standardized conditions has demonstrated to be an efficient serum supplement for *in vitro* expansion of hNDP-SCs.

Background

The occurrence of natal and neonatal teeth in humans is an uncommon anomaly of premature tooth eruption. Natal teeth are those present at birth, and neonatal teeth are those that erupt within the first month of life (1). Both, however, might further accompany various difficulties, such as pain on sucking and refusal to feed faced by mother or child (1, 2). Although the eruption of the lower deciduous incisors is normal at birth in many mammals, natal and neonatal teeth are rare in humans. The incidence of natal teeth ranges from 1:2000 to 1:3500 live births (1–5). Only 1–10% of natal and neonatal teeth are supernumerary; more than 90% of natal teeth and neonatal teeth are prematurely erupted primary teeth (6). Natal teeth are three times more common than neonatal teeth. They most frequently occur in the mandibular central incisor region (Fig. 1).

They manifest variable shapes and sizes, ranging from small and conical to normal primary-tooth shape. However, natal teeth usually have hypoplastic enamel and poor or absent root development, suggesting an immature nature (1).

The pulp of natal teeth is a source of a unique type of dental pulp-related stem cells that display some of the characteristics of pluripotency (7, 8). In comparison with dental pulp stem cells isolated from the pulp of permanent teeth (DPSCs) or of deciduous teeth (SHED), human natal dental pulp stem cells (hNDP-SCs) have a higher proliferation rate and express similar surface markers CD13, CD29, CD44, CD73, CD90, CD146, and CD166. Interestingly, hNDP-SCs express detectable levels of factors such as Nanog, octamer-binding transcription factor 4 (Oct-4), transcription factors Sox-2, and forkhead box protein FoxD3 (7, 8). They also show positivity for osteogenic, myogenic, chondrogenic, and neural markers under cultivation in the standard conditions (7). This determines their immature nature and wide differentiation potential, properties that are considered the most valuable in regenerative or reparative cell therapies.

Most cell-based therapies required a high cell number. Therefore, after being isolated the stem cells must be amplified *ex vivo* under controlled conditions without any effects on their “stemness” characteristics. The majority of laboratory protocols for stem cell expansion use a cultivation medium supplemented with fetal bovine serum (FBS). FBS contains essential components for cell proliferation and maintenance such as hormones, vitamins, transport proteins, trace elements, spreading, and growth factors (9). Unfortunately, the use of FBS is associated with several problematic issues. Scientific disadvantages are the unknown exact composition, serious safety concerns in terms of endotoxins, mycoplasma, and viral contaminants or prion proteins, as well as ethical concerns (10–13). In light of previously mentioned scientific, consistency, and ethical issues, the search for alternatives to FBS has become a major goal in recent years. However, chemically-defined, serum-free media have not yet been developed for every cell line or primary culture due to issues (such as heterogeneity) among cell types and potential effects on cell phenotype or reproducibility. A human platelet lysate (hPL) has been identified as an autologous replacement for animal-derived supplements such as FBS in the expansion of stem and progenitor cells for tissue-engineering applications and cell therapies (14, 15). hPL is prepared from platelet-rich plasma (PRP), either derived from pooled buffy coat-derived platelet concentrates of whole blood or from apheresis. In contrast to PRP, which requires a more complicated manufacturing process, hPL can be generated from common platelet units by a simple freeze-thawing procedure. To avoid the extensive aggregate formation and to deplete potential antigens, the platelet fragments are removed by centrifugation (16, 17). Due to the fact that hPL is a platelet-free element, immunological reactions connected with allogeneic products can be obviated. On the other hand, hPL contains a high level of several growth factors including insulin-like growth factor 1 (IGF-1), transforming growth factor-beta (TGF-β1, TGF-β2), platelet-derived growth factor (PDGF), fibroblast growth factors (FGF), vascular endothelial growth factor (VEGF), and epidermal growth factor (EGF) (18, 19). Some of these signal regulators have also been identified as playing a role in the regulation of osteogenesis of dental pulp stem cells or stem cells from the gingiva or periodontal ligaments (20–23). Therefore, hPL has been shown to promote cell growth; its results have been superior to standard cultures supplemented with FBS,

while the differentiation capacity of the progenitor cells seems not to be affected (24–26). Unfortunately, it must be taken into consideration that hPL composition varies in the amount of plasma and range of growth factors (26). The research studies investigating hPL in stem cell cultivation bring conflicting results. Variation between individual platelets can be reduced by pooling platelet units for the use of hPL under good manufacturing practice conditions. However, it has been reported that hPL can be used for cultivating (from various tissues) a broad range of different cell types, such as human endothelial cells (27), human fibroblasts (28), and mesenchymal stem cells (MSCs) (16, 17, 29).

Methods

This study was aimed at evaluating the influence hPL, as a nutrient supplement, has on the proliferation rate, viability, phenotype profile, several progenitor cell marker expression, relative telomere length difference, and differentiation potential of hNDP-SCs cultivated *in vitro*.

Study guidelines were approved by the Ethics Committee of the University Hospital Hradec Kralove (201812 S07P, approved 08 November 2018). Legal representatives of donors signed informed written consent before natal tooth collection.

Natal teeth collection and hNDP-SCs isolation

Four lineages of hNDP-SCs were isolated from natal teeth obtained from healthy newborns – female (11 days old) and male (4 days old) born at the Department of Obstetrics and Gynecology, University Hospital Hradec Kralove, Czech Republic. Once collected, the natal teeth were immediately immersed in a 0.2% chlorhexidine gluconate solution for 30 seconds for decontamination. Subsequently, the teeth were transported in a tube containing a transport medium consisting of water for injection (Bieffe Medital, Grosotto, Italy), 10% Hank's balanced salt solution (HBSS) (Invitrogen, Waltham, MA, USA), 200 µg / mL gentamicin (Invitrogen), 200 U / mL penicillin (Invitrogen), 200 µg / mL streptomycin (Invitrogen), and 1.25 µg / mL amphotericin (Sigma-Aldrich, St. Louis, MO, USA). During the transportation, the teeth were fully immersed in a medium, and the temperature was kept at 4°C. The hNDP-SC isolation was performed at a sterile laminar box at the Department of Histology and Embryology, Charles University, Faculty of Medicine in Hradec Kralove on the same day as the teeth were harvested. Due to absent roots, the pulp tissues were simply removed from pulp cavities using a sterile dental probe and tweezers. After they were minced and enzymatically digested as previously described (30), the cell pellet was separated from the supernatant using centrifugation (2000 rpm/5 minutes, 600g). Figure 2 shows the steps of isolation.

Culture media

Table 1 illustrates the composition of each culture medium.

Table 1
Overview of cultivation media content.

Components	FBS-culture medium	hPL-culture medium
Eagle Minimum Essential Medium Alpha (α- Mem, Sigma-Aldrich)	94.96 ml	
Fetal bovine serum (PAA Laboratories, Dartmouth, MA, USA)	1.94 ml (**2%**)	x
human Platelet lysate (Transfusion Department, University Hospital Hradec Kralove, Czech Republic)	X	1.94 ml (**2%**)
Insulin-Transferrin-Selenium (ITS, Invitrogen)	10 µl/ml	
EPGF (PeproTech, London, UK)	10 ng/ml	
PDGF (PeproTech)	10 ng/ml	
Dexamethasone (Bieffe Medital)	8 µl/ml	
L-ascorbic acid (Bieffe Medital)	1 ml	
Glutamine (Invitrogen)	1.9 ml	
Streptomycin/Penicillin (Invitrogen)	0.6 ml	
Gentamycin (Invitrogen)	0.5 ml	
Amphotericin (Sigma-Aldrich)	2.5 µg/ml	

Cells seeded in adherent surface culture dishes (TPP, Sigma-Aldrich) were cultivated under standard conditions in a humidified atmosphere containing 5% CO_2 at 37°C. Every three days, we removed the detritus and non-adherent elements by washing dishes with a phosphate-buffered saline (PBS, (Sigma-Aldrich) and exchanged each culture medium. Each lineage was detached from the adherent surface using 0.05% Trypsin-EDTA (Gibco, Thermo Fisher Scientific, Foster City, CA, USA) and passaged when hNDP-SCs reached approximately 70% confluence. Subsequently, cells were reseeded in a final concentration of 5000 cells/cm^2. All cell lines were terminated in the 14th passage (14p).

The hPL was obtained from the Transfusion Department, University Hospital Hradec Kralove, Czech Republic. The preparation protocol started with platelet-rich plasma (PRP) units derived from buffy coats. After a sterility check, PRP units were frozen down to at least − 20°C in the original storage bags. When the bacterial test was negative, hPL units were thawed at 37°C (water bath) until the ice clots disappeared. To decrease individual platelet variation, one hPL unit was pooled from five randomly chosen units donated from healthy individuals according to guidelines for selecting blood donors. To increase the rate of platelet fragmentation and the number of released growth factors, hPL units were

further re-frozen and re-thawed. Subsequently, the hPL units were centrifuged to discard the platelet pellets, thus ensuring a supernatant rich in factors.

Effect on hNDP-SC proliferation and viability

We calculated the total cell count in each passage using a Z2-Counter (Beckman Coulter, Miami, FL, USA). Proliferation capacity was evaluated as population doublings (PDs) and population doubling time in hours (formula 1). Proliferation activity was compared from the 1st passage when hNDP-SCs were seeded into two different growth media.

$$\mathrm{PD} = \log 2(\mathrm{N}_x/\mathrm{N}_1)$$

Formula 1. *Cumulative population doubling count. N_x is the total passage cell count calculated using the Z2-Counter (Beckman Coulter), and N_1 is the initial cell count seeded into the culture dish (5000 cells/cm^2).*

To determine the number of viable hNDP-SCs cells from each sample in the 3rd and 11th passage, we used the trypan dye exclusion method by Vi-Cell analyzer (Beckman Coulter). This method is based on the principle that viable cells do not take up the trypan blue dye, whereas non-viable cells do due to disturbed cell membranes.

Effect on hNDP-SC phenotype profile and specific factor expressions

To show that hNDP-SCs maintain their phenotypic characteristics after growth in different media, undifferentiated hNDP-SCs were subjected to flow cytometric analysis using a Cell Lab Quanta analysis (Beckman Coulter). hNDP-SCs from the 5th passage were stained with primary immunofluorescence antibodies conjugated with phycoerythrin (PE) or fluorescein (FITC) against the following markers: CD10 (Neprilysin: membrane metallo-endopeptidase; CB-CALLA, eBioscience, San Diego, CA, USA), CD13 (Alanyl aminopeptidase; WM-15, eBioscience), CD29 (Integrin beta-1; TS2/16, BioLegend, San Diego, CA, USA), CD34 (Transmembrane phosphoglycoprotein; 581 (Class 287 III), Invitrogen), CD44 (Cell-surface glycoprotein; MEM 85, Invitrogen), CD45 (Protein tyrosine phosphatase; HI30, Invitrogen), CD71 (Transferrin receptor protein 1; BioLegend, MEM-75), CD73 (5'-nucleotidase; AD2, BD Biosciences Pharmingen, Erembodegen, Belgium), CD90 (Thy-1; F15-42-1-5, Beckman Coulter), CD105 (Endoglin; SN6, 289, Invitrogen), CD146 (Melanoma cell adhesion molecule; TEA1/34, Beckman Coulter), CD166 (Activated leukocyte cell adhesion molecule; 3A6, Beckman Coulter), CD271 (Low-affinity nerve growth factor receptor; ME20.4, BioLegend), MHC class II (Major histocompatibility complex II; Tü36, Invitrogen). The percentage of positive cells was determined as a percentage of cells with higher fluorescence intensity than the upper 0.5% isotype immunoglobulin control.

To detect specific biomolecules within hNDP-SCs, we performed immunofluorescent staining. For this analysis, hNDP-SCs from the 7th passage were seeded in a concentration of 5000 cells per cm^2 and cultivated in chamber slides (Nalge Nunc International Corporation, Rochester, NY, USA) for two days.

Before immunostaining, the adherent cells were fixed using 10% formaldehyde. After thorough washing with phosphate-buffered saline (PBS), a 0.5% solution of Triton (250 mL Triton (Sigma-Aldrich) and 500 mL PBS) was used for 10 min to facilitate antibody penetration. Subsequently, samples were stained with primary antibodies against Beta3-tubulin (TU-20, 1:50, Exbio), Nestin (10C2, 1:200, Chemi-Con, Nuremberg, Germany), neurofilaments (DA2, FNPT, prediluted, Zymed), and Nanog (rabbit polyclonal, 1:200, Abcam). After washing, pellets were incubated with appropriate secondary antibodies conjugated with fluorochromes for 30 min to visualize the antigen-binding sites. Cell nuclei were counterstained with 4'-6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) for 5 min, and the samples were observed with a BX51 Olympus microscope. Images were overlapped using Adobe® Photoshop CC 2021 (Adobe Systems, San Jose, CA, USA).

Effect on relative telomere length

To assess the effect of cultivation with hPL on the relative telomere length, we took measurements using real-time polymerase chain reaction (qPCR) in the 3rd and 14th passages (formula 2). The analysis protocol was the same used in previous studies (30–32). After the DNA isolation using a DNeasy Tissue Kit (Hilden, Germany), we calculated its concentration in each sample using a spectrophotometer Nanodrop 1000 (Thermo Fisher Scientific, Waltham, MA, USA).

$$T/S = 2^{-\Delta Ct}$$

Formula 2. *The formula for the relative telomere length calculation, where* $\Delta Ct = Ct_{telomere} - Ct_{„single\ copy" gene}$. *The single gene (housekeeping gene) was a coding acidic ribosomal phosphoprotein 36B4.*

We performed the qPCR in 96-well plates, and we analyzed each sample in triplicates at the same well position on an ABI 7500 HT detection system (Applied Biosystems, Foster City, CA, USA). Each 20 µl reaction consisted of 20 ng DNA, 1 x SYBR Green master mix (Applied Biosystems), 200 nM forward telomere primer (CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT), and 200 nM reverse telomere primer (GGC TG TCT CCT TCT CCT TCT CCT TCT CCT TCT CCT). We used the following primer pairs for the housekeeping gene analysis: 36B4u, CAG CAA GTG GGA AGG TGT AAT CC; 36B4d, CCC 135 ATT CTA TCA TCA ACG GGT ACA A. The cycling of each qPCR analysis (for both telomere and housekeeping gene) started with a ten-minute cycle at 95°C, followed by 15-second cycles at 95 ° C, ending with a one-minute cycle at 60°C.

Effect on hNDP-SC multipotency

For osteogenic, chondrogenic, or adipogenic induction, hNDP-SCs from the 4th passage were seeded into separated culture dishes and grown to 80–100% confluence. Subsequently, they were incubated in the differentiation media.

Osteogenic differentiation in vitro

After hNDP-SCs reached confluence, we induced osteogenic differentiation with the FBS-free differentiation medium containing α-MEM (Sigma-Aldrich), 0.5 mM ascorbic acid (Bieffe Medital), 10 mM of β-glycerophosphate (Sigma-Aldrich), 0.1 μM of dexamethasone (Bieffe Medital), and 10% hPL (Transfusion Department, University Hospital Hradec Kralove, Czech Republic). As a standard osteogenic differentiation medium, we used Differentiation of Basal Medium-Osteogenic (Lonza, Basel, Switzerland). Twice a week we washed both media with PBS and exchanged them. hNDP-SCs were cultivated under differentiation conditions for 3 weeks. At the end of the third week, the pellets were fixed using 10% formalin, dehydrated in ascending concentrations of ethanol, embedded in paraffin, and cut into sections 7-μm thick. Osteogenic differentiation was assessed via staining with Alizarin Red S and von Kossa staining to locate calcium deposits in the extracellular matrix. Osteocalcin was detected using immunocytochemistry. After deparaffination, samples were exposed to a primary mouse IgG antibody (1:50, Millipore, Burlington, MA, USA) and donkey anti-mouse secondary IgG antibody (1:250, Jackson ImmunoResearch Labs, West Grove, PA, USA).

Chondrogenic differentiation in vitro

In hNDP-SCs cultivated in an FBS-free cultivation medium, we induced chondrogenic differentiation using a medium containing α-MEM (Sigma-Aldrich), 0.5 mM of ascorbic acid (Bieffe Medital), 10 mM of β-glycerophosphate (Sigma-Aldrich), 0.1 μM of dexamethasone (Bieffe Medital), and 50 ng/mL TGF-β1 (Stem Cell Technologies, Canada). The Differentiation Basal Medium-Chondrogenic (Lonza) was used as a control medium supplemented with FBS. We exchanged both media twice a week and cultivated cells for three weeks. After three weeks, we prepared paraffin sections 7-μm thick from fixed differentiated cell pellets. Afterwards, we detected collagen and procollagen in the extracellular matrix using blue Masson's trichrome stain and specified type II collagen with immunochemical detection. Slices were incubated with a primary mouse IgM antibody (1:500, Sigma-Aldrich, USA) and Cy^{TM}3-conjugated goat anti-mouse secondary IgM antibody. Cell nuclei were counterstained with 4'-6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich). Furthermore, we also stained acid mucopolysaccharides in the chondrogenic matrix using Alcian blue histological staining.

Adipogenic differentiation in vitro

To induce pro-adipogenic conditions, HNDP-SCs grown without FBS were cultivated using the Mesenchymal Stem Cell Adipogenic Differentiation Medium kit (Cyagen Biosciences, Santa Clara, CA, USA) and differentiation Basal Medium A and B containing hPL instead of FBS. The hMSC Adipogenic medium kit (Lonza), Induction medium, and Maintenance medium induced adipogenesis in cells cultivated in a medium with FBS. Cells were exposed to pro-adipogenic conditions for four weeks. Two different media from each kit were used subsequently and switched every three days for three weeks. In the final week, hNDP-SCs were cultivated only in the hMSC Adipogenic Maintenance medium or Differentiation Basal Medium B. Cultures were then fixed with 10% formalin and rinsed with 50% ethanol. The presence of intracellular lipid droplets, which indicates the occurrence of adipogenic differentiation, was confirmed by Oil Red O staining.

Statistical analysis

The data are presented as the mean ± SD. All statistical analyses were performed using the statistical software GraphPad Prism 9.3.0 (San Diego, USA). The Shapiro-Wilk test or Kolmogorov–Smirnov test was used for normal distribution evaluations. The statistical significances (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$) were calculated using either the paired t-test for continuous variables or the Wilcoxon matched-pairs test for nonparametric variables.

Results

Independently of the culture medium, hNDP-SCs initially had a rounded spindle-like shape with elongated processes reaching the surrounding cells (Fig. 3). After several passages, hNDP-SCs became prolonged and more spindled.

Effect on hNDP-SC proliferation and viability

Interestingly, during initial passages, (mainly 2p – 5p) hNDP-SCs grown in the hPL-culture medium proliferated faster than their counterparts. The average PDT (2p – 5p) per passage for hNDP-SCs grown in the hPL-culture medium was 22.65 ± 0.10 hours (average PD per passage (2p – 5p) was 3.97 ± 0.35), whereas the figure for hNDP-SCs grown in the FBS-culture medium was 44.69 ± 1.36 hours (PD = 3.98 ± 0.38). This difference was statistically significant ($p > 0.0001$). The same trend (but less significant) was seen through the 8th passage, when the proliferation rate of both groups became nearly the same. The proliferation rate of hNDP-SCs cultured in the hPL-culture medium was faster again between the 10th and 12th passage. However, there was a lower figure for PD in the 12th passage (cumulative PD in the 12th passage was 53.94 ± 0.89) compared with the hNDP-SCs cultured in the FBS-culture medium (55.87 ± 0.95). At the end of cultivation, the proliferation rate for hPL-cultivated hNDP-SCs slowed down. Figures 4A and 4B illustrate the proliferation capacity of both groups displaying cumulative PDs and PDT in hours.

The percentages of viable cells were established using the trypan blue exclusion method in the 3rd and 11th passages. The figures for both hPL-cultivated and FBS-cultivated hNDP-SCs were over 90% for the entire time of cultivation (Fig. 5).

Effect on hNDP-SC phenotype profile and specific factor expressions

Defined markers exist that specifically and uniquely identify mesenchymal stem cells. The flow cytometry analysis of all common mesenchymal, hematopoietic stem cell markers indicated that hNDP-SCs grown in the hPL-culture medium were highly positive (> 95%) to the majority of tested markers. On the other hand, the same trend was not observed in FBS-cultivated hNDP-SCs. We did not observe statistically significant variances in CD markers defined as mesenchymal stem cell markers CD29, CD44, CD73, CD90, or the stromal associated markers CD13 and CD166. Both groups showed a high average expression of these markers (< 90%). The protein tyrosine phosphatase (CD45) was highly expressed in hPL-cultivated

hNDP-SCs but lowly expressed in FBS-cultivated hNDP-SCs (> 10%). The markers CD34, CD105, CD146, and MHC class II differed statistically significantly when comparing groups (Fig. 6A, B).

To determine whether this spontaneous ability is independent of culture medium composition, we performed immunocytochemistry. Undifferentiated hNDP-SCs were stained with primary antibodies against Beta3-tubulin (an early neuronal marker (33)), Nestin (neural progenitor marker(33)), neurofilaments (a late neuronal marker (33)), and Nanog (a marker known for its functions in ESC pluripotency, maintenance, and self-renewal (34)). The following pictures show that the above-mentioned markers were positively expressed in most of the cells independently of whether we used FBS- or hPL-supplemented medium for cultivation (Figs. 7–10).

Effect on relative telomere length

Many studies have reported that telomere length shortening is a hallmark of cell senescence, and the maintenance of their length is essential for cell self-renewal and differentiation potential (35, 36). Therefore, we performed qPCR in order to explore whether there are variances in relative telomere length between hNDP-SCs cultivated in two different media. We studied the differences in relative telomere length changes between the 3rd and 14th passages. Independently of the culture medium, we observed a statistically significantly shorter relative telomere length in the 14th passage compared to the 3rd passage. The shortening was more noticeable in the case of hNDP-SCs cultivated in the standard FBS-culture medium (Fig. 11).

Effect on hNDP-SC multipotency

The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy proposes minimal criteria to define human mesenchymal stem cells, the category to which hNDP-SCs belong. One of the criteria is that such cells must be able to differentiate into osteoblasts, adipocytes, and chondroblasts *in vitro*. We proved that hNDP-SCs cultivated in hPL- or FBS-culture medium kept the ability to differentiate into osteogenic, chondrogenic, and adipogenic cell lines. To confirm our results, we used histologic staining and immunocytochemistry. The following figures (12–16) show our results.

Discussion

An increasing interest in mesenchymal stem cells and their role in regenerative and reparative medicine has brought many concerns and limitations that should be taken into consideration before their future broader use, especially in human medicine. One of the limitations is fetal bovine serum, the widely used standard medium supplement and source of growth factors for cell culture. Regenerative cells occur in low doses in origin tissues. Therefore, they must be amplified after their isolation to obtain a suitable dose for clinical application. hNDP-SCs are not an exception. Even though the problems associated with using FBS are well known (risks of xenoimmunization against bovine antigens, the transmission of pathogens, ethical issues associated with FBS collection) (9–11, 37), FBS remains the standard growth factor supplement in most laboratory cultivation protocols. Therefore, it is rationale to find suitable human alternatives for *in vitro* cell propagation. This has become more important with the rapidly

growing field of advanced cell therapy, where the use of FBS should be avoided due to international guidelines.

Over the past two decades, various human alternatives have been tested for their ability to sustain the proliferation and differentiation of cells in culture. The use of human serum might seem the most straightforward solution. Unfortunately, studies have indicated that this method is not reliable, and MSC proliferation is slow – cells reach the desired confluence only with difficulty (38). Platelet-rich plasma has been shown to enhance MSC proliferation (12, 39–41), but the debris present in PRP might disturb cell culture. Furthermore, it is necessary to activate thrombocytes to release growth factors. The use of human platelet lysate was first described in 2005 (16). So far, hPL enriched in growth factors (such as platelet-derived growth factor) is used predominately for human MSCs, endothelial, and fibroblast culture. Whereas FBS is readily available as a by-product of slaughterhouse procedures, hPL is generated by the freeze-thaw process (in which waste products form after the expiration of platelet units). Variations exist between individual hPL, but that issue can be resolved through pooling. One major drawback of hPL, however, is that it is rarely distributed commercially (16, 26). Furthermore, all human blood-derived substituents pose a risk of transmitting human diseases through viruses such as human immunodeficiency virus and human T-lymphotropic virus. Nevertheless, these threats could be decreased by strict adherence to blood bank quality standards.

Our study aimed at verifying the effect of hPL-supplemented culture medium on hNDP-SCs by studying the proliferation capacity, viability, expression of specific markers, and relative telomere length. We sought to determine the consequences of hPL on hNDP-SC multipotency. The study included four lineages of hNDP-SCs isolated from two newborns (one male, one female).

The hPL used in the study was generated from the blood of five healthy donors to eliminate variations. The amount of growth factors in the hPL suggests a possible mechanism of action for cell proliferation. The inconsistent data caused by different preparation protocols, different blood sources, and the different concentrations of platelets or growth factors make it difficult to establish an optimal hPL concentration. However, many recent studies have agreed that increasing the concentration of hPL negatively affects the MSC proliferation rate (42, 43). Chen et al. concluded that, when dental pulp stem cells were cultivated in 10% hPL, significant inhibition of cell proliferation was observed; 1% and 5% hPL enhanced the cell growth, but 5% was the most effective concentration for the proliferation and mineralization of DPSCs (43). We expanded four lineages of hNDP-SCs in α-MEM culture medium supplemented with either 2% FBS or 2% hPL. In one of our previous studies, 2% concentration of human blood components was established as the most effective in dental pulp-related stem cell cultivation (44).

hPL in the culture medium accelerated the proliferation rate of hNDP-SCs at the beginning of the cultivation (2nd passage – 5th passage). hPL-cultivated hNDP-SCs showed approximately two times shorter PDT (22.65 ± 0.10 hours) compared with the FBS-cultivated group (44.69 ± 1.36 hours), while the PDs per passage was approximately the same (3.97 ± 0.35 vs. 3.98 ± 0.38). Our results are comparable with other studies (26, 39, 42, 43, 45, 46). At the end of cell growth, we observed the prolongation of

population doubling time in the hPL-treated group. For potential clinical application, the total cell count would need to be amplified in the initial stages following isolation; this, however, should pose no issue given that hPL-treated cells revealed extensive proliferation capabilities particularly in the beginning of the cultivation process. The initial viability measured using trypan blue exclusion methods was also not statistically significantly higher. In contrast, we observed higher percentages of viable cells cultivated with FBS in one of the later passages (11th passage).

Interestingly, hPL showed a high expression of all tested markers (< 90%). These results are different from other studies where no effect of the medium supplement was observed (46–48). There was no statistical difference in mesenchymal stem cells markers (CD29, CD44, CD73, CD90) and stromal associated markers (CD13 and CD166). These markers were also highly positively expressed on hNDP-SCs cultivated in FBS. However, markers CD10, CD34, CD45, CD105, CD146 varied significantly between groups. Since our results are different from other studies (49), we can only hypothesize the reasons for significant variances in the expression of tested CD markers. On the other side, phenotype changes were seen in recent study focusing on hPL in the medium culture for mesenchymal progenitors derived from human-induced pluripotent stem cells (50).

A recent study determined that higher CD10 expression identifies high proliferation in perivascular progenitor cells (51). The CD34 marker is taken as a hematopoietic stem cell marker; however, certain studies have called this into question (52, 53), and further inquiry is needed. CD105, also known as endoglin, is a type I membrane glycoprotein that functions as an accessory receptor for TGF-beta superfamily ligands. Higher expression of CD105 might be explained by the fact that hPL is rich in several growth factors, including insulin-like growth factor 1 (IGF-1) and transforming growth factor-beta (TGF-β1, TGF-β2). Ma et al. concluded that the expression level of CD146 showed a positive correlation with proliferation, differentiation, and immunomodulation, suggesting that CD146 can serve as a surface molecule to evaluate the potency of human dental pulp stem cells cultivated in the serum-free medium (54). To summarize all the above, it seems that hNDP-SCs are more affected by changes in serum-free growth medium than other mesenchymal stem cells, and that hPL keeps hNDP-SCs less differentiated and prepared for wider differentiation into mature cells lines. Nevertheless, since the disadvantages of using flow cytometry as a tool for immunophenotyping have already been published (55), and using two methods for phenotype analysis is recommended, further investigation is needed before we would be able to reach such a conclusion.

Undifferentiated hNDP-SCs kept their ability to express specific markers (Beta3-tubulin, Nestin, neurofilaments, and Nanog) independently of the nutrient supplement used in the cultivation medium, suggesting these cells display some of the characteristics for pluripotency.

We studied the effect of hPL in the cultivation medium on relative telomere length. We evaluated cells in the 3rd and 14th passages using qPCR. We observed shorter relative telomere lengths in hNDP-SCs grown in hPL in the 3rd passage than in hNDP-SCs grown in FBS. In our previous study, we observed that the compensatory mechanism of telomerase activity might be time-dependent. The necessary and

excessive *in vitro* cultivation leads to telomere attrition. This idea is supported in the current study as well; we observed significant telomere attrition in both groups of cells when comparing figures between the 3rd and 14th passages. However, hNDP-SCs cultivated in hPL proliferated faster in initial passages, and the compensatory effect of telomerase was not efficient due to lack of time. The proliferation rate slowed down at the end of the cultivation and, therefore, the compensatory effect was sufficient in comparison with hNDP-SCs cultivated in the standard cultivation medium.

We also triggered osteogenesis, chondrogenesis, and adipogenesis in both groups of cells. We determined the successful differentiation using histological staining and immunocytochemistry. We did not observe any variances. All hNDP-SCs were able to keep their multipotency and differentiate into mature cell lines independent of the nutrient supplement used in the growth medium.

Conclusion

Our study aimed to evaluate the effect of hPL as a nutrient supplement during *in vitro* expansion of hNDP-SCs in comparison with standard FBS. Both supplements were at a total concentration of 2% in the cultivation medium. hNDP-SCs cultivated in hPL showed a statistically significantly higher proliferation rate in initial passages. We did not observe a statistically significant effect on mesenchymal stem cells marker (CD29, CD44, CD73, CD90) or stroma-associated marker (CD13, CD166) expression. Cell viability, relative telomere length, and multipotency of HNDP-SCs remained unaffected during cultivation at 2% hPL-medium. In conclusion, hPL which is produced under controlled and standardized conditions has shown to be an efficient serum supplement for *in vitro* expansion of hNDP-SCs.

Abbreviations

α-Mem – Eagle Minimum Essential Medium Alpha

CD – cluster of differentiation

DAPI – 4’-6-diamidino-2-phenylindole

DPSCs – dental pulp stem cells

EDTA – Ethylenediaminetetraacetic acid

EGF – epidermal growth factor

FBS – fetal bovine serum

FGF – fibroblast growth factors

FITC – fluorescein

HBSS – Hank's balanced salt solution

hNDP-SCs – human natal dental pulp stem cells

hPL – human platelet lysate

Ig – immunoglobulin

IGF-1 – insulin-like growth factor 1

ITS – Insulin-Transferrin-Selenium

Oct-4 – octamer-binding transcription factor 4

MHC – major histocompatibility complex

MSCs – mesenchymal stem cells

p – passage

PBS – Phosphate-buffered saline

PDGF – platelet-derived growth factor

PDs – population doublings

PDT – population doubling time

PE – phycoerythrin

PRP – platelet-rich plasma

qPCR – real-time polymerase chain reaction

SD – standard deviation

SHEDs – stem cells from human exfoliated deciduous teeth

TGF-β1, TGF-β2 – transforming growth factor-beta

VEGF – vascular endothelial growth factor

Declarations

Ethics approval and consent to participate

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by The Ethical Committee in Hradec Kralove, Czech Republic (ref. no. 201812 S07P, approved 08 November 2018). Legal representatives of donors signed informed written consent before natal tooth collection.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

Funding

The study was financially supported by the Charles University and Charles University's COOPERATIO Program, Research Area DENTAL MEDICINE 207030.

Authors' contributions

All authors contributed to this study: conceptualization: T.S. and J.S. (Jakub Suchanek); methodology: T.SK.; software: J.S. (Jan Schmidt); validation: N.P. and T.SK.; formal analysis: J.S. (Jan Schmidt); investigation: N.P. and T.SK.; resources: J.S. (Jan Schmidt); data curation: N.P. and T.SK.; writing—original draft preparation: N.P.; writing—review and editing: T.SK., J.S. (Jan Schmidt), T.S., J.S. (Jakub Suchanek); visualization: N.P, T.SK., and J.S. (Jan Schmidt); supervision: J.S. (Jakub Suchanek); project administration: T.S.; funding acquisition: T.S. and J.S. (Jakub Suchanek). All authors have read and agreed to the published version of the manuscript.

Acknowledgements

The authors thank Mesdames Helena Ruckerova, Simona Vrchotova, Petra Hajzlerova from the Department of Histology and Embryology at Charles University, Faculty of Medicine in Hradec Kralove, for their skillful technical assistance; prof. MUDr. Stanislav Micuda, Ph.D. and Ing. Hana Lastuvkova for their assistance in the telomere length measurement. The authors also thank Matthew Shane Renfro, BA for English language editing.

References

1. Mhaske S, Yuwanati MB, Mhaske A, Ragavendra R, Kamath K, Saawarn S. Natal and neonatal teeth: an overview of the literature. ISRN Pediatr. 2013;2013:956269.

2. Leung AK, Robson WL. Natal teeth: a review. J Natl Med Assoc. 2006;98(2):226–8.
3. Leung AK. Natal teeth. Am J Dis Child. 1986;140(3):249–51.
4. Seminario AL, Ivancaková R. Natal and neonatal teeth. Acta Medica (Hradec Kralove). 2004;47(4):229–33.
5. Kates GA, Needleman HL, Holmes LB. Natal and neonatal teeth: a clinical study. J Am Dent Assoc. 1984;109(3):441–3.
6. Sureshkumar R, McAulay AH. Natal and neonatal teeth. Arch Dis Child Fetal Neonatal Ed. 2002;87(3):F227.
7. Karaöz E, Doğan BN, Aksoy A, Gacar G, Akyüz S, Ayhan S, et al. Isolation and in vitro characterisation of dental pulp stem cells from natal teeth. Histochem Cell Biol. 2010;133(1):95–112.
8. Akpinar G, Kasap M, Aksoy A, Duruksu G, Gacar G, Karaoz E. Phenotypic and proteomic characteristics of human dental pulp derived mesenchymal stem cells from a natal, an exfoliated deciduous, and an impacted third molar tooth. Stem Cells Int. 2014;2014:457059.
9. van der Valk J, Bieback K, Buta C, Cochrane B, Dirks WG, Fu J, et al. Fetal Bovine Serum (FBS): Past - Present - Future. Altex. 2018;35(1):99–118.
10. Heiskanen A, Satomaa T, Tiitinen S, Laitinen A, Mannelin S, Impola U, et al. N-glycolylneuraminic acid xenoantigen contamination of human embryonic and mesenchymal stem cells is substantially reversible. Stem Cells. 2007;25(1):197–202.
11. Sundin M, Ringdén O, Sundberg B, Nava S, Götherström C, Le Blanc K. No alloantibodies against mesenchymal stromal cells, but presence of anti-fetal calf serum antibodies, after transplantation in allogeneic hematopoietic stem cell recipients. Haematologica. 2007;92(9):1208–15.
12. Lang S, Loibl M, Herrmann M. Platelet-Rich Plasma in Tissue Engineering: Hype and Hope. Eur Surg Res. 2018;59(3–4):265–75.
13. Hawkes PW. Fetal bovine serum: geographic origin and regulatory relevance of viral contamination. Bioresources and Bioprocessing. 2015;2(1):34.
14. Bieback K. Platelet lysate as replacement for fetal bovine serum in mesenchymal stromal cell cultures. Transfus Med Hemother. 2013;40(5):326–35.
15. Burnouf T, Strunk D, Koh MB, Schallmoser K. Human platelet lysate: Replacing fetal bovine serum as a gold standard for human cell propagation? Biomaterials. 2016;76:371–87.
16. Doucet C, Ernou I, Zhang Y, Llense JR, Begot L, Holy X, et al. Platelet lysates promote mesenchymal stem cell expansion: a safety substitute for animal serum in cell-based therapy applications. J Cell Physiol. 2005;205(2):228–36.
17. Schallmoser K, Bartmann C, Rohde E, Reinisch A, Kashofer K, Stadelmeyer E, et al. Human platelet lysate can replace fetal bovine serum for clinical-scale expansion of functional mesenchymal stromal cells. Transfusion. 2007;47(8):1436–46.
18. Marx RE, Carlson ER, Eichstaedt RM, Schimmele SR, Strauss JE, Georgeff KR. Platelet-rich plasma: Growth factor enhancement for bone grafts. Oral Surg Oral Med Oral Pathol Oral Radiol Endod.

1998;85(6):638–46.
19. Blair P, Flaumenhaft R. Platelet alpha-granules: basic biology and clinical correlates. Blood Rev. 2009;23(4):177–89.
20. Kettunen P, Karavanova I, Thesleff I. Responsiveness of developing dental tissues to fibroblast growth factors: expression of splicing alternatives of FGFR1, -2, -3, and of FGFR4; and stimulation of cell proliferation by FGF-2, -4, -8, and – 9. Dev Genet. 1998;22(4):374–85.
21. Shiba H, Fujita T, Doi N, Nakamura S, Nakanishi K, Takemoto T, et al. Differential effects of various growth factors and cytokines on the syntheses of DNA, type I collagen, laminin, fibronectin, osteonectin/secreted protein, acidic and rich in cysteine (SPARC), and alkaline phosphatase by human pulp cells in culture. J Cell Physiol. 1998;174(2):194–205.
22. Onishi T, Kinoshita S, Shintani S, Sobue S, Ooshima T. Stimulation of proliferation and differentiation of dog dental pulp cells in serum-free culture medium by insulin-like growth factor. Arch Oral Biol. 1999;44(4):361–71.
23. Marcopoulou CE, Vavouraki HN, Dereka XE, Vrotsos IA. Proliferative effect of growth factors TGF-beta1, PDGF-BB and rhBMP-2 on human gingival fibroblasts and periodontal ligament cells. J Int Acad Periodontol. 2003;5(3):63–70.
24. Fekete N, Gadelorge M, Fürst D, Maurer C, Dausend J, Fleury-Cappellesso S, et al. Platelet lysate from whole blood-derived pooled platelet concentrates and apheresis-derived platelet concentrates for the isolation and expansion of human bone marrow mesenchymal stromal cells: production process, content and identification of active components. Cytotherapy. 2012;14(5):540–54.
25. Griffiths S, Baraniak PR, Copland IB, Nerem RM, McDevitt TC. Human platelet lysate stimulates high-passage and senescent human multipotent mesenchymal stromal cell growth and rejuvenation in vitro. Cytotherapy. 2013;15(12):1469–83.
26. Hemeda H, Giebel B, Wagner W. Evaluation of human platelet lysate versus fetal bovine serum for culture of mesenchymal stromal cells. Cytotherapy. 2014;16(2):170–80.
27. Kilian O, Flesch I, Wenisch S, Taborski B, Jork A, Schnettler R, et al. Effects of platelet growth factors on human mesenchymal stem cells and human endothelial cells in vitro. Eur J Med Res. 2004;9(7):337–44.
28. Mirabet V, Solves P, Miñana MD, Encabo A, Carbonell-Uberos F, Blanquer A, et al. Human platelet lysate enhances the proliferative activity of cultured human fibroblast-like cells from different tissues. Cell Tissue Bank. 2008;9(1):1–10.
29. Lange C, Cakiroglu F, Spiess AN, Cappallo-Obermann H, Dierlamm J, Zander AR. Accelerated and safe expansion of human mesenchymal stromal cells in animal serum-free medium for transplantation and regenerative medicine. J Cell Physiol. 2007;213(1):18–26.
30. Pilbauerova N, Soukup T, Suchankova Kleplova T, Suchanek J. Enzymatic Isolation, Amplification and Characterization of Dental Pulp Stem Cells. Folia Biol (Praha). 2019;65(3):124–33.
31. Mokry J, Soukup T, Micuda S, Karbanova J, Visek B, Brcakova E, et al. Telomere attrition occurs during ex vivo expansion of human dental pulp stem cells. J Biomed Biotechnol. 2010;2010:673513.

32. Pilbauerova N, Soukup T, Suchankova Kleplova T, Schmidt J, Suchanek J. The Effect of Cultivation Passaging on the Relative Telomere Length and Proliferation Capacity of Dental Pulp Stem Cells. Biomolecules. 2021;11(3):464.
33. Foudah D, Monfrini M, Donzelli E, Niada S, Brini AT, Orciani M, et al. Expression of neural markers by undifferentiated mesenchymal-like stem cells from different sources. J Immunol Res. 2014;2014:987678.
34. Pan G, Thomson JA. Nanog and transcriptional networks in embryonic stem cell pluripotency. Cell Res. 2007;17(1):42–9.
35. Jeon BG, Kang EJ, Kumar BM, Maeng GH, Ock SA, Kwack DO, et al. Comparative analysis of telomere length, telomerase and reverse transcriptase activity in human dental stem cells. Cell Transpl. 2011;20(11–12):1693–705.
36. Serakinci N, Graakjaer J, Kolvraa S. Telomere stability and telomerase in mesenchymal stem cells. Biochimie. 2008;90(1):33–40.
37. Guiotto M, Raffoul W, Hart AM, Riehle MO, di Summa PG. Human platelet lysate to substitute fetal bovine serum in hMSC expansion for translational applications: a systematic review. J Transl Med. 2020;18(1):351.
38. Bieback K, Hecker A, Kocaömer A, Lannert H, Schallmoser K, Strunk D, et al. Human alternatives to fetal bovine serum for the expansion of mesenchymal stromal cells from bone marrow. Stem Cells. 2009;27(9):2331–41.
39. Li J, Xiang L, Guan C, Yang X, Hu X, Zhang X, et al. Effects of Platelet-Rich Plasma on Proliferation, Viability, and Odontogenic Differentiation of Neural Crest Stem-Like Cells Derived from Human Dental Apical Papilla. Biomed Res Int. 2020;2020:4671989.
40. Lee UL, Jeon SH, Park JY, Choung PH. Effect of platelet-rich plasma on dental stem cells derived from human impacted third molars. Regen Med. 2011;6(1):67–79.
41. Stute N, Holtz K, Bubenheim M, Lange C, Blake F, Zander AR. Autologous serum for isolation and expansion of human mesenchymal stem cells for clinical use. Exp Hematol. 2004;32(12):1212–25.
42. Ballesteros OR, Brooks PT, Haastrup EK, Fischer-Nielsen A, Munthe-Fog L, Svalgaard JD. Adipose-Derived Stromal/Stem Cell Culture: Effects of Different Concentrations of Human Platelet Lysate in Media. Cells Tissues Organs. 2020;209(4–6):257–65.
43. Chen B, Sun HH, Wang HG, Kong H, Chen FM, Yu Q. The effects of human platelet lysate on dental pulp stem cells derived from impacted human third molars. Biomaterials. 2012;33(20):5023–35.
44. Suchánková Kleplová T, Soukup T, Řeháček V, Suchánek J. Human plasma and human platelet-rich plasma as a substitute for fetal calf serum during long-term cultivation of mesenchymal dental pulp stem cells. Acta Medica (Hradec Kralove). 2014;57(3):119–26.
45. Karadjian M, Senger AS, Essers C, Wilkesmann S, Heller R, Fellenberg J, et al. Human Platelet Lysate Can Replace Fetal Calf Serum as a Protein Source to Promote Expansion and Osteogenic Differentiation of Human Bone-Marrow-Derived Mesenchymal Stromal Cells. Cells. 2020;9(4).

46. Vennila R, Raja Sundari MS, Selvaraj S, Srinivasan P, Pathak S, Rupert S, et al. Effect of Human Platelet Lysate in Differentiation of Wharton's Jelly Derived Mesenchymal Stem Cells. Endocr Metab Immune Disord Drug Targets. 2019;19(8):1177–91.
47. Muraglia A, Todeschi MR, Papait A, Poggi A, Spanò R, Strada P, et al. Combined platelet and plasma derivatives enhance proliferation of stem/progenitor cells maintaining their differentiation potential. Cytotherapy. 2015;17(12):1793–806.
48. Mohammadi S, Nikbakht M, Malek Mohammadi A, Zahed Panah M, Ostadali MR, Nasiri H, et al. Human Platelet Lysate as a Xeno Free Alternative of Fetal Bovine Serum for the In Vitro Expansion of Human Mesenchymal Stromal Cells. Int J Hematol Oncol Stem Cell Res. 2016;10(3):161–71.
49. Fernandez-Rebollo E, Mentrup B, Ebert R, Franzen J, Abagnale G, Sieben T, et al. Human Platelet Lysate versus Fetal Calf Serum: These Supplements Do Not Select for Different Mesenchymal Stromal Cells. Sci Rep. 2017;7(1):5132.
50. McGrath M, Tam E, Sladkova M, AlManaie A, Zimmer M, de Peppo GM. GMP-compatible and xeno-free cultivation of mesenchymal progenitors derived from human-induced pluripotent stem cells. Stem Cell Res Ther. 2019;10(1):11.
51. Ding L, Vezzani B, Khan N, Su J, Xu L, Yan G, et al. CD10 expression identifies a subset of human perivascular progenitor cells with high proliferation and calcification potentials. Stem Cells. 2020;38(2):261–75.
52. Lin CS, Ning H, Lin G, Lue TF. Is CD34 truly a negative marker for mesenchymal stromal cells? Cytotherapy. 2012;14(10):1159–63.
53. Lin CS, Xin ZC, Dai J, Lue TF. Commonly used mesenchymal stem cell markers and tracking labels: Limitations and challenges. Histol Histopathol. 2013;28(9):1109–16.
54. Ma L, Huang Z, Wu D, Kou X, Mao X, Shi S. CD146 controls the quality of clinical grade mesenchymal stem cells from human dental pulp. Stem Cell Res Ther. 2021;12(1):488.
55. Kulikova B, Kovac M, Bauer M, Tomkova M, Olexikova L, Vasicek J, et al. Survivability of rabbit amniotic fluid-derived mesenchymal stem cells post slow-freezing or vitrification. Acta Histochem. 2019;121(4):491–9.

Figures

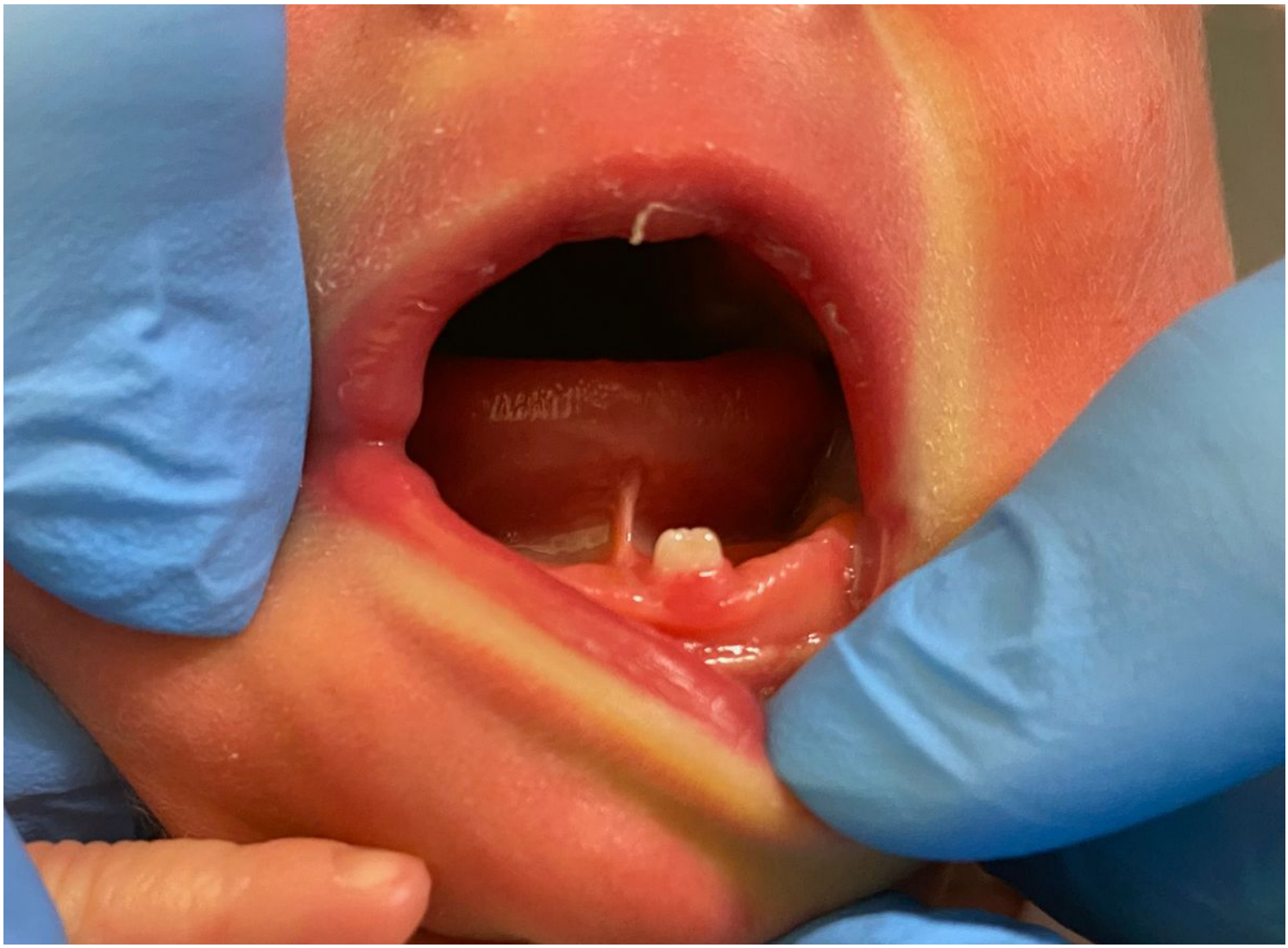

Figure 1

The natal tooth localized in the mandibular central incisor region in a newborn.

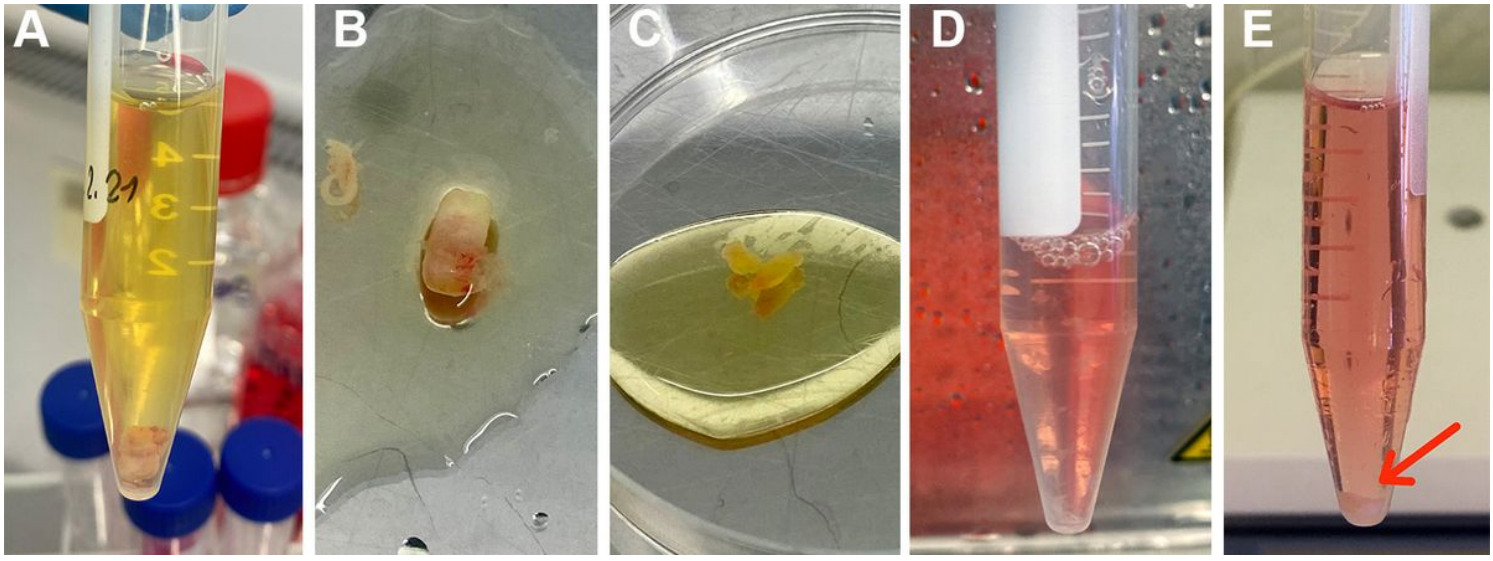

Figure 2

Enzymatic isolation of hNDP-SCs. A) the natal tooth in the transportation tube containing the transport medium, B) the natal tooth with absent root, C) separated pulp tissues, D) minced pulp tissues digested in a solution of 0.05% trypsin-EDTA solution (Gibco) in order to obtain single-cell suspensions, E) the cell pellet created after centrifugation (red arrow).

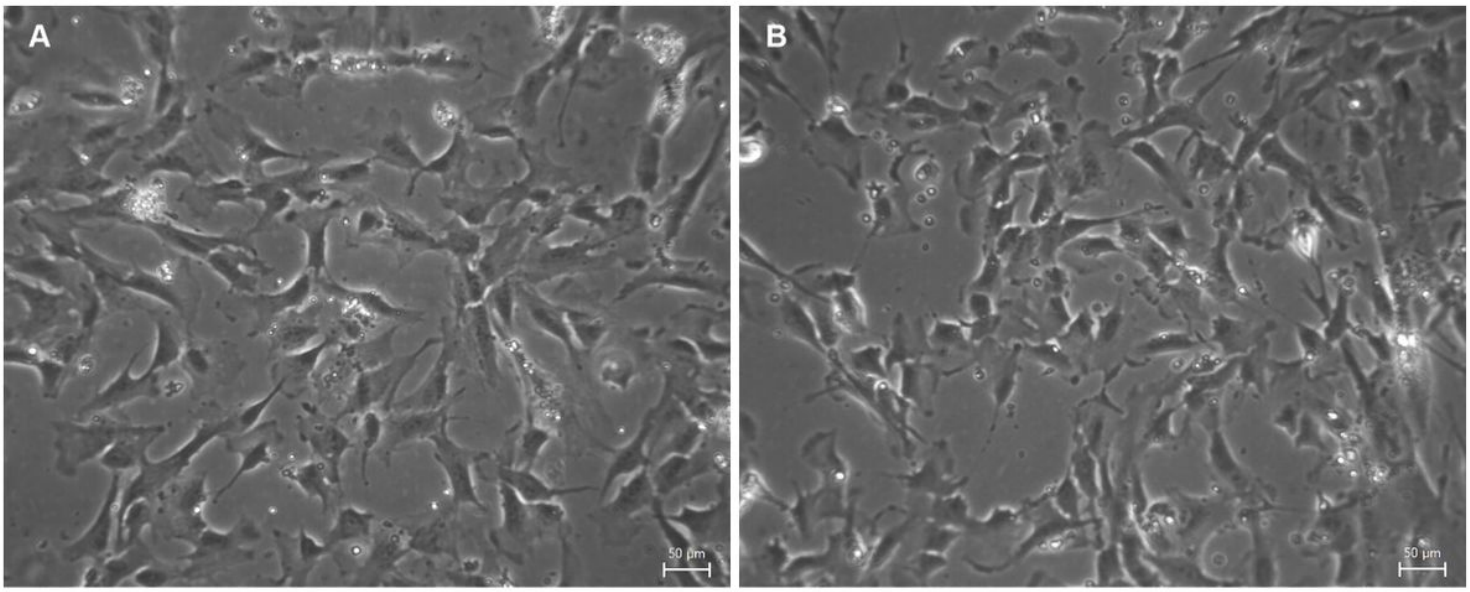

Figure 3

The rounded spindle-like shape of hNDP-SCs, cultivated for 14 days upon their isolation. Scale bar 50 µm. A) hNDP-SCs cultivated in the hPL-culture medium, B) hNDP-SCs cultivated in the FBS-culture medium.

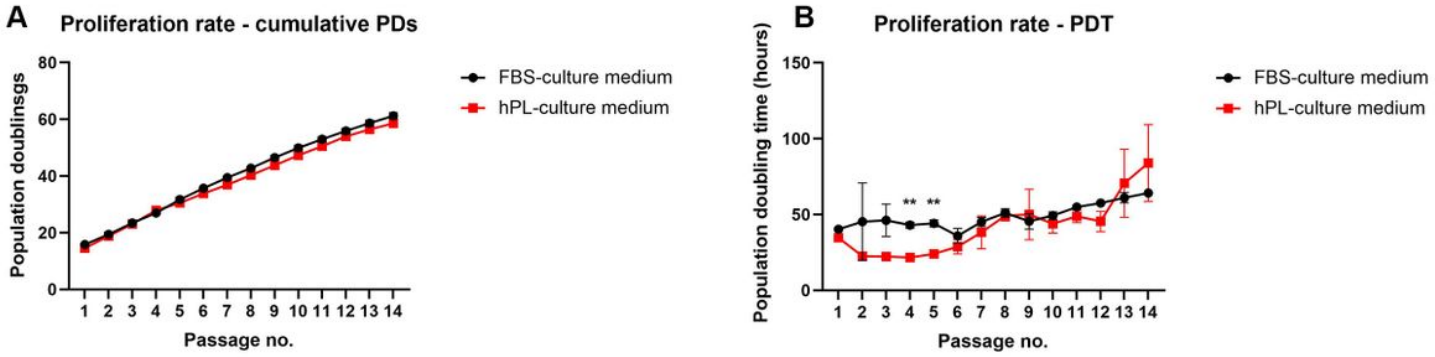

Figure 4

*The proliferation rate of hNDP-SCs. The first passage was considered as the initial for comparison of hNDP-SCs grown in two different media. The data are presented as the mean ± SD. The Shapiro-Wilk test or Kolmogorov–Smirnov test was used for normal distribution evaluations. The statistical significances (** $p < 0.01$) were calculated using the paired t-test. A) cumulative populations doubling; B) doubling time in hours.*

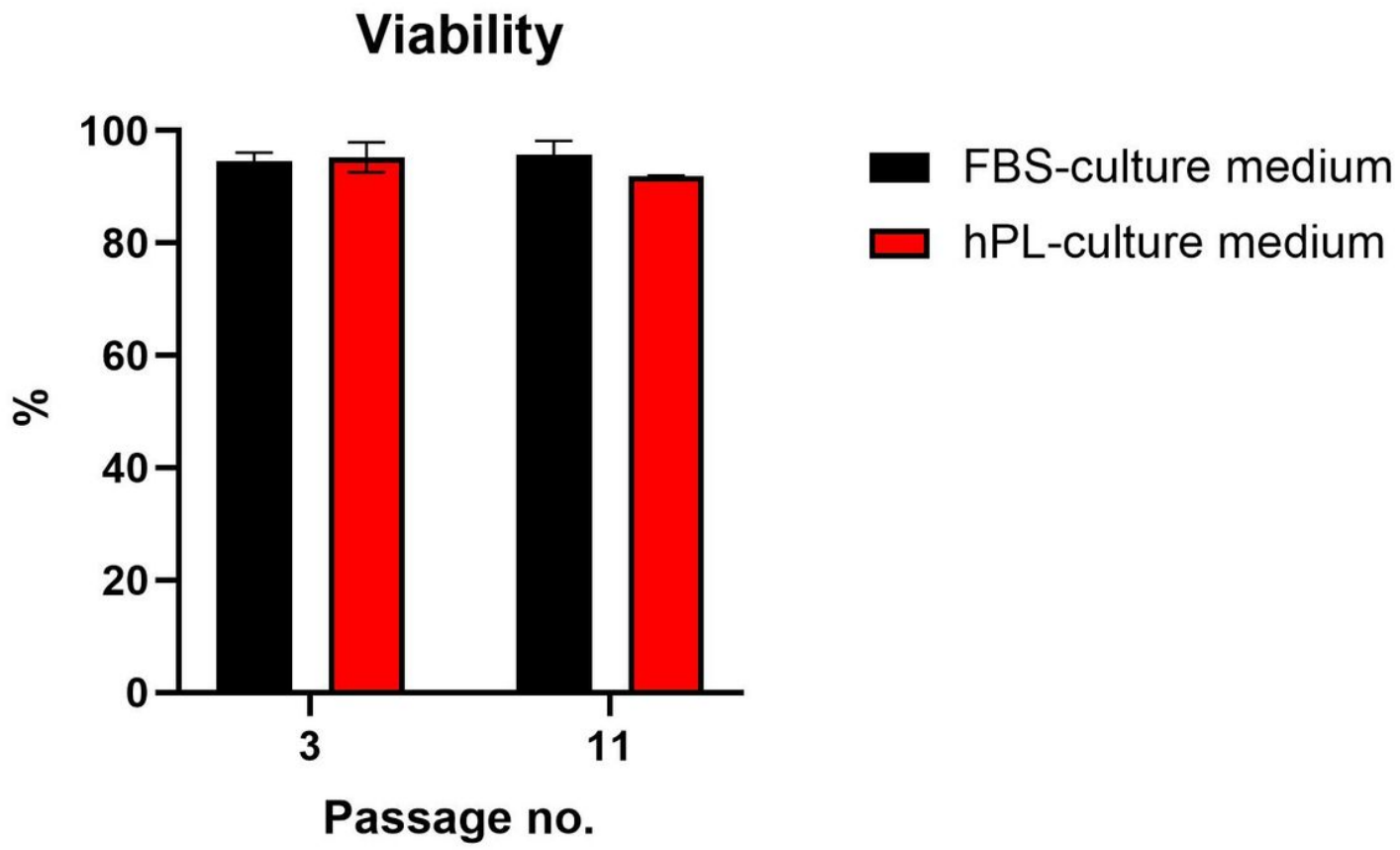

Figure 5

Viability of hNDP-SCs cultivated 2% of hPL or FBS supplement measured in the 3rd and 11th passage. The data are presented as the mean ± SD. The Shapiro-Wilk test or Kolmogorov–Smirnov test was used for normal distribution evaluations. The statistical analysis was calculated using the paired t-test. The difference was not statistically significant.

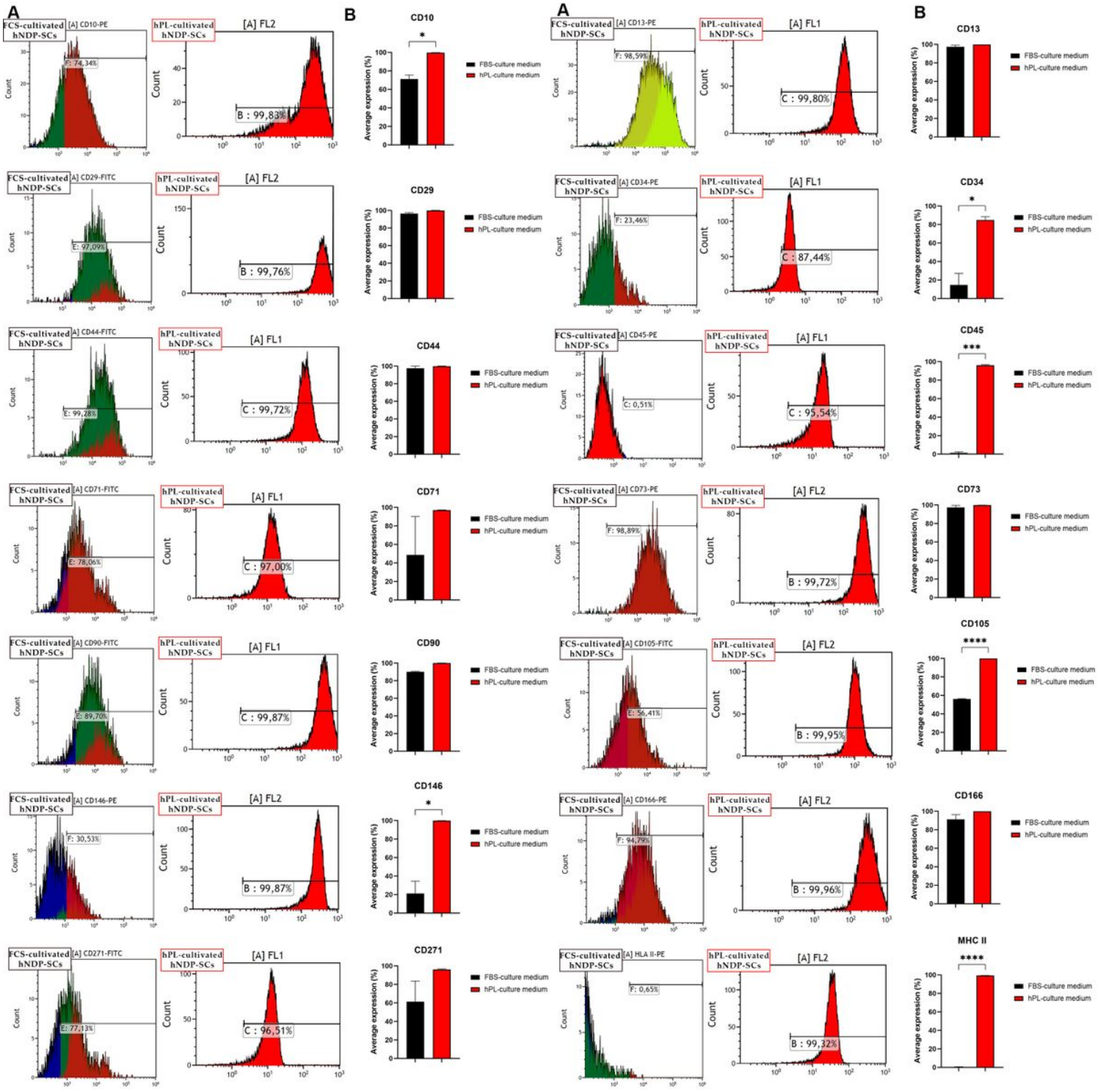

Figure 6

Phenotype profile measured in the 5th passage using flow cytometry. hNDP-SCs from both groups were stained with primary immunofluorescence antibodies conjugated with PE or FITC against the analyzed CD markers. The percentage of positive cells was determined as a percentage of cells with higher fluorescence intensity than the upper 0.5% isotype immunoglobulin control. A) Histograms of one particular cell line showing its expression of analyzed CD markers, B) graphs depicting the average expression of all analyzed CD markers. The data are presented as the mean ± SD. The Shapiro-Wilk test or

Kolmogorov–Smirnov test was used for normal distribution evaluations. The statistical analyses were calculated using the paired t-test (p < 0.05, *** p < 0.001, **** p < 0.0001).*

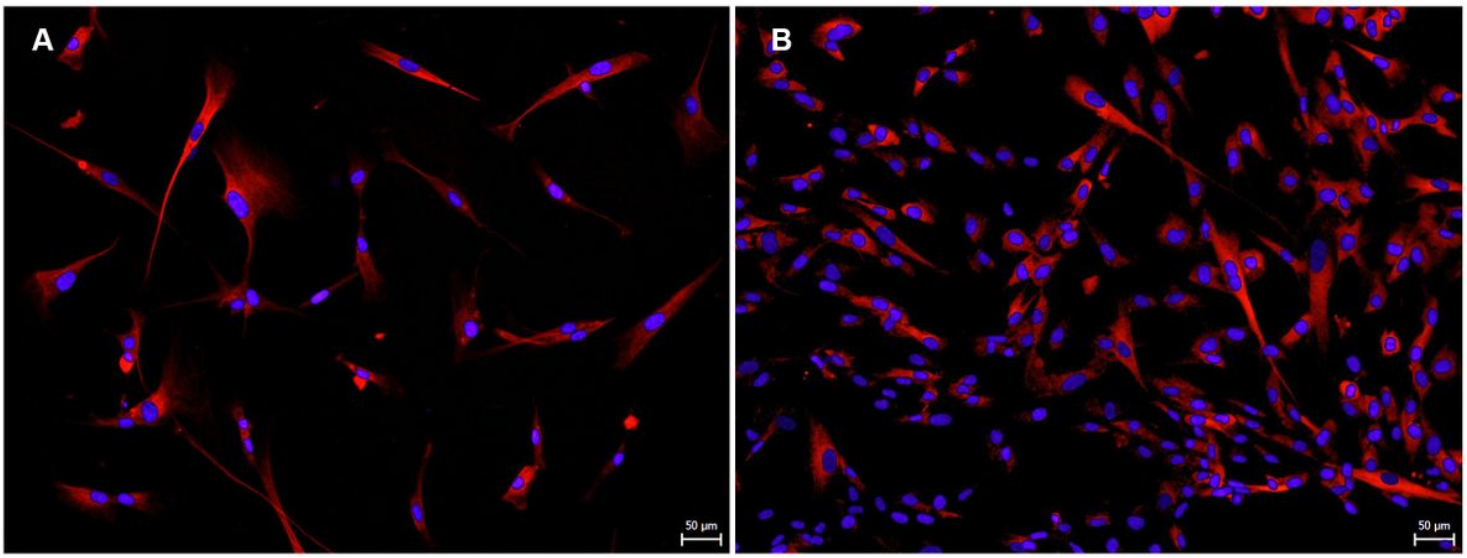

Figure 7

Immunocytochemical detection of Beta-3-tubulin, the early neuronal marker, in undifferentiated hNDP-SCs harvested in 7th passage. Most cells were positive for Beta-3-tubulin (red fluorescence). Cell nuclei display as fluorescent blue. Scale bare 50 µm. A) hNDP-SC line cultivated hPL supplemented medium. B) hNDP-SC line cultivated FBS supplemented medium.

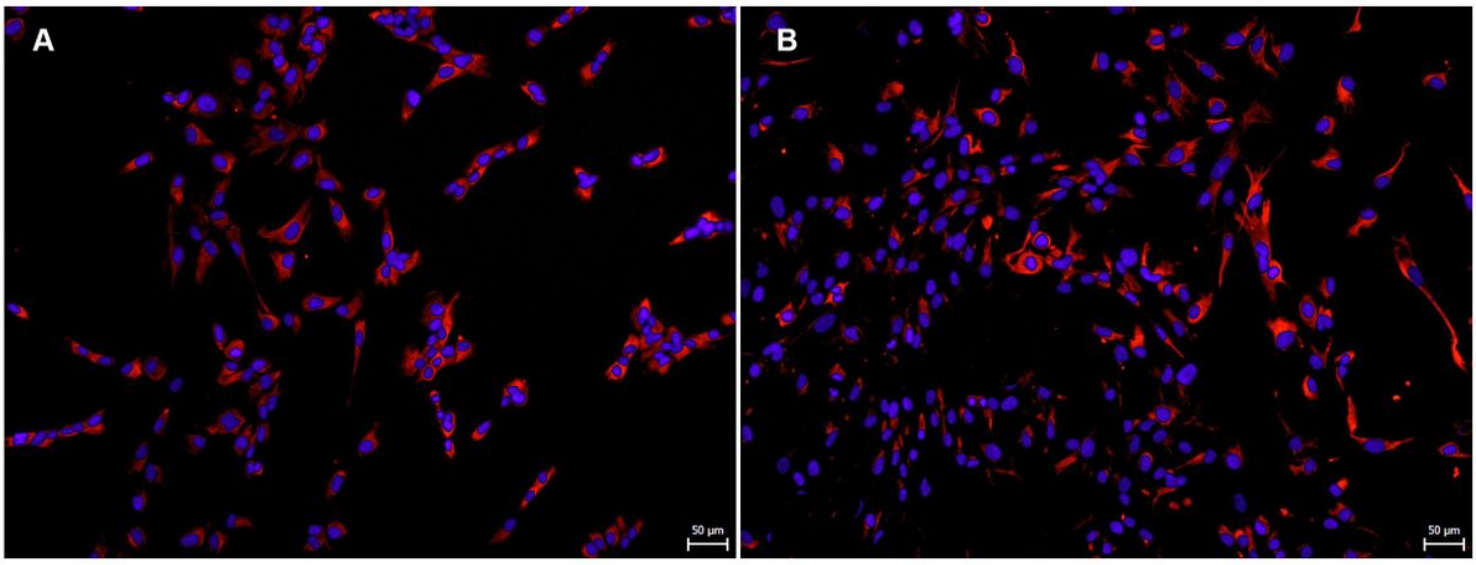

Figure 8

Immunocytochemical detection of Nestin, the neural progenitor marker, in undifferentiated hNDP-SCs harvested in 7th passage. Most cells were positive for Nestin (red fluorescence). Cell nuclei display as

fluorescent blue. Scale bare 50 µm. A) hNDP-SC line cultivated hPL supplemented medium. B) hNDP-SC line cultivated FBS supplemented medium.

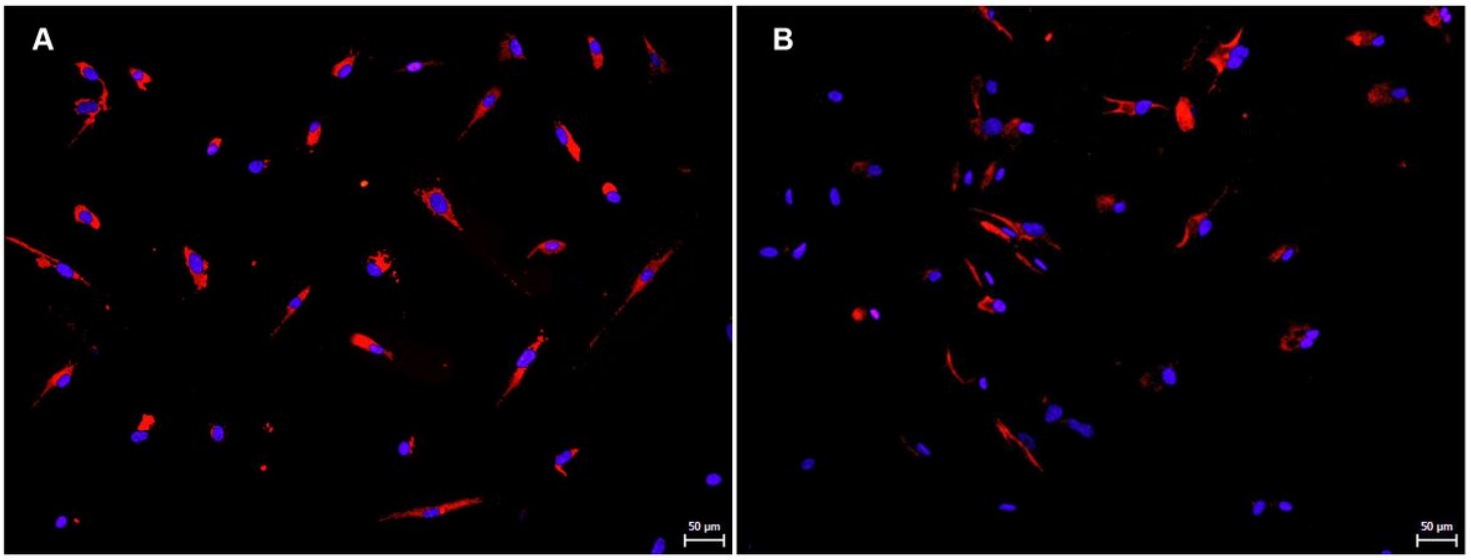

Figure 9

Immunocytochemical detection of neurofilaments, the late neuronal marker, in undifferentiated hNDP-SCs harvested in 7th passage. Most cells were positive for neurofilaments (red fluorescence). Cell nuclei display as fluorescent blue. Scale bare 50 µm. A) hNDP-SC line cultivated hPL supplemented medium. B) hNDP-SC line cultivated FBS supplemented medium.

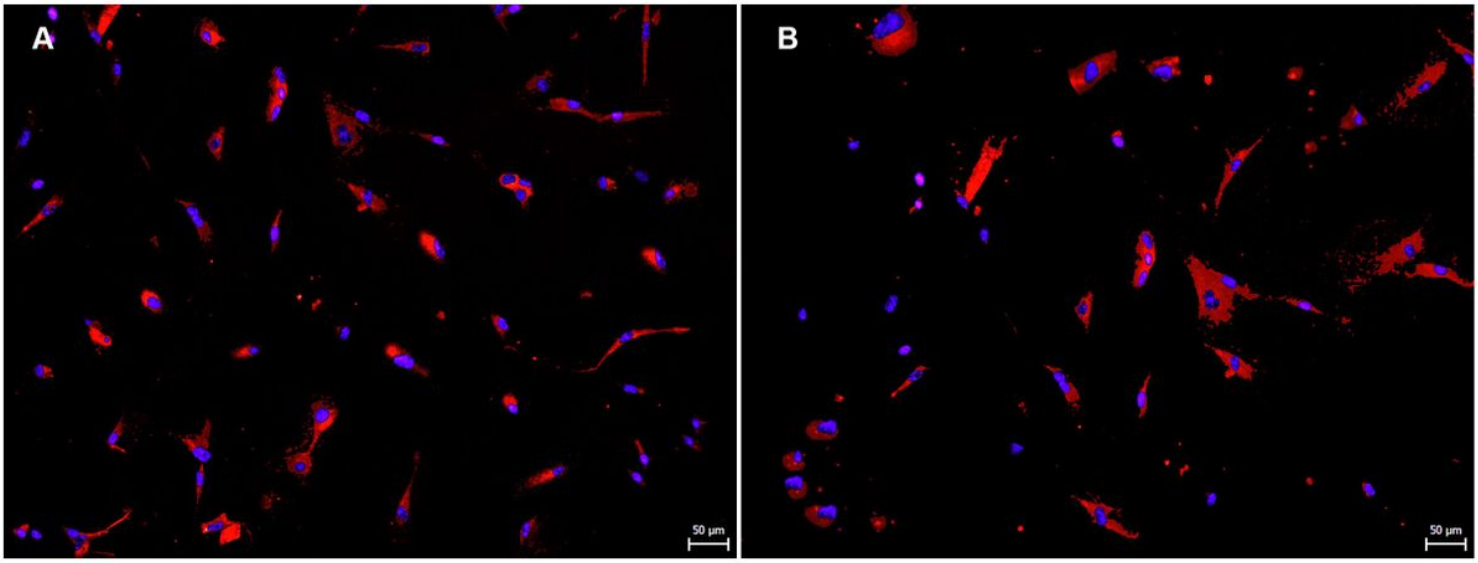

Figure 10

Immunocytochemical detection of Nanog, marker playing a role in ESC pluripotency, maintenance, and self-renewal. Most cells were positive for Nanog (red fluorescence). Cell nuclei display as fluorescent blue.

Scale bare 50 µm. A) hNDP-SC line cultivated hPL supplemented medium. B) hNDP-SC line cultivated FBS supplemented medium.

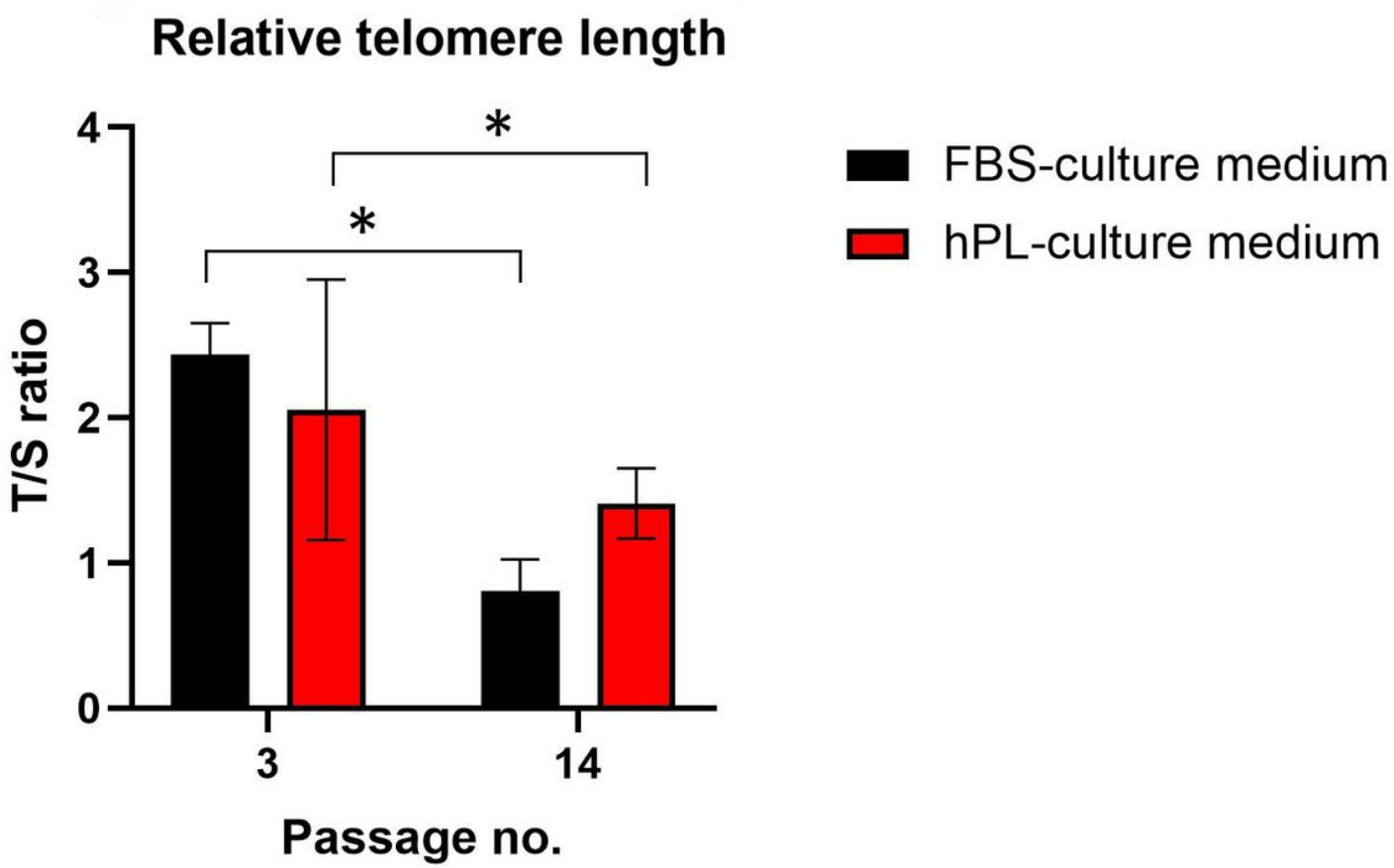

Figure 11

Average relative telomere length measured between 3rd and 14th passages using qPCR. Both groups of hNDP-SCs experienced the shortening of relative telomere length in the 14th passage ($p < 0.05$), but it was more noticeable in the case of hNDP-SCs cultivated in medium with 2% FBS. The data are presented as the mean ± SD. The Shapiro-Wilk test or Kolmogorov–Smirnov test was used for normal distribution evaluations. The statistical analyses were calculated using the paired t-test.*

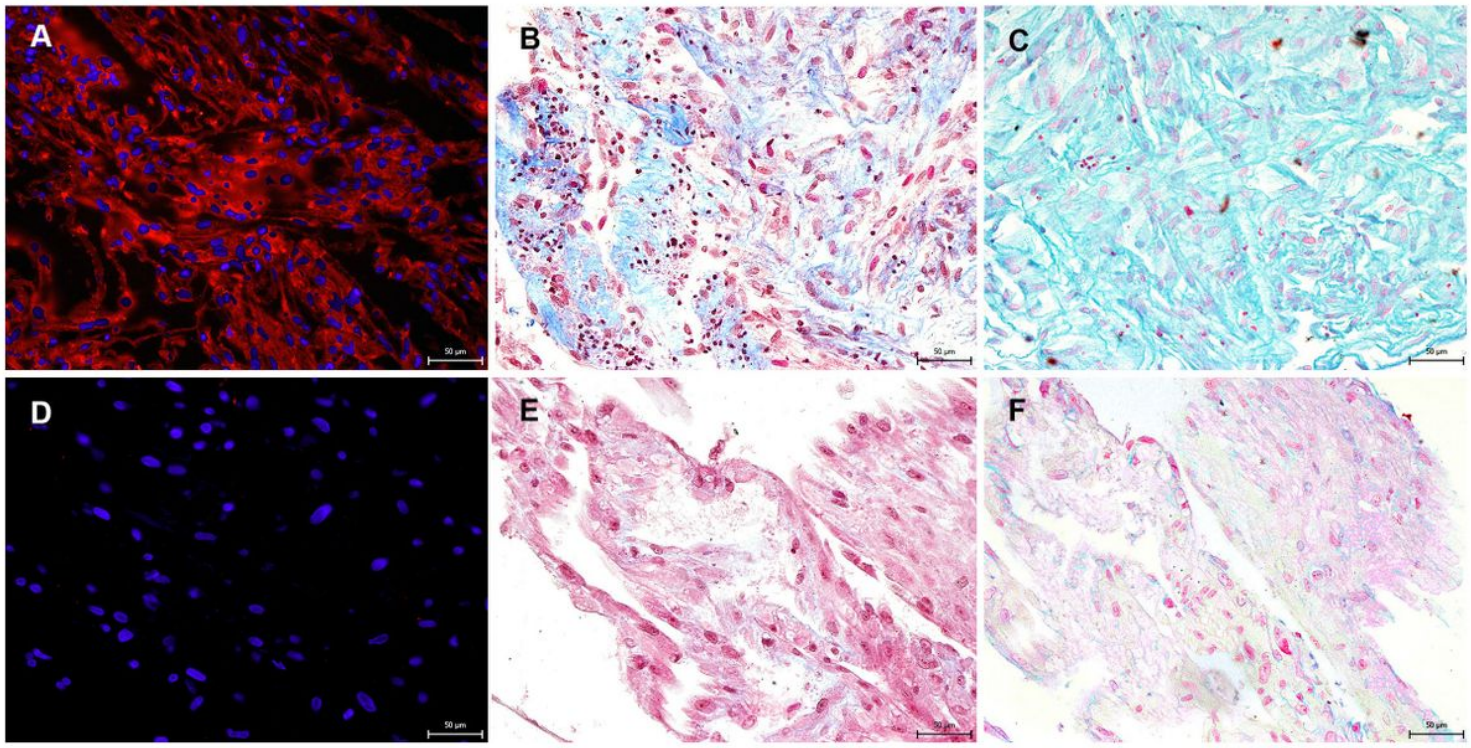

Figure 12

Detection of chondrogenic differentiation in the extracellular matrix of hNDP-SCs cultivated in the FBS-culture medium; scale bar 50 µm; A-C) after 3-week cultivation in chondrogenic differentiation medium; D-E) undifferentiated hNDP-SCs; A,D) immunocytochemical detection of collagen type II (red fluorescence); B,E) histological detection of collagen and procollagen after blue Masson's trichrome stain (blue areas); C,F) histological detection of acid mucopolysaccharides after Alcian blue stain (turquoise areas).

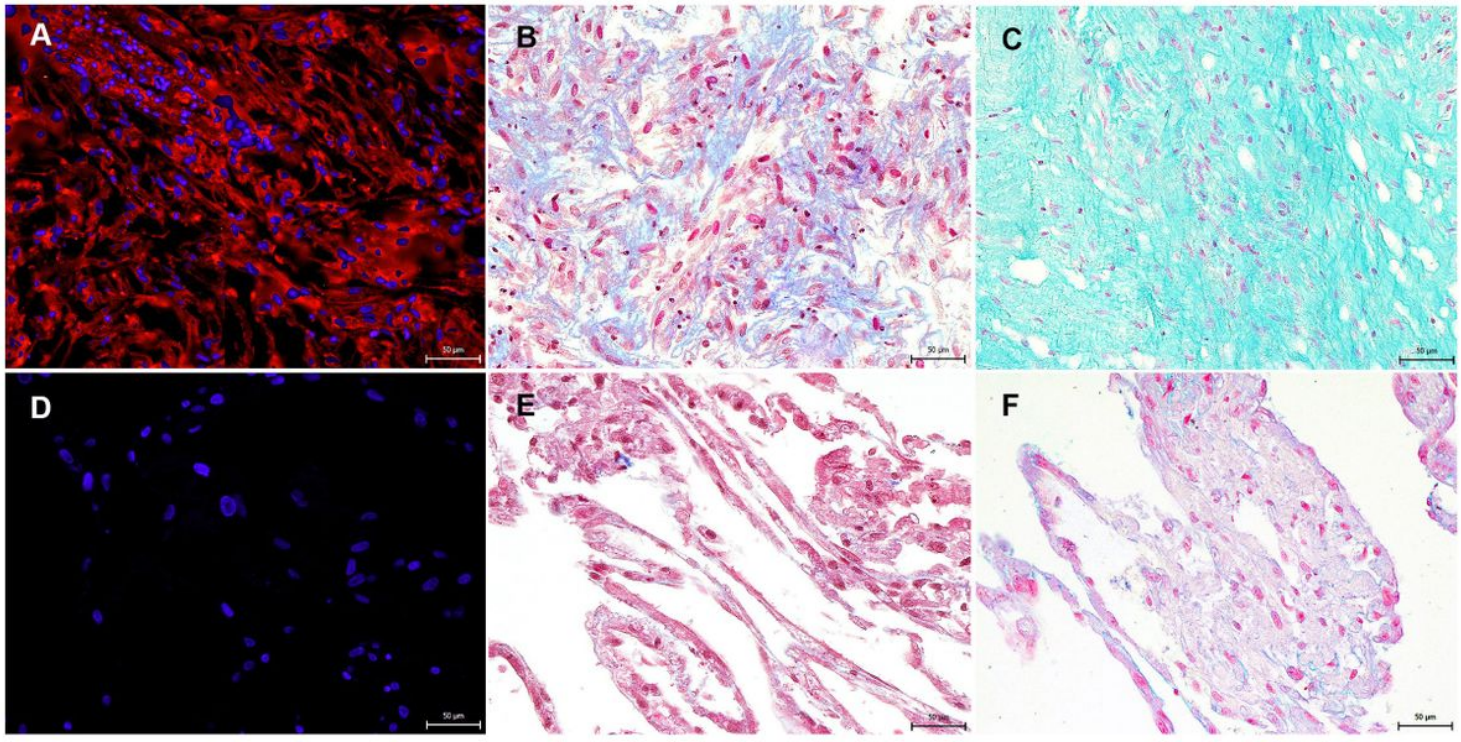

Figure 13

Detection of chondrogenic differentiation in the extracellular matrix of hNDP-SCs cultivated in the hPL-culture medium; scale bar 50 µm; A-C) after 3-week cultivation in chondrogenic differentiation medium; D-E) undifferentiated hNDP-SCs; A,D) immunocytochemical detection of collagen type II (red fluorescence); B,E) histological detection of collagen and procollagen after blue Masson's trichrome stain (blue areas); C,F) histological detection of acid mucopolysaccharides after Alcian blue stain (turquoise areas).

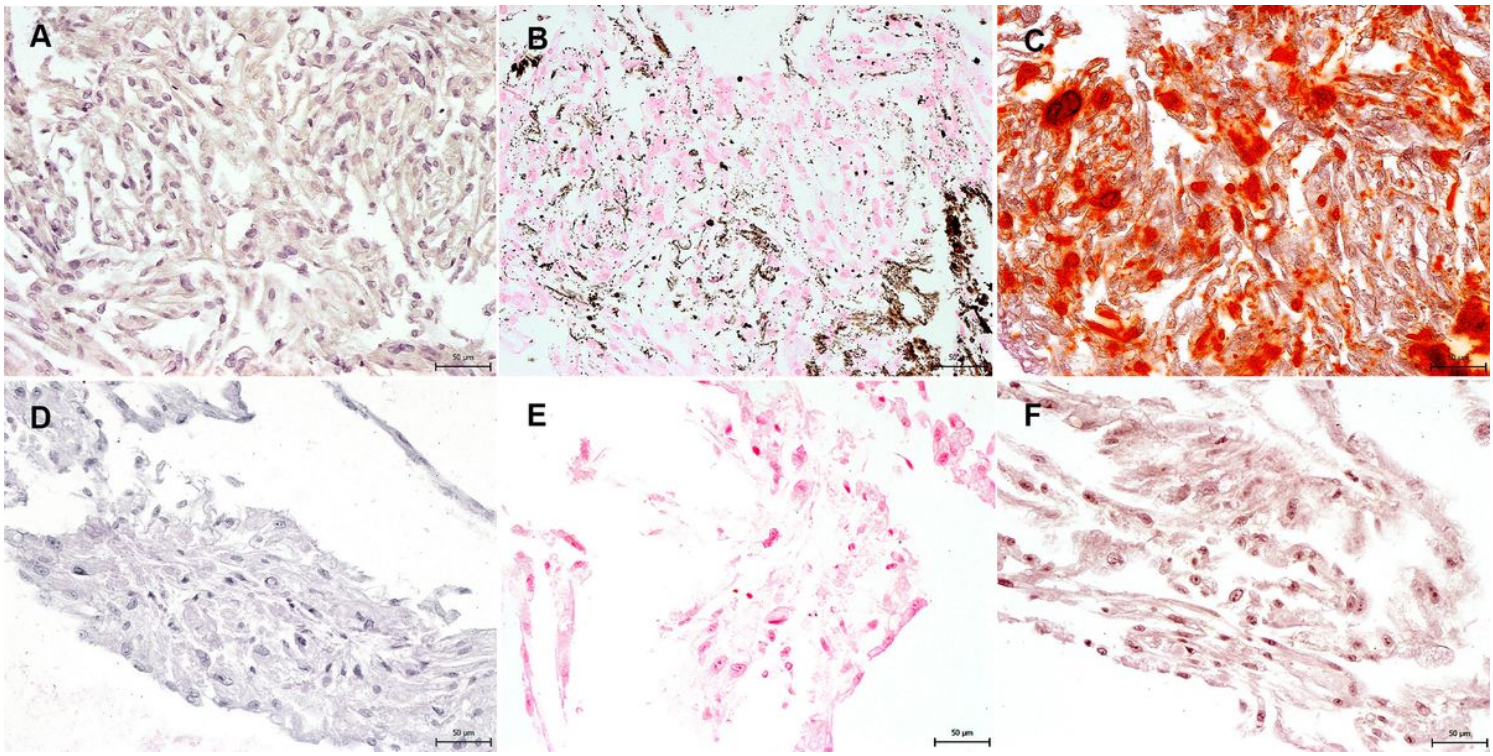

Figure 14

Detection of osteogenic differentiation in the extracellular matrix of hNDP-SCs cultivated in the FBS-culture medium; scale bar 50 µm; A-C) after 3-week cultivation in osteogenic differentiation medium; D-E) undifferentiated hNDP-SCs; a,d) immunocytochemical detection of osteocalcin (brown or rusty areas); B,E) histological detection of calcium phosphate deposits after von Kossa stain (dark brown or black spots); C,F) histological detection of calcium deposits after Alizarin Red stain (red areas).

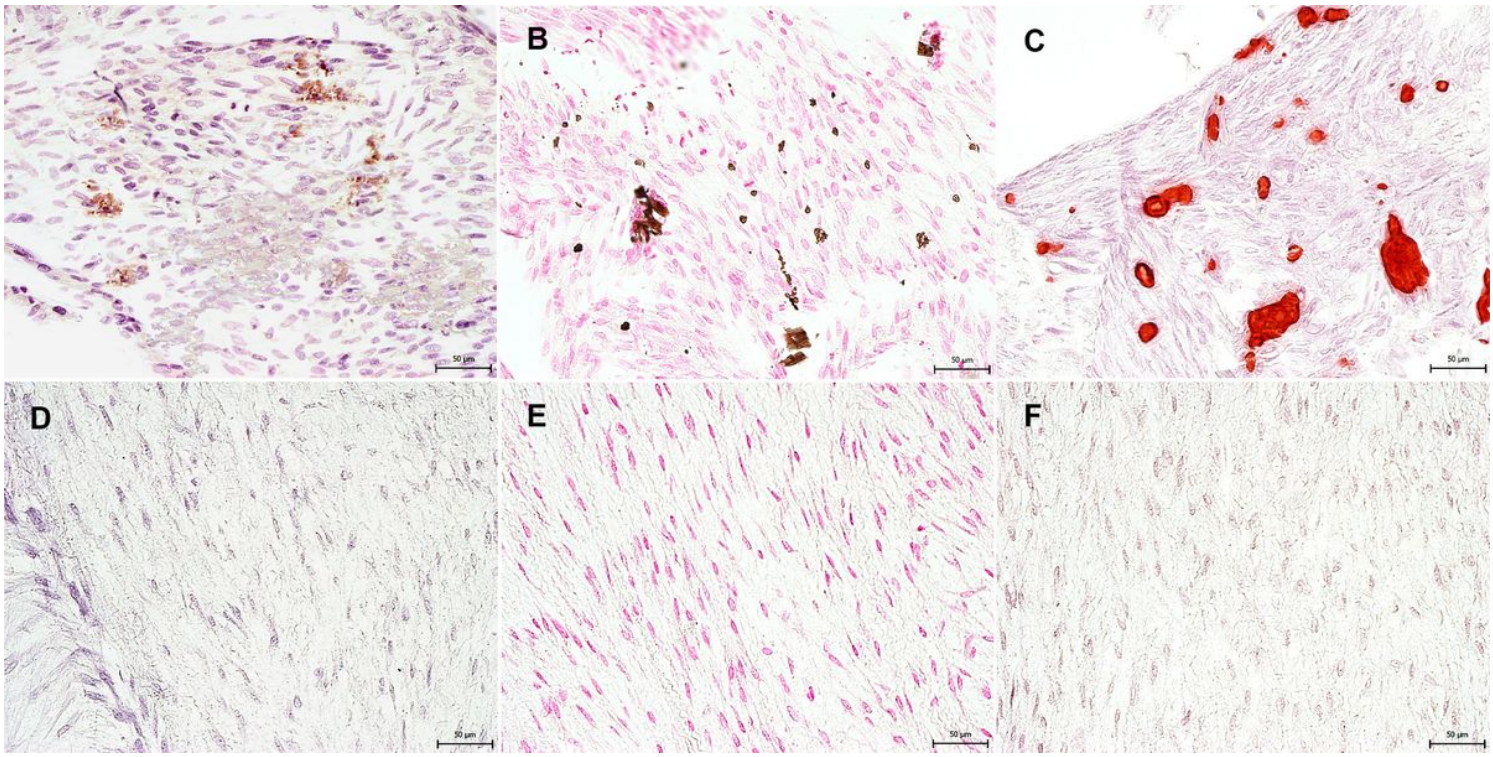

Figure 15

Detection of osteogenic differentiation in the extracellular matrix of hNDP-SCs cultivated in the hPL-culture medium; scale bar 50 µm; A-C) after 3-week cultivation in osteogenic differentiation medium; D-F) undifferentiated hNDP-SCs; A,D) immunocytochemical detection of osteocalcin (brown or rusty areas); B,E) histological detection of calcium phosphate deposits after von Kossa stain (dark brown or black spots); C,F) histological detection of calcium deposits after Alizarin Red stain (red areas).

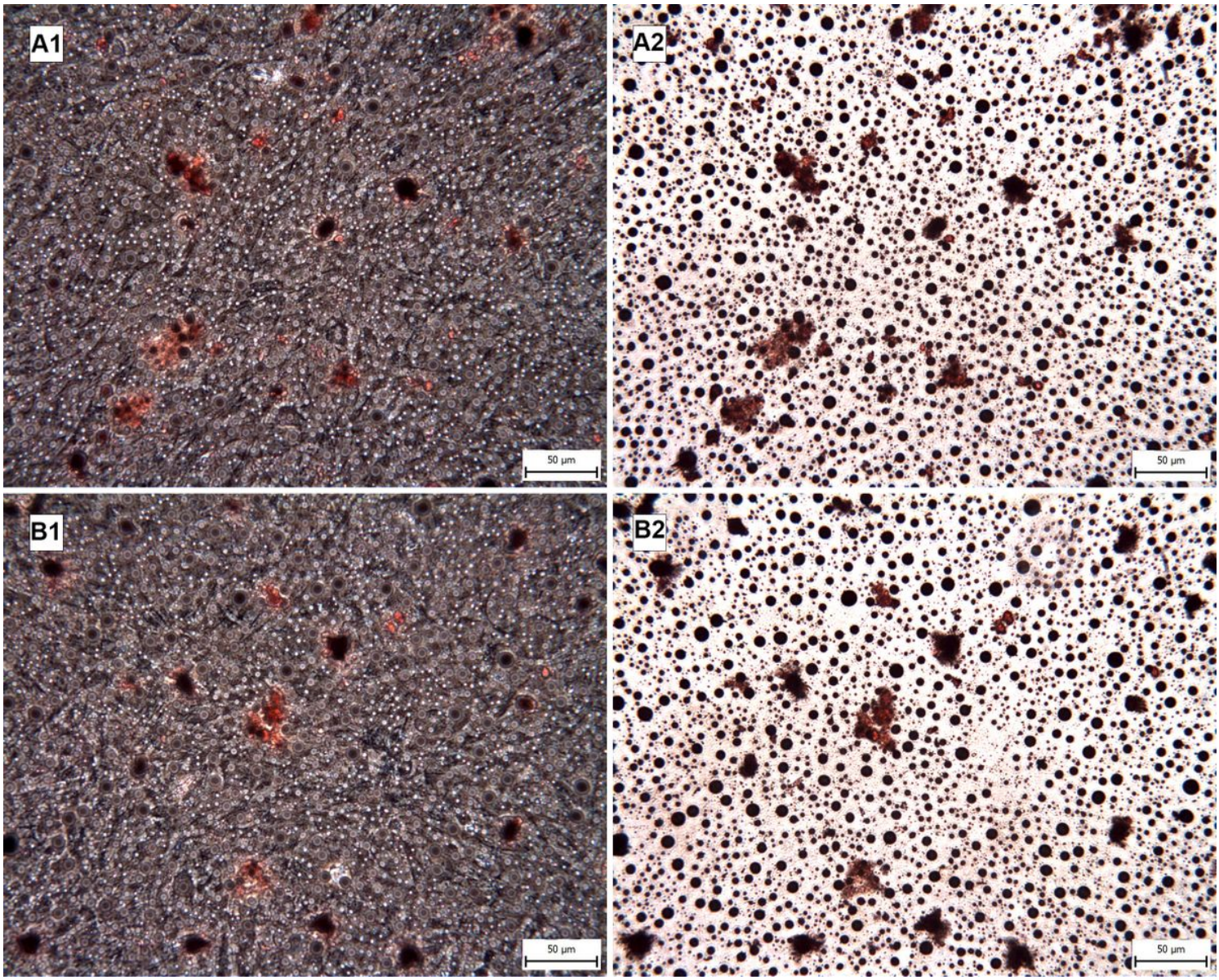

Figure 16

Detection of adipose droplets and vacuoles in the extracellular matrix of hNDP-SCs after 4-week cultivation in adipogenic differentiation medium. After Oil Red staining, the adipose vacuoles are revealed as red areas. Scale bar 50 µm. A) FBS-cultivated hNDP-SCs: (A1) phase contrast optical microscope, A2) inverted optical microscope), B) hPL-cultivated hNDP-SCs: (B1) phase contrast optical microscope, B2) inverted optical microscope).