Original Article

Proliferative Capacity and Phenotypical Alteration of Multipotent Ecto-Mesenchymal Stem Cells from Human Exfoliated Deciduous Teeth Cultured in Xenogeneic and Allogeneic Media

(mesenchymal stem cells / allogeneic serum / human blood plasma / platelet-rich plasma / SHED / dental pulp / xenogeneic serum)

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Abstract. Foetal calf serum (FCS) is a standard supplement used in media for *in vitro* stem cell cultivation. This xenogeneic supplement remains widely used for its favourable growth-promoting properties and ease of accessibility; however, it is inherently not fit for human medicine due to its capacity to temper with the cultured cell quality. For this reason, the international community encourages research and development of allogeneic sera, which would expunge this issue. This study aims to investigate the differences in proliferative capacity, phenotype, and differentiation capacity of ecto-mesenchymal stem cells from human exfoliated deciduous teeth (SHED) cultured *in vitro* in media supplemented with allogeneic and xenogeneic sera. To address these aims, we cultured three lineages of stem cells in media supplemented with FCS in a concentration of 2% + growth factors; human blood plasma and platelet-rich plasma in concentrations of 2% + growth factors, and 10%. Here, the xenogeneic cultivation was considered as a basis for comparison because this serum is commonly used in studies concerning ecto-mesenchymal stem cells. The study shows that multipotent ecto-mesenchymal SHED can be feasibly cultivated in media where the xenogeneic FCS is substituted by allogeneic platelet-rich plasma, considering the cultured cell proliferative and differentiation capacities. We have also proved that different sera impact the cultured cells' phenotype differently, which has major implications for previous and future stem cell research and regenerative therapy.

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Abbreviations: α-MEM – α modification of minimum essential medium, AvCPD – average cumulative population doublings, bFGF – basic fibroblast growth factor, CD – cluster of differentiation, DP – dental pulp, DPSC – dental pulp stem cell, FBS – foetal bovine serum, FCS – foetal calf serum, HP – human plasma, IL – interleukin, ITS – insulin-transferrin-sodium-selenite supplement, MSC – mesenchymal stem cells, OCT – octamer-binding transcription factor, PAF – platelet-activating factor, PBS – phosphate-buffered saline, PD – population doublings, PDGF – platelet-derived growth factor, PRP – platelet-rich plasma, Serum+ – serum enriched with growth factors, SHED – stem cells from human exfoliated deciduous teeth, TGF – transforming growth factor, VEGF – vascular endothelial growth factor.

Introduction

Mesenchymal stromal/stem cells (MSC) are multipotent stromal cells that can differentiate *in vitro* or *in vivo* into a variety of mature cell types (Nardi and da Silva Meirelles, 2006; Knoblich, 2008; Keating, 2012). The exact definition of what properties MSC should manifest remains somewhat fluent with the growing body of research and due to the ever increasing number of sources of MSC (Keating, 2012). Nevertheless, the most recent definition, as proposed by the International Society for Cellular Therapy in 2006, states the following minimum denomination criteria and requirements: 1) MSC must manifest the ability to self-renew, plastic adherence and *in vivo* trilineage differentiation to osteogenic, adipo-

genic and chondrogenic cells; and 2) they have to express cell surface markers CD105, CD73 and CD90 and lack the haematopoietic markers CD45, CD19, CD19 or CD79, CD14 or CD11b, and HLA-DR (Dominici et al., 2006). MSC retain these unique properties from ontogenesis to adulthood (Ackema and Charité, 2008), which effectively broadens the development potential of multicellular organisms and finds use ranging from tissue regenerative therapy and engineering to cell therapy (Hartenstein, 2013; Abdelalim, 2014), drug testing and targeted drug delivery, and in disease and immunomodulation research (Lai et al., 2013). The ease and minimal ethical challenges involved in obtaining the primary MSC presents additional major benefit, as they are documented to be derived from multiple sources such as various tissues (Shih et al., 2005), bone marrow (Caplan, 1991), umbilical cord blood (Sarugaser et al., 2005), breast milk (Patki et al., 2010) and dental pulp (DP, Gronthos et al., 2000). While the bone marrow MSC are the most frequently used MSC in human regenerative medicine today, their collection meets multiple shortcomings; according to Huang et al. (2009), such shortcomings include "pain, morbidity, and low cell numbers upon harvest". These authors point out that MSC can also be obtained from other sources causing minimal impact to the donor and allowing for higher cell numbers, such as dental tissues. The least invasive source of dental MSC are then deciduous teeth, providing us with the stem cells from human exfoliated deciduous teeth (SHED).

SHED are postnatal MSC by phenotype (Huang et al., 2009; Potdar and Jethmalani, 2015) as well as by their capacity of self-renewal and multilineage differentiation (Gronthos et al., 2002; Miura et al., 2003; Zhang et al., 2006). Due to their origin in neural crest, SHED, also labelled as ecto-mesenchymal SC, may possess different characteristics to other MSC (Huang et al., 2009). According to Kerkis et al. (2006), SHED-specific surface markers include OCT3/4, CD29, CD31, CD44, CD73, CD90, CD105, CD146 a CD166, and exclude CD14, CD34, CD43 or CD45. Furthermore, SHED have also been proved to be clonogenic (Muira et al., 2003) and capable of differentiation into adipogenic, neural (Gronthos et al., 2002) and myogenic cells (Zhang et al., 2006). According to Muira et al. (2003), SHED are an accessible resource of high-quality human postnatal stem cells that are "capable of providing enough cells for potential clinical application" (Yamada et al., 2011). In comparison with MSC isolated from the pulp of adult teeth (DPSC), SHED show favourable research and use-relevant difference in decreased population doubling time (Kerkis et al., 2006; Morsczeck et al., 2010; Nourbakhsh et al., 2011) and increased telomerase activities (Akpinar et al., 2014). For the above reasons, SHED are one of the most promising sources of MSC for human stem cell therapy/regenerative medicine (Huang et al., 2009).

Stem cell therapy/regenerative medicine is an enthusiastic strategy for regeneration, rather than reparation, of lost or damaged tissues making use of cultured MSC. While this field's scientific knowledge base has accumulated since 1968, it still deals with an unfavourable amount of unknowns that prohibit safe clinical studies. In order to avoid therapy failure and undesirable side effects, the MSC properties under various external environmental conditions have to be perfectly understood (Liu et al., 2014). At present, we recognize that the cultured MSC properties are influenced by two major factors: 1) the inter-individual characteristics of the donor (Katsara, 2011), and 2) the culture medium composition (Suchánek et al., 2013). It is mostly the latter that presents ethical and medical objections to application of xenogeneic supplements in culture media for human use (Mannello and Tonti, 2007)

The use of xenogeneic supplements is not only inherently inconsistent with the good laboratory and manufacturing practice (Dimarakis and Levicar, 2006; Sekiya et al., 2002; Bieback et al., 2010); there is also a body of evidence proving that the xenogeneic cultured MSC can open the human recipient to serious medical issues. Kocaoemer et al. (2007) caution of the cultured MSC internalizing the bovine serum proteins, which can result in severe immunological reaction (Tuschong et al., 2002; Spees et al., 2004; Martin et al., 2005; Heiskanen et al., 2007). Suchanek et al. (2013) also caution of the cultured MSC karyotypic instability, which can cause cell-malignant transformation; Bruinink (2004) and Dictus (2007) caution of metabolic and morphological changes, which can cause a large variety of cultured MSC behavioural changes; and Halme and Kessler (2006) and WHO (1997) caution of the danger to transfer zoonoses, mostly viral and prion diseases, onto the human recipient (Chachques et al., 2004; Spees et al., 2004). For the aforementioned reasons, the international medical community has aimed to forbid the use of these supplements in human clinical trials and medicine (WHO, 1997; Sekiya et al., 2002; Dimarakis and Levicar, 2006). Nevertheless, the xenogeneic supplement foetal calf/bovine serum (FCS/FBS) (Honn et al., 1975) remains the most widely used supplement in experimental *in vitro* MSC cultivation, mostly for its high concentration of growth factors (Weyard et al., 2013), and so most of the scientific observations of cultured MSC properties are based on MSC cultured in FCS-supplemented media (Dimarakis and Levicar, 2006). Logically, to be able to build up human medicine-relevant knowledge about the properties of cultured SHED, we have to avoid using FCS-supplemented media and replace it with either allogeneic serum-supplemented media or serum-free media.

Nevertheless, even the allogeneic and serum-free sera are not issue free. While serum-free media composition can be standardized, allowing for chemically defined and controlled culture conditions and elimination of potential sources of zoonoses (Froud, 1999; Spees et al., 2004; Marti et al., 2005; Halme and Kessler, 2006; Kocaoemer et al., 2007), these sera are not universally applicable to culturing all types of MSC, and their effi-

ciency and accessibility remains low and non-transparent (Brunner, 2010). The other xeno-free culturing supplement is allogeneic serum, which is the cheaper of the two and is universally applicable to cultivation of any type of MSC (Weyand et. al., 2013). These sera are human blood derivatives including autologous or pooled human serum, cord blood serum and platelet derivatives (Weyand et. al., 2013), which makes them easily accessible; however, Zhang et al. (2015) point out that "the allogeneic immune response, contamination, and donor-to-donor and donor-to-receptor differences do obstruct the application of the cultured MSC". All issues considered, numerous researchers have confirmed that the allogeneic sera are the most viable substitutes of FCS (Stute et al., 2004; Kobayashi et al., 2005; Lin et al., 2005; Shahdadfar et al., 2005; Stojkovic et al., 2005; Mizuno et al., 2006; Müller et al., 2006). There are multiple types of allogeneic sera from which human blood plasma (HP) and platelet-rich plasma (PRP) are the two easiest to obtain.

HP and PRP sera contain the same growth factors and cytokines but differ in their concentration due to the platelet degranulation in PRP plasma, which releases extra factors [HP < (3,4 or 5)*PRP] (Dohan et al., 2006; Marx, 2001). These growth factors enumerate: platelet-derived growth factor (PDGF), transforming growth factors β 1 and β 2 (TGF β1, TGF β2), vascular endothelial growth factor (VEGF), platelet-derived endothelial cell growth factor, interleukin 1 (IL-1), basic fibroblast growth factor (bFGF), platelet-activating factor 4 (PAF-4) and many others (Dohan et al., 2006a,c). According to Bernardo et al. (2007) and Kilian et al. (2004), the plasma additive to human serum in PRP also provides increased stability of the properties of the cultured cells, their intensified migration ability and immune suppressivity.

We were interested in finding an allogeneic serum that could replace the xenogeneic serum FCS in further research on SHED applications in human regenerative medicine. This serum would have to prove effective in supporting the high proliferative capacity of SHED and would not, or minimally, impact the cultured SHED phenotype and so their ability to differentiate. Our research aims were, successively:

I. Compare the cultured SHED proliferative capacity/cumulative population doubling (6^{th} – 10^{th} passage), morphology (at 10^{th} passage) and viability (at 10^{th} passage) by sera (2% FCS+, 2%+ & 10% HP, 2%+ & 10% PRP)
II. Compare cluster of differentiation (at 10^{th} passage) of allogeneic-media cultured SHED and xenogeneic-media cultured SHED
III. Evaluate the allogeneic-media cultured SHED capacity to differentiate (from the 10^{th} passage, cells cultivated in allogeneic serum-supplemented medium that most promoted the proliferation rate)

We chose to use the exfoliated deciduous teeth-derived SHED and three different sera: xenogeneic FCS and allogeneic pooled HP and PRP, for their properties and ease of access. We used SHED from three different donors to support the statistical validity of our research results; nevertheless, we were aware that the three lineages might manifest vast heterogeneity due to their genetic background, the stage of the cell cycle during sample collection, the gender and age of the donor, and the donor health status (Wagner et al., 2006). All the primary SHED were first cultivated in 2% FCS enriched with growth factors EGF, PDGF and ITS (2% FCS+) medium, for the duration of five passages, to ensure a sufficient amount of coherent biological material for the experimental phase. To reduce the possible impact of FCS on the cultured cell population during the first five passages, we used the lowest possible concentration of 2% FCS. The sera used as the test supplements in media for 6^{th} to 10^{th} passage were: the 'control' 2% FCS+ and allogeneic sera HP and PRP in concentrations of 2%+ (enriched with growth factors EGF, PDGF and ITS) and 10%. The allogeneic medium that promoted the SHED proliferation rate the most was used for differentiation analysis into osteogenic, adipogenic and chondrogenic cells (MSC definition), and additionally into myogenic and neurogenic cells (a goal outlined in the study-supporting grant). All the cultivation/differentiation experiments were carried out in quadruplets for every lineage; the results were averaged and noted with standard deviation. The results for cluster of differentiation of all tested lineages were also averaged. The final main limitation to our research and discussion is non-existence of the literature that examines cultivation of SHED in allogeneic sera; therefore, we compared our results with either SHED cultivated in xenogeneic sera or other dental tissue research including allogeneic sera experiments.

The results of this study contributed to evaluation of the relevancy of MSC research based on cultivation in xenogeneic (FCS) supplemented media, where the deciding factor is the similarity/difference in the xenogeneic and allogeneic cultured cells' properties.

Ethical guidelines statement

This study is based on biological material obtained for medical reasons and with the subjects' legal (representatives) informed consent, after they have been given all the research relevant information. The extraction of the deciduous teeth was indicated for either untreatable caries lesion or orthodontic treatment. The allogeneic sera were obtained from a group of donors who fulfilled medical requirements to donate blood components for medical use. The supplements were further prepared in collaboration with the Transfusion Department of the University Hospital Hradec Králové, pooled from material of at least five different donors, to be statistically relevant and to avoid the risk of bias. The method of this experiment, the use of the relating biological material and the associated legal requirements were discussed and approved by the Ethical Committee of the University Hospital Hradec Králové, ref. 200712 S01P; and the Ethical Committee of the University Hospital Hradec Králové, ref. 201011 S14P.

To address the aims of this study, the following study structure was established [Fig. 1]:

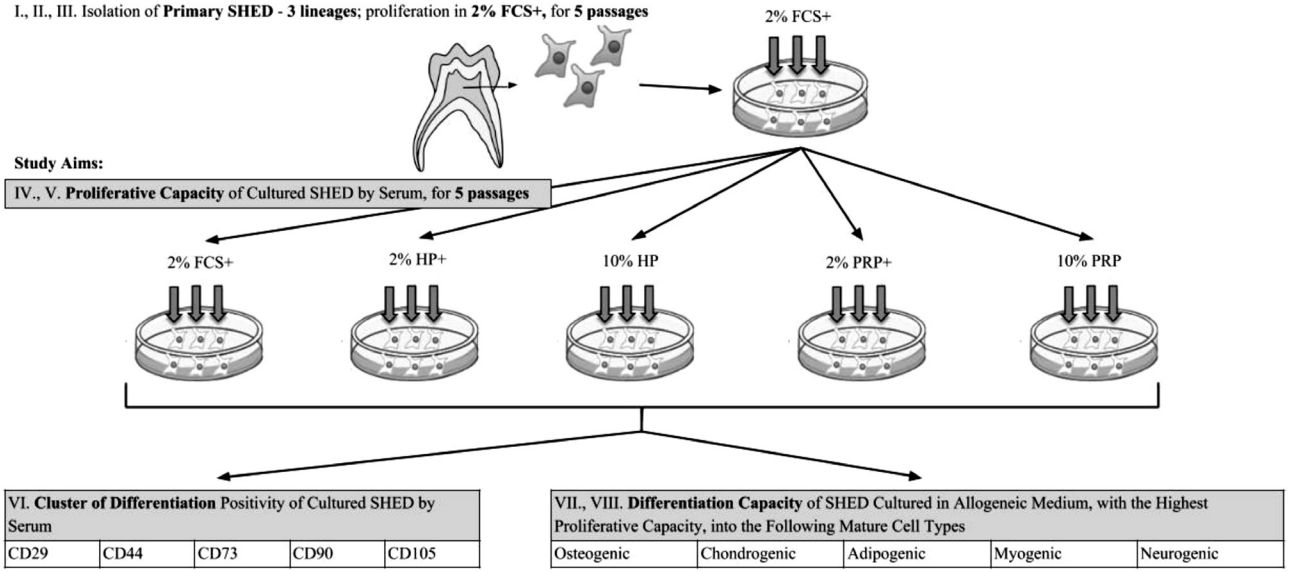


Fig. 1. Graphic representation of the experimental material and methods

Material and Methods

To address the aims of this study, the following study structure was established (Fig. 1):

I. Extraction of exfoliated deciduous teeth and isolation of primary SHED

The study experiment presents three lineages of SHED obtained from three healthy donors (7 years old male Ze001, 3 years old male Ze002 and 9 years old female Ze003). Tooth extractions were performed under standard conditions in local anaesthesia. The extracted teeth were treated with disinfectant and then stored, fully immersed, in a transport medium containing 1 ml of HBSS (Invitrogen, Carlsbad, CA), 9 ml water for inj. (Bieffe Medital, Capannori, Italy), 200 µl/10 ml gentamycin (Invitrogen), 200 µl/10 ml streptomycin (Invitrogen), 200 µl/10 ml amphotericin (Sigma, St. Louis, MO) and 200 µl/10 ml penicillin (Invitrogen) at 4 °C. DP was extracted under sterile conditions. It was first cut into pieces of about 1 mm^3, then enzymatically dissociated for 40–60 min at the temperature of 37.0 °C in a solution of enzyme collagenase type I (Sevapharma, Praha, Czech Republic), dispase (Invitrogen), PBS (Invitrogen) and HBSS in the 1 : 1 : 1 : 1 ratio (Jo et al., 2007).

II. Verification of the extracted SHED ability to self--renew (in 2% FCS+ over Hayflick's limit)

To prove the capacity of the extracted SHED to surpass the Hayflick's limit (Hayflick, 1965), the ability to self-renew, was essential for the success of our study as we needed to be sure there would be enough biological material to work with; we therefore performed the following experiment. The obtained cells and remnants of soft tissue suspension were mixed with the α modification of minimum essential medium (α-MEM, Invitrogen) supplemented with 20 % of FCS (PAA, Dartmouth, MA) to inactivate the enzymes. The suspension was then centrifuged for 5 min at 600 *g* to clean the cells of the supernatant. The resulting cell pellet was re-suspended and seeded into a culture flask Cell+ (Sarstedt, Newton, NC) containing standard cultivation media composed of α-MEM, 2% FCS, 10 ng/ml EGF (PeproTech, London, UK), 10 ng/ml PDGF (PeproTech), L-ascorbic acid (Sigma), 2% glutamine (Invitrogen), penicillin/streptomycin (Invitrogen), gentamycin (Invitrogen), dexamethasone (Sigma) and was supplemented with insulin-transferrin-sodium-selenite supplement (ITS) (Sigma) at a concentration of 10 µl/ml. The cells were cultivated in an incubator at 37 °C, in an atmosphere with 5% CO_2. The prime culture cells were harvested after 5 days and seeded into passage No. 1. Every other passaging was done after reaching 70 % confluence and the cells were seeded in a density of 5,500 cells/cm^2. We terminated this experiment after reaching 40 PD/Hayflick's limit.

III. Preparation of experimental SHED lineages (cryopreservation of the 2% FCS+ cultivated SHED harvested from the 5th passage)

To individuate the three SHED lineages throughout the study text, they were labelled Ze001, Ze002, and Ze003. The cells from the 5th passage were cryopreserved and stored as an input cell population for the next experimental phase.

IV. Experimental test media-cultivation – seed the 2% FCS+ cultured SHED in five different media (2% FCS+, 2%+ & 10% HP, 2%+ & 10% PRP) for another five passages (6th–10th)

The cells cryopreserved in the 5th passage, as described above, were then thawed and seeded into five media supplemented with sera that differed in both composition and concentration. The following media were chosen on the basis of a preliminary study by Suchánek (2009). The xenogeneic serum we appointed as control serum was 2% FCS enriched with growth factors EGF, PDGF and ITS (2% FCS+), which, according to the aforementioned study, promoted the cell proliferative capacity more than 10% FCS (Suchánek, 2009); a supplement which is commonly used in the dental pulp stem cell (DPSC) literature (Smith et al., 2012; Nyman et al., 2013). The composition of the blood derivative-based-allogeneic test media was then elected accordingly as 2%+ (2% HP+ and 2% PRP+, both enriched with EGF, PDGF and ITS) to provide comparative results with the xenogeneic serum. Besides these 2% enriched media, we also added 10% HP and 10% PRP media, as they have been suggested as feasible substitutes to the 2% FCS+ by multiple sources (Lucarelli et al., 2003; Kanno et al., 2005; Mishra et al., 2009). We did not test any media with concentrations of HP or PRP higher than 10 % as from this point on they become increasingly toxic, and at 20% concentration arrest cell culture growth (preliminary study – unpublished data).

The full composition of the test media was as follows:
[2% FCS+] (control medium): α-MEM, 2% FCS, L-ascorbic acid, 2% glutamine, penicillin/streptomycin, gentamycin, dexamethasone and 10 µl of ITS/ml, 10 ng/ml EGF, 10 ng/ml PDGF
[2% HP+]: α-MEM, 2% HP, L-ascorbic acid, 2% glutamine, penicillin/streptomycin, gentamycin, dexamethasone and 10 µl of ITS/ml, 10 ng/ml EGF, 10 ng/ml PDGF
[2% PRP+]: α-MEM, 2% PRP, L-ascorbic acid, 2% glutamine, penicillin/streptomycin, gentamycin, dexamethasone and 10 µl of ITS/ml, 10 ng/ml EGF, 10 ng/ml PDGF
[10% HP]: α-MEM, 10% HP, L-ascorbic acid, 2% glutamine, penicillin/streptomycin, gentamycin, dexamethasone
[10% PRP]: α-MEM, 10% PRP, L-ascorbic acid, 2% glutamine, penicillin/streptomycin, gentamycin, dexamethasone

V. Expressing cultured SHED proliferative capacity/cumulative population doubling (6th–10th passage), morphology (at 10th passage) and viability (10th passage) by sera

The 2% FCS+ medium-cultivated SHED (for 5 passages) were reseeded and further cultivated in the above test media for another five passages. During this test cultivation, we regularly measured the cell proliferative capacity (cumulative population doublings and population doubling time) using a Z2 Counter (Beckman Coulter, Miami, FL). Cell morphology was recorded by a BX51 Olympus microscope (Olympus, Tokyo, Japan) equipped with digital camera Olympus DP71 (Olympus). Their viability was measured using a Vi-Cell analyser (Beckman Coulter) at the 10th passage.

VI. Expressing cultured SHED cluster of differentiation (at 10th passage) by sera

The phenotypical analysis was performed on undifferentiated cultured SHED from the 10th passage. These cells were detached and stained sequentially with various immunofluorescent primary antibodies to detect the presence of surface molecules/CDs/MSC markers. The percentage of cells manifesting positivity to a marker was determined on the basis of their fluorescence intensity > 0.5 % (which is the percentage of cells that stain even without the possession of the pursued marker). The following classification criteria to detect the various markers were: < 10 % no expression, 11–40 % low expression, 41–70 % moderate expression, and > 71 % high expression. The analysis was performed in Cell Lab Quanta (Beckman Coulter) using the following antibodies: anti-CD29 (BD Biosciences, Heidelberg, Germany), anti-CD44 (BD Biosciences), anti-CD73 (BD Biosciences), anti-CD90 (BD Biosciences) and anti-CD105 (BD Biosciences). The positivity of these CD markers should be ≥ 90 % in MSC (Dominici et al., 2006; Akpinar et al., 2014). The lineages cultured in the allogeneic media that most promoted cell growth were further tested for the presence of OCT3/4 PE IgG 2a, clone EM92 (eBioscience, San Diego, CA). This marker is generally used as a marker of undifferentiated cells. The presence of this protein is essential for undifferentiated embryonic stem cell self-renewal. These antibodies were chosen as they are the best studied throughout the literature concerning stem cells derived from DP (Kerkis, 2006).

VII. Allogeneic medium-cultured SHED differentiation capacity (of undifferentiated SHED from the 10th passage cultivated in allogeneic serum-supplemented medium that most promoted the cell proliferation rate)

Three lineages of undifferentiated 10th passage SHED cultured in allogeneic media with the highest proliferative capacity-boosting ability [1st–5th passage in 2% FCS+] differentiated into the following cell types using the following media and tools of evaluation (Karbanová et al., 2011). We seeded approximately 5,500 cells/cm^2 to induce the various types of differentiation, changed the media every 3–4 days and cultivated the cells for a minimum of four weeks (Akpinar et al., 2014).

VII.A. Osteogenic differentiation

SHED growing in a confluent cell monolayer were cultivated in osteogenic differentiation medium composed of the tested media enriched with 10 mM glycerophosphate (Sigma). After four weeks the cells were stained for osteonectin.

VII.B. Chondrogenic differentiation

SHED growing as micromass culture were cultivated in a non-adherent test tube using chondrogenic differentiation medium composed of the tested medium enriched with TGF-β1 (R&D Systems, Minneapolis, MN) in a concentration of 50 ng/ml. After four weeks the pellet of the differentiated cells was evaluated.

VII.C. Adipogenic differentiation

SHED growing in confluent cell monolayers were differentiated in MSC cell adipogenic differentiation medium (Cyagen Biosciences Inc, Silicon Valley, CA). After four weeks, the cells were fixed and stained to detect intracellular lipid droplets in mature adipocytes with 0.18% Oil Red O (Sigma-Aldrich, St. Louis, MO).

VII.D. Myogenic differentiation

SHED growing in a confluent cell monolayer were exposed to myogenic differentiation medium composed of 5-azacytidine (Sigma-Aldrich), in a concentration of 3 μM/l, for the period of 24 h. After 24 h the differentiation medium was removed and the cells were cultivated in media containing the tested allogeneic serum. After four weeks the cells were stained for α-actinin, cardiac troponin and desmin.

VII.E. Neurogenic differentiation

SHED growing in a confluent cell monolayer were cultivated in neurogenic differentiation medium composed of 50 ml DMEM (Gibco, Paisley, UK), 50 ml F12 HAM (Gibco), 2 ml B27 Supplement (Gibco), 1 ml N2 Supplement (Gibco), 10 ng/ml bFGF (PeproTech, London, UK), 20 ng/ml EGF, 1 ml L-glutamin (Gibco), 1 ml penicilin. After four weeks the cells were stained for nestin.

VIII. Tools for evaluation of allogeneic media-cultured SHED differentiation

VIII.A. Oil Red O staining – evaluation of adipogenic differentiation

Cultures of adipogenic-differentiated cells were fixed for at least 1 h with 10% formalin in an isotonic phosphate buffer, then washed with distilled water and immersed for 2 h in a solution of 0.18% Oil Red O and extensively rinsed with water. Excess water was evaporated by placing the stained cultures under a heater with a temperature of about 32 °C. In order to determine the extent of adipose conversion, 1 ml of isopropyl alcohol was added to the stained culture dish, the extracted dye was immediately removed by gentle pipetting and its absorbance monitored by a spectrophotometer (Thermo Scientific, Waltham, MA) at 520 nm. The results were expressed as the arithmetic means of six independent experiments, bars indicated S.E.M. Absorbance was normalized to the amount of 1×10^6 cells.

VIII.B. Observation – evaluation of chondrogenic differentiation

Cultures of chondrogenic-differentiated cells were observed and assessed on the basis of their physical qualities.

VIII.C. Cytochemistry – evaluation of osteogenic, myogenic and neurogenic differentiation

Cultures of osteogenic/myogenic and neurogenic-differentiated cells and 3D nodules growing on 2-well Permanox Chamber Slides (Nunc, Roskilde, Denmark) were washed three times in phosphate-buffered saline (PBS) and then fixed with 10% formalin at 4 °C for 10 min. After further thorough wash with PBS, cells were permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) for 10 min at room temperature, blocked in PBS containing 5% normal goat (Sigma) or donkey serum (Jackson ImmunoResearch Labs, West Grove, PA) and then incubated with the primary antibody (Ab) (Table 1) at 4 °C for 12 h. As a negative control, the primary Ab was omitted. After washing, cells were either incubated with the appropriate fluorochrome-conjugated species-specific secondary Ab at room temperature for 45 min, or incubated with reagents from either an anti-mouse or anti-rabbit EnVision peroxidase kit (Dako, Glostrup, Germany), according to the manufacturer's protocol. Colour reactions were performed using the chromogen 2 μg/ml DAB (3,3´-diaminobenzidine tetrahydrochloride; Fluka, Darmstadt, Germany). For fluorescent microscopy, nuclei were counterstained with 4´-6-diamidino-2-phenylindole (DAPI; Sigma). Samples were examined using a BX51 Olympus microscope equipped with digital camera Olympus DP71.

Results

We successfully isolated and cultured three different lineages of SHED in 2% FCS+ supplemented medium over 40 population doublings (PD) (Ze003 – 62.9 PD, Ze001 – 43.4 and Ze002 – 40.2 PD), and so surpassing the Hayflick's limit, proved their ability to self-renew

Table 1. Primary Ab with manufacturer and clone

Primary Ab	Manufacturer	Clone
Anti-α-actinin (sarcomeric)	Sigma, St. Louis, MO	EA-53
Anti-cardiac troponin I	HyTest, Turku, Finland	560
Anti-desmin	Epitomics, Burlingame, CA	Y66
Anti-nestin	Chemicon, Temecula, CA	10C2
Anti-osteonectin	Novocastra Laboratories Ltd, Newcastle upon Tyne, UK	15G12

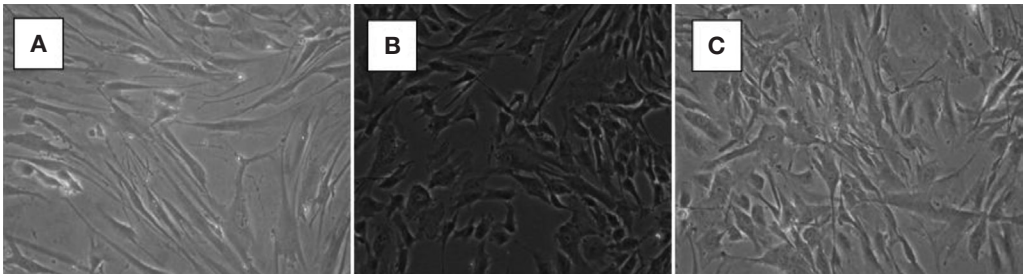


Fig. 2. Primary SHED lineages in 4th passage/2% FCS+ medium
A) Ze001 **B**) Ze002 and **C**) Ze003 primary SHED lineages in 4th passage/after 40 PD cultivated in medium supplemented with 2% FCS enriched with growth factors (2% FCS+). At 40 PD cells kept their typical morphology – spindle-shaped with prolonged processes that keep them in contact with other cells. Phase contrast microscopy, magnification 200×

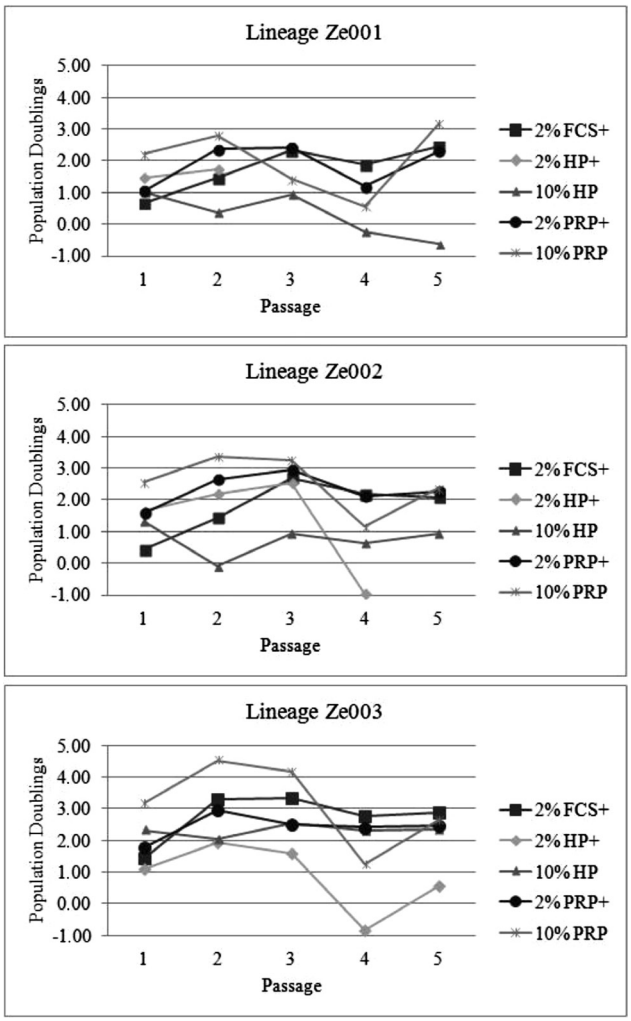
(Fig. 2), which assured us of the proliferative capacity of our samples. We did not observe any spontaneous differentiation or chromosomal instability during the long-term cultivation. Reassured by these results, we cultured the pre-experimental primary SHED in the same medium for only five passages, which allowed us to obtain large amounts of coherent biological material for all the experiments. Unfortunately, at the end we lacked material to test the cultured cells for CD105 positivity.

I. Comparison of cultured SHED proliferative capacity/cumulative population doubling (6th – 10th passage) and viability (at 10th passage) by sera (2% FCS, 2% & 10% HP, 2% & 10% PRP)

I.A. Cultured SHED proliferative capacity/cumulative population doubling (6th–10th passage) by sera (2% FCS+, 2%+ & 10% HP, 2%+ & 10% PRP)

The 10% PRP enriched medium boosted the cultured SHED proliferative capacity the most, from the start to the end, reaching approximately 12.89 average cumulative PD (AvCPD) of all lineages/5 passages). The 2% PRP+ was second best, with approx. 10.99 AvCPD of all lineages/5 passages, and 2% FCS+ was third with approx. 10.5 AvCPD of all lineages/5 passages. The HP sera performed poorly with 10% HP AvCPD, levelling horizontally in the 4th experimental passage (Graph I) and finishing the 5th passage with 5.57 AvCPD of all lineages/5 passages and 2% HP+ showing no to minimum growth all along. The 2% HP+ trial was terminated early on, as it had shown such unpromising results (Graphs I, II, Table 2).

While the cumulative PD of the 2% FCS+ grew almost linearly, the human derivatives showed more variable trends. All the sera performance then dropped in the 4th passage, with 10% PRP falling the most significantly (–1.94 AvCPD), followed by 2% PRP+ (–0.71 AvCPD), 10% HP (–0.57 AvCPD), and 2% FCS+ (–0.5 AvCPD) (Graph II). The PRPs showed the highest AvCPD during the 2nd passage, while the FCS and HP during the 3rd passage.

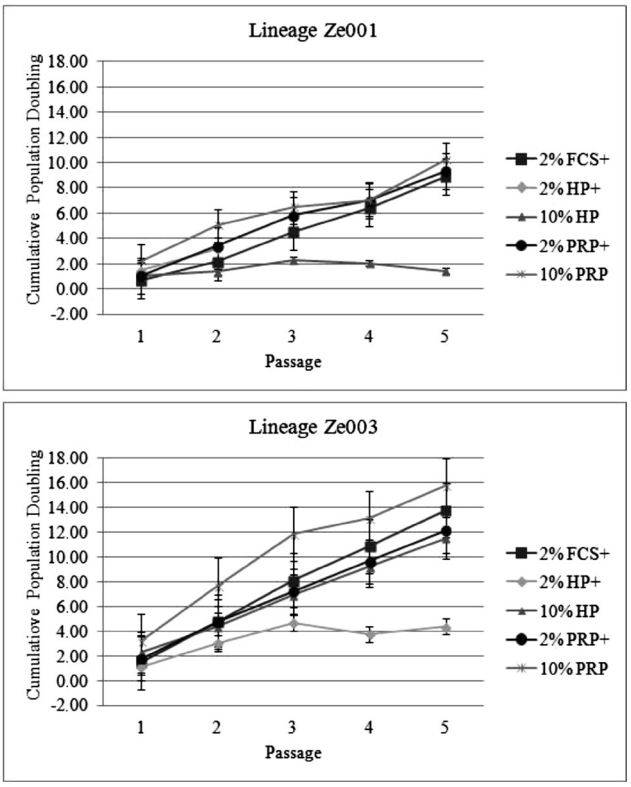


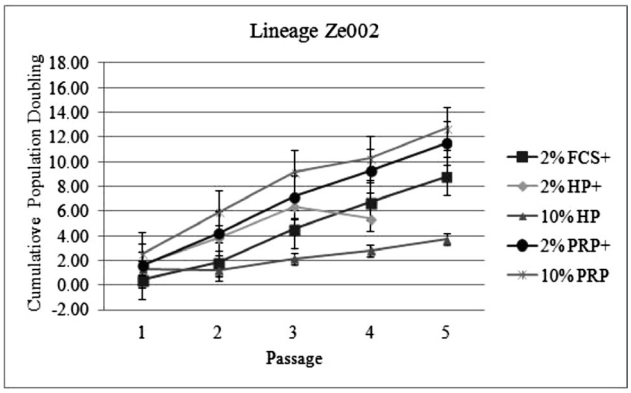
Graph I. Cumulative population doubling/passage by serum

The highest proliferation rate in the tested samples was detected in medium containing 10% PRP in all three lineages. The weakest or no proliferative capacity was observed in medium with 2% HP+. The performance of the remaining three media was comparable in all three lineages.

I.B. Cultured SHED morphology (at 10th passage)

We noticed that the cells cultivated in 10% HP changed their morphology as they got attached to the bottom of the cultivation dish and became splashed (Fig. 2C). All the other cultured SHED retained their original spindle-shaped morphology (Fig. 3A,B,D,E).





Graph II. Proliferative capacity/passage by serum
The amount of PD per passage. Standard deviation (SD) as per Table 2. SHED cultivated in 2% PRP medium showed the most stable proliferative capacity throughout the passages.

Table 2. Population doubling/passage by serum

Passage		6		7		8		9		10	
Medium	**Lineage**	**PD**	**SD**	**PD**	**SD**	**PD**	**SD**	**PD**	**SD**	**PD**	**SD**
2% FCS+	Ze001	0.68	± 0.24	1.48	± 0.97	2.35	± 0.30	1.90	± 0.17	2.47	± 0.83
	Ze002	0.43	± 0.09	1.44	± 1.41	2.69	± 0.11	2.19	± 0.35	2.90	± 0.66
	Ze003	1.46	± 0.22	3.31	± 0.12	3.35	± 0.25	2.78	± 0.08	2.88	± 0.53
2% HP+	Ze001	1.46	± 0.26	1.72	± 0.32	N/A	N/A	N/A	N/A	N/A	N/A
	Ze002	1.64	± 0.10	2.19	± 0.32	2.52	± 0.24	-0.95	± 1.27	N/A	N/A
	Ze003	1.11	± 0.61	1.94	± 0.42	1.60	± 0.85	-0.85	± 0.66	0.57	± 0.63
10% HP	Ze001	1.00	± 0.37	0.37	± 0.19	0.92	± 0.50	-0.24	± 0.41	-0.64	± 0.66
	Ze002	1.31	± 0.23	-0.10	± 0.18	0.94	± 0.38	0.63	± 0.19	0.94	± 0.64
	Ze003	2.33	± 0.60	2.40	± 0.30	2.55	± 0.27	2.30	± 0.21	2.34	± 0.31
2% PRP+	Ze001	1.60	± 0.09	2.36	± 0.26	2.41	± 0.19	1.19	± 0.07	2.32	± 0.65
	Ze002	1.58	± 0.18	2.64	± 0.44	2.94	± 0.08	2.11	± 0.15	2.25	± 0.34
	Ze003	1.78	± 0.16	2.96	± 0.52	2.49	± 0.37	2.41	± 0.27	2.47	± 0.24
10% PRP	Ze001	2.23	± 0.25	2.81	± 0.23	1.43	± 0.40	0.57	± 0.28	3.18	± 1.91
	Ze002	2.55	± 0.20	3.37	± 0.28	3.25	± 0.37	1.15	± 0.51	2.36	± 0.15
	Ze003	3.18	± 0.06	4.53	± 0.15	4.15	± 0.44	1.26	± 0.24	2.65	± 0.27.

I.C. Cultured SHED viability (at 10th passage)

The viability of the cultured SHED in the media with 2% FCS+, 2% PRP+ and 10% PRP was higher than 90 %; in the medium with 10% HP it was higher than 85 % and in the medium with 2% HP+ (in lineage Ze003, the only lineage cultured in 2% HP that reached the 10th passage), it was 75.93 %.

II. Comparison of cluster of differentiation (at 10th passage) of allogeneic media-cultured SHED and xenogeneic media-cultured SHED

Because of the lack of biological material we were not able to evaluate the cultured SHED positivity for CD105. The other SHED CD positivity per serum/lineage was established (Table 3).

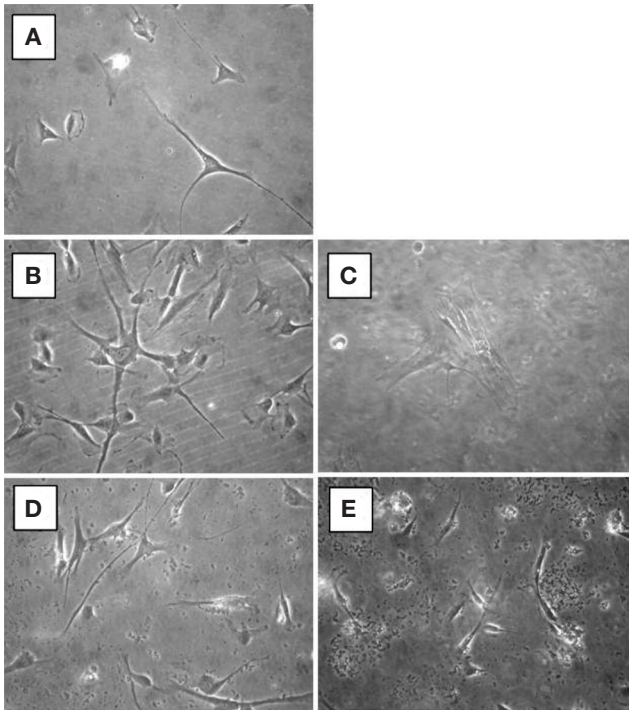


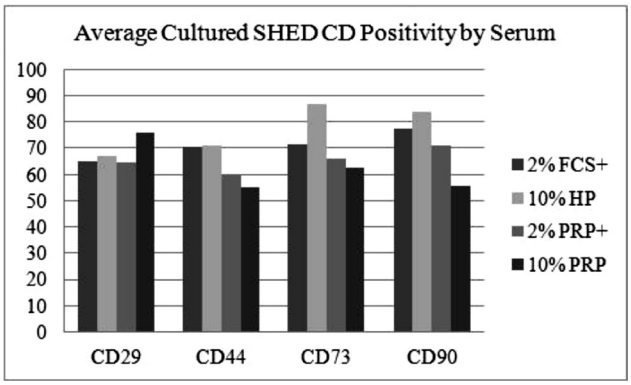
Fig. 3. Test media-cultured SHED at 10th passage
A) Ze001 cultivated in 2% FCS+ medium – image captured in 9th passage, one day after seeding into the culture vessel. **B**) Ze001 cultivated in 2% HP+ medium – image captured in 6th passage, one day after seeding into the culture vessel. **C**) Ze001 cultivated in 10% HP medium – image captured in 9th passage, one day after seeding into the culture vessel. The cultured SHED adhered to the surface of the culture vessel more than in other media. **D**) Ze001 cultivated in 2% PRP+ medium – image captured in 9th passage, one day after seeding into the culture vessel. **E**) Ze001 cultivated in 10% PRP medium – image captured in 9th passage, one day after seeding into the culture vessel. In panels A, B, D, E we can see the typical SHED morphology. In panels D and E, we can see tiny particles that float freely in the culture media. All images were captured with a phase contrast microscope, original magnification 200×.

Phenotypical analysis, performed at the 10th passage (after five passages in the test media), showed that human blood components, mostly the PRP, influenced the phenotypical profile of the cultured SHED. This analysis does not include the data for the 2% HP+ medium as the experiment was terminated preliminarily for the lack of cultured SHED proliferation activity.

Assuming that CD markers should reach positivity equal to or higher than 90 %, these results do not fulfil the MSC requirements (SHED-specific requirements do not exist yet). On average, the SHED cultivated in 10% HP were the closest to the ideal, followed by 2% FCS+, 2% PRP+ (Graph III). The 10% PRP supported the expression of the tested CD the least. The results for different CDs were not consistent through a medium; while in 10% HP SHED showed the highest positivity for CD44, CD73 and CD90, CD29 reached the highest positivity in 10% PRP (Table 3). Additionally, SHED cultivated in

Table 3. Cultured SHED CD positivity by serum

	CD29	CD44	CD73	CD90
Lineage Ze001				
2% FCS+	68.73	72.18	71.63	64.53
10% HP	70.34	62.83	83.79	80.60
2% PRP+	76.16	53.95	54.30	63.07
10% PRP	69.74	42.57	48.94	42.05
Lineage Ze002				
2% FCS+	69.13	72.76	83.58	84.44
10% HP	80.56	79.54	95.62	96.20
2% PRP+	62.10	60.80	68.49	77.19
10% PRP	83.84	56.68	61.08	57.39
Lineage Ze003				
2% FCS+	57.09	66.45	58.85	82.98
10% HP	50.38	70.67	80.60	75.45
2% PRP+	55.69	63.40	75.65	72.44
10% PRP	73.52	66.62	77.45	67.03
Average SHED CD Positivity by Serum				
2% FCS+	64.98	70.46	71.35	77.32
10% HP	67.09	71.01	86.67	84.08
2% PRP+	64.65	59.38	66.15	70.90
10% PRP	75.70	55.29	62.49	55.49



Graph III. Average cultured SHED CD positivity by serum

the medium supplemented with 10% PRP showed positivity for OCT3/4 (Ze001, Ze002, and Ze003) at 57.99 % with SD ± 2.89 %. Overall, only the 10% HP-cultured SHED promoted the CD positivity more than 2% FCS+. PRP, in both concentrations, had a predominantly negative impact on the CD positivity.

III. Evaluation of allogeneic media-cultured SHED capacity to differentiate (from the 10th passage cultivated in allogeneic sera supplemented medium that most promoted the proliferation rate)

All the three lineages of SHED, cultured in 10% PRP for five passages, were tested for their ability to differentiate into the below-specified cell types, with the following results:

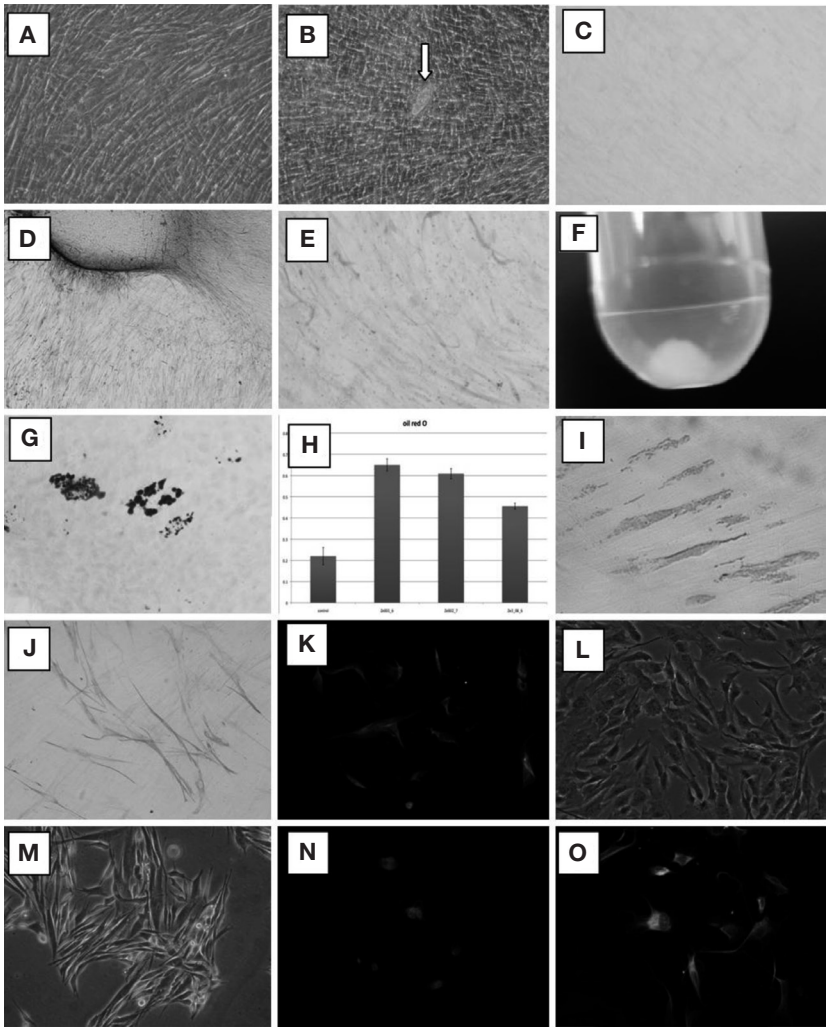


Fig. 4. 10% PRP medium-cultured SHED differentiation capacity
A) Ze001 lineage in cultivation medium supplemented with 10% PRP, after four weeks of cultivation. **B**) Ze001 lineage in osteogenic medium, after four weeks of cultivation. We can observe small non-homogeneous particles on the top of the cells, and moreover one larger 3-D particle marked with arrow. **C**) Immunohistochemistry detection of osteonectin in Ze001 lineage cultivated in 10% PRP-supplemented medium. **D**) and **E**) immunohistochemistry detection of osteonectin in Ze001 lineage cultivated in osteogenic medium. **F**) Cell pellet of Ze003 cultivated in chondrogenic medium, after three weeks of cultivation. **G**) Oil Red O staining of Ze002 lineage after 10 days in adipogenic medium with positive presence of fat droplets/vacuoles inside the cultured cells. **H**) Spectrophotometrical comparison of absorbance of Oil Red O extracted from lineages exposed or not exposed to adipogenic medium. **I)** Immunohistochemistry detection of cardiac troponin protein in Ze001 lineage exposed to 5-azacytidine for 24 h. **J**) Immunohistochemistry detection of desmin in Ze002 lineage exposed to 5-azacytidine for 24 h. **K**) Immunofluorescence detection of α-actinin in Ze003 that was exposed to myogenic differentiation medium. **L**) Negative control for neurogenic differentiation of Ze003 lineage, after four weeks of cultivation. **M**) Ze003 lineage cultivated in neurogenic medium after four weeks of cultivation. Cultured SHED did not proliferate, were more spindle-shaped and kept close contacts with other cells. **N**) Negative control for immunofluorescence detection of nestin in Ze003 lineage after four weeks of cultivation. **O**) Immunofluorescence detection of nestin in Ze003 lineage after four weeks of cultivation in neurogenic media.

III.A. Osteogenic differentiation

SHED exposed to the osteogenic differentiation medium started to produce the extracellular matrix (Fig. 4A,B) with larger 3-D particles after four weeks of cultivation. We proved the presence of osteonectin using immunohistochemical detection (Fig. 4C,D,E).

III.B. Chondrogenic differentiation

SHED exposed to the chondrogenic differentiation medium in a non-adherent test tube formed 3-D aggregates (Fig. 4F), which continuously kept enlarging for the next four weeks.

III.C. Adipogenic differentiation

SHED exposed to the adipogenic differentiation medium created intracellular lipid droplets/vacuoles, which

were confirmed by Oil Red O staining (Fig. 4G). Using quantification methods and negative control we confirmed significantly higher concentrations of Oil Red O staining in all three differentiated lineages of SHED compared to the negative control (Fig. 4H).

III.D. Myogenic differentiation

SHED exposed to the myogenic differentiation medium were tested using immunohistochemical detection of desmin, cardiac troponin and α-actinin; the results proved a positivity for the above-mentioned proteins in all the three differentiated lineages (Fig. 4I,J,K).

III.E. Neurogenic differentiation

SHED exposed to the neurogenic differentiation medium changed their morphology. They became even more spindle shaped, slowed down in proliferation and kept closer to each other (Fig. 4L,M). Using immunofluorescence detection we proved high positivity for nestin within these cells (Fig. 4N,O).

Discussion

Overall, the results of this study are influenced by the pre-experimental cultivation of the input biological material (primary SHED) in xenogeneic serum (2% FCS+). From the set of data we obtained we are not able to draw any conclusion as to how this cultivation impacted the resulting proliferative capacity, CD, and the differentiation potential of the SHED cultivated in the test media. While this issue disqualifies the exact numerical values of our results, the results remain valid in indicating the trends in which the tested sera influence the examined SHED population. The pooled SHED and sera also bring up the issue of heterogeneity of biological material, which we know influences the cell behaviour and capacities (Wagner et al., 2006).

In our previous study we proved that DPSC cultivated in medium containing 2% FCS and enriched with growth factors EGF, PDGF and ITS promoted the proliferative capacity of cultured cells more than the medium containing 10% FCS (Suchanek et al., 2009). Considering that SHED are very similar to DPSC, the 10% PRP test sera boosted the SHED proliferative capacity more than 2% FCS+, 10 % FCS, 2% HP+, & 10% HP. This result correlates with similar studies on proliferation of DPSC from adult donors (Govindasamy et al., 2011; Lee et al., 2011; Chen et al., 2012). Another interesting observation, concerning the sera influence on the proliferation rate, is that while 2% PRP+ performed worse than 10% PRP, the 2% FCS+ performed better than 10% FCS. We believe, because of the lack of relevant pre-existing scientific evidence, that FCS, being xenogeneic, might be slightly toxic to human stem cells, which correlates with the aforementioned result. Furthermore, the HP-based media promoted the proliferative capacity of the cultured cells only slightly, if at all. This can be explained by insufficient concentration of the growth factors. These findings correlate with the studies of bone marrow stem cells by Müller (2006), Mojica-Henshaw et al. (2013) and Gstraunthaler et al. (2014). Their proliferation arrest also explains the change in morphology of the SHED cultivated in 10% HP, as for the lack of proliferative activity they settled on the bottom of the cultivation flask. Suchanek (2009) observed this behaviour of DPSC in 10% FCS as well.

The tested SHED phenotypes provided us with extremely valuable information. While we cannot compare our results with any other study because of the absence of studies on SHED cultured in allogeneic sera and the use of FCS in the first five passages, we can securely state that the cultivation sera do impact the cultivated cell phenotype. This information raises a major question about the validity of MSC/SC phenotypical definition, which is based on cells cultivated in xenogeneic sera. This is where we see a huge opportunity for future research that would be relevant to human regenerative therapy. Overall, the data on the test media-cultured SHED phenotype show that the standard CD positivity lowered in PRP and increased in 10% HP, compared to the 2% FCS+ media.

Despite that, we cannot label the allogeneic sera-supplemented media-cultured SHED as MSC for their 'inadequate' phenotypical positivity (Dominici et al., 2006). We have proved that some allogeneic sera can feasibly replace xenogeneic sera due to their sufficient proliferation-stimulating capacity (PRP). The SHED cultured in 10% PRP medium have also retained their positivity for OCT 3/4 and remained multipotent, capable of adipo/osteo/chondro/myo/neurogenic differentiation (Miura, 2003; Karbanova, 2011). We have also confirmed that media supplemented with HP are not suitable for cultivation of SHED. Finally, our study recognizes there is a huge gap in research due to the ability of sera to temper with the cultured cell phenotype.

Conclusion

We conclude that multipotent ecto-mesenchymal stem cells from human exfoliated deciduous teeth can be feasibly cultivated in media in which the xenogeneic FCS is substituted by allogeneic PRP, as they retain their proliferative capacity and differentiation capacity into osteogenic, chondrogenic, adipogenic, myogenic and neurogenic cell types. We have also proved that different sera impact the cultured cell phenotype differently, which has major implications for previous and future stem cell research and regenerative therapy. Due to the experiment limitations, the study results should be viewed as suggestive of a trend and should be used as a basis for future related research.

Acknowledgement

The authors have no conflict of interest to declare.

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