VILNIUS UNIVERSITY

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NEURONAL DIFFERENTIATION AND NEUROPROTECTIVE PROPERTIES OF MESENCHYMAL STEM CELLS

Summary of doctoral thesis

Biomedical Sciences, Biophysics (02 B)

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This study was carried out at the Department of Neurobiology and Biophysics of Vilnius University during 2010 – 2014 years

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VILNIAUS UNIVERSITETAS

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MEZENCHIMINIŲ KAMIENINIŲ LĄSTELIŲ NERVINĖS DIFERENCIACIJOS IR NEUROPROTEKCINIŲ SAVYBIŲ TYRIMAI

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Introduction

New stem cell-based therapeutic strategies for human neurological disorders have attracted a great deal of interest in recent years, but clinically useful therapies are still not available for most patients. The main reason for this is insufficient understanding of fundamental molecular processes regulating growth, differentiation and survival of human neural cells (1). These issues may be resolved only with adequate and effective ex vivo experimental models (2). The majority of *in vitro* neural differentiation protocols have been developed using human embryonic stem cells (ESC), and to date, these have been successfully differentiated into spinal motor neurons (3), midbrain dopamine neurons (4), neuroepithelial cells (5), γ-butyric acid (GABA) neurons (6), neural crest cells (7), astrocytes and oligodendrocytes (8). Major limitations of this model are associated with ethical issues, immunogenicity and contamination of neural cultures with non-differentiated, or not fully differentiated progenitors, which may yield unwanted proliferation and tumour formation after transplantation. Although, further refinements of protocols are needed before clinical use (9), studies using experimental model of ESCs have been extremely useful for understanding molecular mechanisms of neural differentiation, disease modelling and drug screening. Using inducible pluripotent stem cells (iPS) is ethically acceptable, but is associated with incomplete differentiation (possible formation of teratomas) and the presence of viral transgenes in the genomes of reprogrammed cells (10). A combined expression of neural lineage specific factors can directly, without intermediate state of pluripotence, convert fibroblasts into neurons (11), dopaminergic neurons (12) and neural stem cells (NSCs) (13).

Tissue-specific stem cells represent another important source for *ex vivo* experimental modeling of human neural cells, among which the most promising are NSCs and mesenchymal stromal cells (MSCs). NSCs have an indefinite proliferation potential and can differentiate into neurons, astrocytes and oligodendrocytes, but the isolation and use of human foetal NSCs is ethically problematic. However, with the use of such cells, large and homogeneous NSC populations have been expanded in a short time (14).

MSCs can differentiate under appropriate stimuli into lineages of mesenchymal tissues like bone, cartilage, muscle and fat, and recently, cells with similar traits, i.e. morphology, differentiation potential, immunophenotype have been identified in virtually all tissues of the adult organism and suggested to reside in a perivascular niche (15). There have been numerous attempts to differentiate MSCs into neural cells, indicating that the optimal protocol has yet to be established. In our opinion, there are two major reasons for this: first, MSCs derived from mesodermal tissues (bone marrow, adipose tissue) have been used requiring transdifferentiation of MSCs to ectodermal neural lineages. It seems likely, that neural differentiation instead of transdifferentiation would be more effective. In our laboratory we have isolated and characterised stem cells derived from human exfoliated deciduous teeth (SHEDs) (16). SHEDs originate from

cranial neural crest cells which are precursors of both neural and skeletal tissues (17). Thus, in contrast to MSCs from other mesodermal tissues, they could be particulary suitable for the induction of neural differentiation. Second, standard culture conditions (10 % of fetal bovine serum) favours the selection of mesenchymal instead of neural progenitors.

Considering this, our goal **in the first part of this study** was oriented towards development of new *in vitro* experimental model for neuronal differentiation using stem cells derived from human exfoliated deciduous teeth. Specifically, we aimed to:

1. Determine optimal conditions suitable for the selection of neural progenitors in cultures of stem cells derived from human exfoliated deciduous teeth (SHEDs).
2. Develop new protocol for neural differentiation of SHEDs.
3. Evaluate neural differentiation efficiency using immunocytochemistry and gene expression analyses.

The second part of doctoral thesis was dedicated to the investigation of neuroprotective properties of SHED cells.

Several recent studies demonstrate that in contrast to the MSCs derived from other tissues, SHEDs have unique neurogenic properties which could be potentially exploited for therapeutic use (18). However, the exact mechanisms behind these effects are not clear, and it is not known whether exosomes and microvesicles derived from the SHEDs may have neuroprotective properties.

Considering this, our goal **in the second part of this study** was to investigate the effects of exosomes and microvesicles derived from SHEDs during 6-hydroxydopamine-induced apoptosis in human dopaminergic neurons. Specifically, we aimed to:

1. Isolate and characterize exosomes and microvesicles secreted by SHEDs.
2. Compare how standard culture conditions and culture on microcarriers in bioreactor affect exosomes secreted by SHEDs.
3. Investigate how exosomes and microvesicles derived from SHEDs grown under different culture conditions affect 6-hydroxydopamine-induced apoptosis in human dopaminergic neurons.

Scientific Novelty

In the present study, we have established a novel three-stage protocol for neural differentiation of stem cells derived from human exfoliated deciduous teeth (SHEDs) cells. We show that SHEDs differentiated according to our new protocol represent an ensemble of cells with neuronal and glial traits. This opens new perspectives for more in-depth studies of the specification of neuronal and glial subtypes from differentiating SHEDs. Additionally, our study provides new information about the importance of major

developmental signalling pathways (BMP and Notch) during neuronal and glial differentiation of SHEDs. Our new experimental model may be utilised as a versatile tool for future tissue engineering and cell therapies in human neurological disorders.

This dissertation also provides first evidence that exosomes derived from SHEDs can rescue human dopaminergic neurons from 6-hydroxy-dopamine-induced apoptosis. These findings could be potentially exploited for the development of new therapeutic strategies against Parkinson's disease.

The defensive statements

1. Stem cells derived from human exfoliated deciduous teeth (SHEDs) display properties of neural progenitor cells.
2. SHEDs could be effectively differentiated into neurons and glia under *in vitro* conditions.
3. Culture conditions have an impact on proteomic composition of the exosomes secreted by SHEDs.
4. Exosomes from SHEDs affect 6-OHDA-induced apoptosis in human dopaminergic neurons.

Materials and Methods

Isolation and Culture of Stromal Cells from the Dental Pulp of Human Exfoliated Deciduous Teeth (SHEDs)

Cells were obtained from human exfoliated deciduous teeth of children (female, 7 years old, and male, 6 years old, Caucasians), whose parents had signed an informed consent. Material was collected under the approval of the Lithuanian Bioethics committee. Cell isolation and characterization was performed as described previously (16). Briefly, the collected tooth was washed in phosphate-buffered saline (PBS) and incubated in L-15 medium with 200 U/ml penicillin, 200 μg/ml streptomycin and 2.5 μg/ml of amphotericin B (all from Biochrom, Berlin, Germany). Pulp tissue was scraped out and placed in collagenase type I (Biochrom) solution, the latter of which consisted of 0.2% collagenase in DMEM (Biochrom) with 1 % BSA (Applichem, Darmstadt, Germany), 100 U/ml penicillin and 100 μg/ml streptomycin, and finally incubated for 1 h at +37 °C in an orbital shaker platform. After digestion, the cell suspension was diluted in PBS and centrifuged at 250 g for 5 min. The supernatant was discarded, and cells were resuspended in low glucose (LG) (1000 mg/ml) DMEM (Biochrom) supplemented with 10 % FBS (Biochrom), glutamine and antibiotics and plated. Cultures were maintained at 37 °C in a humidified atmosphere containing 5 % CO_2 and were subsequently tested for their proliferation rate, surface markers and ability to differentiate towards osteogenic and adipogenic lineages in vitro.

Neural Differentiation

Four-well plates (Nunc, Roskilde, Denmark) with coverslips were coated with 5 μg/ml laminin (Invitrogen, Carlsbad, CA, USA) solution overnight at 37 °C in a humidified atmosphere containing 5% CO_2.Wells were washed with PBS and SHEDs (passage 4–5) seeded at a density of 1×10^4 cells per well (1.65 cm^2) in DMEM-LG supplemented with 10 % FBS, L-glutamine and antibiotics. Alternatively, for gene expression studies, we used factory-coated poly-lysine/laminin 24-well plates (Biocoat, BD Biosciences, Erembodegen, Belgium). After 24 h, standard medium was changed to serum-free medium. We used DMEM-LG supplemented with 15 % Stempro MSC SFM supplement (Invitrogen), 2 mM-glutamine and antibiotics. After 1 week, cells were moved to the neurogenic medium, which consisted of Neurobasal A (Invitrogen) medium supplemented with 1 × B27 (Invitrogen), 20 ng/ml basic fibroblast growth factor (bFGF, Biological Industries, Kibbutz Beit, Haemek, Israel) and 20 ng/ml epidermal growth factor (EGF, Biological Industries). Neural differentiation was induced after 7–10 days by exposing cells to 1.5 mM dibutyryl cAMP (Sigma-Aldrich, St. Louis, MO, USA), 10 ng/ml nerve growth factor (NGF, Biological Industries), 10 ng/ml glial cell line-derived neurotrophic factor (GDNF, Biological Industries) and 10 ng/ml brain-derived neurotrophic factor (BDNF, Biological Industries) in Neurobasal A medium supplemented with 1 × B27 for 14–21 days. Under all conditions, the medium was replaced twice a week.

Bright-field images of control and differentiating SHED cultures were acquired with Motic AE31 microscope equipped with a Moticam 2500 camera and Motic Images Plus 2.0 software.

Automated 3D microcarrier cell culture of SHEDs

We used BioLevitator™ (Hamilton Bonaduz AG), a commercially available three-dimensional culturing platform and alginate microcarrier cell culture system (Global Cell Solutions, Charlottesville, VA) for the propagation of SHEDs. The BioLevitator™ system utilizes protein-coated magnetic microcarriers as attachment surfaces that are gently rotated to provide suspension-like cell culture. The Global Eukaryotic Microcarriers (GEM™) (Global Cell Solutions) are composed of alginate core embedded with paramagnetic particles and coated with adhesion molecules. Cells attach to the GEMs and are simultaneously moved in the vertical direction by a magnet and horizontally by rotating the culture vessel, providing a suspension-like culture system that promotes gas exchange and maintains a homogeneous medium composition.

In our experiments we used laminin-coated GEMs. Inoculation and culture of SHEDs on GEMs in the Biolevitator was performed according to the manufacturer‘s recommendations with some modifications. Briefly, 250 μl of pre-washed, laminin-coated GEMs was added to the LeviTube (Hamilton Bonaduz AG) containing 1.5 ml MSC NutriStem XF medium. Then single cell suspension of SHEDs (8×10^5 cells in 1.5 ml MSC NutriStem XF) was added to the GEM/medium preparation in the LeviTube and inoculation programme was initiated for 4 h. After inoculation, GEMs were checked microscopically, 10 ml of MSC NutriStem XF was added to the each LeviTube, then programme for cell culture was initiated. Next day, additional 10 ml of MSC NutriStem

XF was added to the each LeviTube. Supernatants for differential centrifugation were collected twice a week.

Cultivation and dopaminergic differentiation of ReNcell VM human neural stem cells

ReNcell VM immortalized human neural stem cell line was purchased from EMD Millipore Corporation, Temecula, CA. Cells were expanded on tissue culture flasks (Orange Scientific, Braine-l'Alleud, Belgium) coated with 20 µg/ml laminin (Invitrogen, Carlsbad, CA) in Neurobasal A medium (Invitrogen) supplemented with 1 x B27 (Invitrogen), 2 mM L-glutamine (Biochrom), 20 ng/ml basic fibroblast growth factor (bFGF, Biological Industries) and 20 ng/ml epidermal growth factor (EGF, Biological Industries). Cells were routinely grown to 70-80 % confluency, then passaged using Accutase (Millipore).

For differentiation studies cells were plated on twenty four-well plates (Greiner Bio-One, Frickenhausen, Germany) with or without glass cover slips coated with 20 µg/ml laminin and grown until 50-60 % confluence under standart conditions (see above). Dopaminergic differentiation was induced by exposing cells to 0.5 mM dibutyryl cAMP (Sigma-Aldrich, St. Louis, MO), 0.2 mM ascorbic acid (Sigma), 20 ng/ml glial cell line-derived neurotrophic factor (GDNF, Biological Industries), 20 ng/ml brain-derived neurotrophic factor (BDNF, Biological Industries) and 1 ng/ml transforming growth factor β1 (TGF-β1, Biological Industries) in Neurobasal A medium supplemented with 1 x B27 for 14-21 days. Under all conditions the medium was replaced twice a week.

RNA Extraction, cDNA Synthesis and Real-Time PCR

Total RNA was isolated from proliferating and differentiating cells at passage 3 using TRIzol (Invitrogen) according the manufacturer's instructions. Two micrograms of RNA extract was converted into first-strand cDNA using the Revert Aid M-MuLV Reverse transcriptase kit (Fermentas) with oligo (dT) primers in a 20-µl reaction mixture. Gene expression studies were performed with RT Profiler™ PCR Arrays from SABiosciences company (96-well plates for Neurogenesis and Neural Stem Cell array (PAHS-404A) and Human cAMP/Ca2+ PathwayFinder array (PAHS- 066A)). We have used Maxima SYBR Green qPCR Master mix (Fermentas) and 0.2 µl of cDNA template per reaction. Real-time polymerase chain reaction was performed using the MX3005P® instrument and MxPro software (version 4) (Stratagene, La Jolla, CA, USA). An initial 10-min denaturation step at 95 °C was followed by 40 cycles of 15 s of denaturation at 95 °C and 1-min annealing-elongation at 60 °C. PCR products were analysed by melting curve programme (95 °C for 1 min, 55 °C for 30 s and 95 °C for 30 s).

Gene expression levels were analysed using RT^2 Profiler PCR Array Data Analysis software (version 3.5, QIAGEN). For normalization of cDNA levels, we used housekeeping genes hypoxanthine phosphoribosyltransferase 1, ribosomal protein L13a and glyceraldehyde-3-phosphate dehydrogenase. We performed three independent experiments for each qPCR analysis with similar results. The p-values were calculated using Student's t-test of the replicate 2^(−Delta Ct) values for each gene in the control group, and treatment groups and differences were considered as significant when $p<0.05$.

Immunocytochemistry

SHED cells grown on coverslips were washed twice with PBS, fixed with 4 % PFA for 20 min at room temperature (RT), washed with PBS, permeabilized with 0.1 % Triton X-100 (in PBS) for 15 min at RT, treated with 1 % bovine serum albumin (BSA) (in PBS) blocking buffer for 30 min at RT and then incubated with one of the following primary antibodies: 15 μg/ml rabbit anti β-III tubulin (Sigma Aldrich), 1:100 goat anti-myelin basic protein (MBP; Santa Crus Biotechnology), 1:100 goat anti peripherin (Santa Crus Biotechnology), 1:100 goat anti-vimentin (Santa Crus Biotechnology), 1:200 rabbit anti Brn3a (Millipore), 1:500 rabbit anti-tyrosine hydroxylase (Millipore) in 1 % BSA for 1 h at RT or overnight at 4 °C. After washing, secondary antibodies (1:1,000 chicken anti-rabbit Alexa Fluor 488 (Invitrogen), chicken anti-rabbit Alexa Fluor 594 or chicken anti-goat Alexa Fluor 488) were added in the blocking solution for 1 h at RT in the dark. Staining with secondary antibodies alone did not produce any significant background, thus obliviating the need for further testing with unrelated antibodies. Finally, all sections were counterstained with 10 μg/ml 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Applichem, Darmstadt, Germany) for 5 min at RT in the dark, washed and mounted in anti-fading fluorescent mounting medium (DakoCytomation, Huddinge, Sweden). Confocal images were acquired with an Olympus Fluoview FV10i and Leica TCS SP8 (Leica Microsystems) confocal laser scanning microscope.

Culture treatments with 6-hydroxydopamine (6-OHDA), exosomes and microvesicles. Real-time monitoring of apoptosis

6-OHDA (Sigma-Aldrich) was dissolved in water containing 0.1 % ascorbic acid. Differentiated ReNcell VM cells were exposed to 100 μM 6-OHDA for 2 h, then fresh medium was replaced and exosomes, or microvesicles were added. We used exosomes derived from SHEDs cultivated under standart conditions and exosomes, or microvesicles isolated from SHEDs grown on the laminin-coated microcarriers in BioLevitator. PBS was used as a vehicle control. At this point CellEvent™ Caspase-3/7 green detection reagent for apoptosis (Life technologies) was also added into the cell cultures. This reagent is fluorogenic substrate for activated caspases 3 and 7 producing a fluorogenic response indicative of apoptosis. The fluorescence emission maximum of the dye is approximately 520 nm. After 30 min of incubation live cell imaging was performed using Leica SP8 (Leica Microsystems) confocal microscope. Time lapse: 20 hours with images taken every 10 minutes.

Image analysis. Quantification of apoptotic cells

Fluorescent-channel images taken from live imaging experiments were analyzed by ImageMaster 2D Platinum 7.0 software (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Fluorescent apoptotic cells were counted as spots in each image using ImageMaster algorithm. The same spot detection parameters (smooth-2; saliency-10; min.area-5) were applied for each image.

Live Cell Imaging

With the Cell-IQ® PC Cell Imaging & Analysis System (CM Technologies), cells were monitored continuously for approximately 120 h and further analysed using the Cell-IQ Imagen and Cell-IQ Analyser software.

Isolation of microvesicles and exosomes

Purification of microvesicular and exosomal fractions was performed using differential centrifugation, according to the (19) with some modifications. All centrifugation steps were performed at 4 °C. Supernatants collected from SHEDs cultivated under standart conditions, or on the laminin-coated microcarriers in BioLevitator were centrifuged successively at increasing speeds (300 g for 10 min, 2000 g for 10 min, then at 20000 g for 30 min). The final supernatant was ultracentrifuged at 100 000 g for 70 min in Sorvall LYNX 6000 ultracentrifuge, with rotor T29-8x50 in oak ridge centrifuge tubes with sealing caps (all from Thermo Fisher Scientific, Rochester, NY), then pellet washed in 40 ml PBS and ultracentrifuged again at 100 000 g for 70 min. Final pellets of exosomal fraction were resuspended in sterile PBS and stored at -80 °C. For isolation of microvesicular fraction, pellets after 20 000g centrifugation step were washed in 40 ml of PBS and centrifuged for another 30 min at 20 000 g. Final pellets of microvesicular fraction were resuspended in sterile PBS and stored at -80 °C.

Protein extraction from cells and microvesicles

Cells were washed with cold PBS (pH 7.3) twice and lysed in lysis buffer (1% NP-40, 1% sodium deoxycholate, 0.1 % SDS, 150 mM NaCl, 10 mM Tris (pH 7.4), 10 mM EDTA, 1 x Complete (protease inhibitor cocktail), 1mM PMSF, 1mM Na_3VO_4, 5 mM NaF) 30 min, at 4 ^{0} C. 2.5 VV/ml benzonase (Merck) was added and cell lysates were homogenised using 21 G syringe and centrifuged at 15 000 g, 30 min, + 4 ^{0} C. Supernatant fraction was collected, aliquoted and stored at -70^{0}C.

After differential centrifugation collected microvesicular fraction (pellets after 20 000g) was lysed in lysys buffer (1% NP-40, 1% sodium deoxycholate, 0.1 % SDS, 150 mM NaCl, 10 mM Tris (pH 7.4), 10 mM EDTA, 1 x Complete (protease inhibitor cocktail), 1mM PMSF, 1mM Na_3VO_4, 5 mM NaF) 30 min at 4^0 C and centrifuged at 15000 x g, 30 min, + 4 ^{0} C. Supernatant fraction was collected, aliquoted and stored at -70^{0}C.

Preparation of supernatants for electrophoresis. Electrophoresis.

Supernatant samples for SDS-polyacrilamide gel electrophoresis (SDS/PAGE) were prepared as following: 12.5 μl 0.5 M Tris/HCl pH 6.8, 30 μl 10 % SDS, 20 μl 0.5 M DTT, 20 μl glycerol and 5 μl 0.5 % bromophenol blue was added to 100 μl of supernatants, samples were heated for 5 min at 95°C and used for electrophoresis or stored at -70 ^{0} C. Exosomes suspension in PBS was mixed with 6x of buffer above and heated for 5 min at 95°C.

SDS/PAGE electrophoresis was runed using 10 % SDS-polyacrilamide gels, electrophoresis buffer (25 mM Tris base, 192 mM glycine, 0.1 % SDS) and Mini Protean

(Biorad) electrophoresis system. After electrophoresis some gels were stained with colloidal Coomassie Blue (PageBlue, Thermo Fisher Scientific) or silver (PageSilver, Thermo Fisher Scientific) according to manufacturers specifications.

Western blotting. Immune analysis of proteins

After electrophoresis SDS-polyacrilamide gels were blotted onto a polyvinylidene fluoride (PVDF) membrane (Biorad) in transfer buffer (25 mM Tris, 190 mM glycine and 20% methanol) using a Bio-Rad blot transfer system.

PVDF membranes were blocked for 1 hour at room temperature in blocking solution (5% milk in PBS-0.18%Tween-20), then incubated 1 hour or at $+4^0$ C overnight with primary antibodies (in 5% Milk-PBS-0.18%Tween-20), washed three times with PBS-0.18%Tween-20 and 1 hour incubated at room temperature with secondary antibodies conjugated with horse radish peroxydase (diluted 1:2000 in PBS-0.18%Tween-20). After incubation membranes were washed five times with PBS-0.18%Tween-20 and analised using ECL reagents (Pierce) according to manufacturers specification.

Before reprobing the same membranes with different antibodies they were stripped for 2 hours in glycine solution (0.1 M glycine, 2.9 pH) at room temperature and washed with PBS-0.18%Tween-20.

Proteomic analysis of supernatants and exosomes

We analised proteomics of supernatant extravesicular fraction (differential centrifugation after 2000g) and exosomes. SHEDs were grown in standart conditions and on microcarriers in bioreactor in same serum-free medium (MSC Nutristem XF Medium). Proteins were prepared as following: reduction: 0.05M Tris(2-carboxyethyl)phosphine for 20min, at 37^0 C; alkylation: 0.15M iodoacetamide for 30min at room temperature in the dark. 0.75 µg trypsin (Sequencing Grade Modified Trypsin, Promega) was added and proteolysis was made for 3 hours at 37^0 C and overnight at 30^0 C. After trypsinolysis peptides were cleaned with C18 colums (Harvard Apparatus) according to the manufacturers specification. Liquid chromatography together with tandem mass spectrometry (LC-MS/MS) was runned using EASY-nLC (Thermo Fisher Scientific) chromatograph integrated with Velos Pro-Orbitrap Elite hibrid mass spectrometer (Thermo Fisher Scientific), which has nanoelectro spray source (Thermo Fisher Scientific). LC-MS/MS samples were separated using two stages column system composed of 2 cm C18-A1 column-catcher and 10 cm C18-A2 analytic column (both from Thermo Fisher Scientific). Linear separation gradient was composed from 5% buffer B for 5 min, 35% buffer B for 60 min, 80% buffer B for 5 min and 100% buffer B for 10 min. Flow speed was 0.3µl/min (buffer A: 0.1% TFA in 1% acetonitrile; buffer B: 0.1% TFA in 98% acetonitrile). 4 µl of sample was injected for one LC-MS/MS analysis. Full MS scan was made using 60000 resolution in orbitrap analyser. Data were collected using LTQ Tune software. Data were analysed in UniProt protein database using Thermo Proteome Discoverer programe Sequest search algorithmes. Proteomic analysis was made in Proteomics departament, Biotechnology institute, University of Helsinki, Finnland.

Results and Discussion

Neural Differentiation of SHEDs

For neural differentiation studies, we used two lines of SHEDs. These consisted of typical small spindle-shaped fibroblastoid cells (Fig. 2c) and displayed similar growth rates with a population doubling time of about 24 h. *In vitro* differentiation revealed a strong osteogenic potential but no capacity to differentiate towards adipocytes. Flow cytometry showed that these cells expressed characteristic antigens of MSC-like cells, including CD73, CD90, CD105, CD146, but not hematopoietic markers CD14, CD34 and CD45.

Interestingly, we observed minor subpopulations of neuron-like cells in all SHED lines. In some cases, these formed small groups (Fig. 2a), but more frequently they appeared as single neuron-like cells with a strikingly different appearance from the surrounding fibroblastoid cells (Fig. 2b). These small-sized round cells had long neurite-like processes with cone-like terminal structures. The presence of the neuron-like cells may reflect the heterogeneity of SHED cultures. A more likely possibility is that these subpopulations and MSC-like cells originate from common progenitors present in the culture. SHEDs originate from cranial neural crest cells (NCC), which are unique among the NCC populations because they can differentiate into precursors of both neural and skeletal tissues (17). The exact nature of *in vitro* human SHEDs remains elusive, but several studies have demonstrated that under standard culture conditions (10 % FBS) they are very similar to MSC-like cells derived from other non-ectodermal tissues, i.e. display typical fibroblastoid morphology, immunophenotype, differentiation potential, immunosuppressive properties and proteomic profile (16). The existence of mesenchymal progenitors among SHEDs may reflect that they were already prespecified towards the mesenchymal fate. Another possible explanation is that standard culture

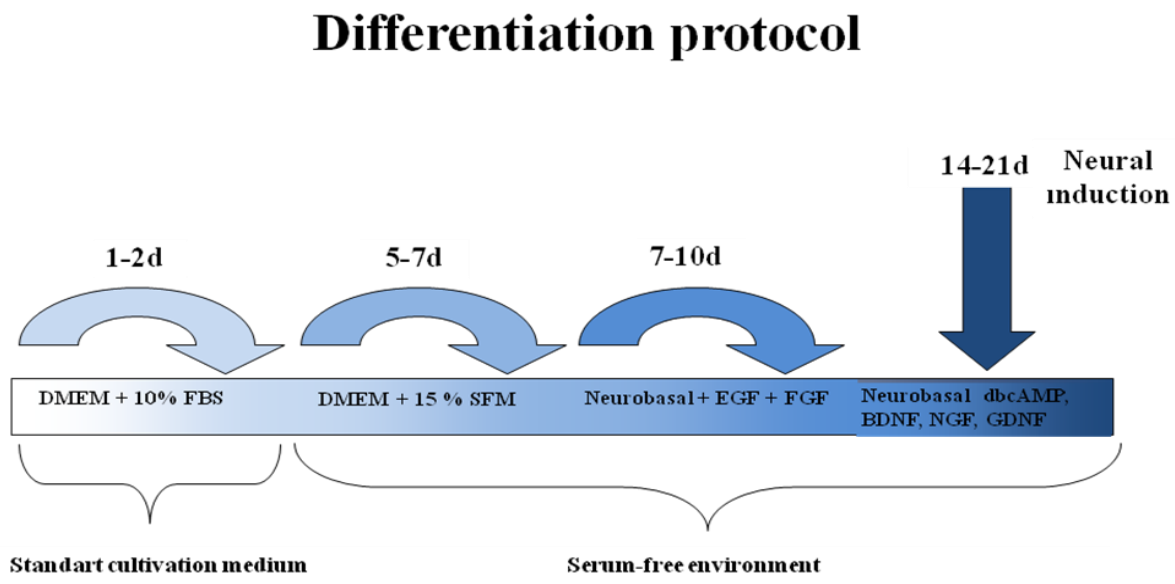

Fig. 1. A Schematic representation of neural differentiation protocol.

conditions favour the selection of mesenchymal instead of neural progenitors. It is known that FBS represents a rich source of cytokines promoting epithelial-to-mesenchymal transition (20). Since serum exposure can induce rapid conversion of NCC into mesenchymal precusors (21), we hypothesized that a gradual adaptation of SHEDs to the serum-free and neurogenic environments could help to reset a programme of neural specification.

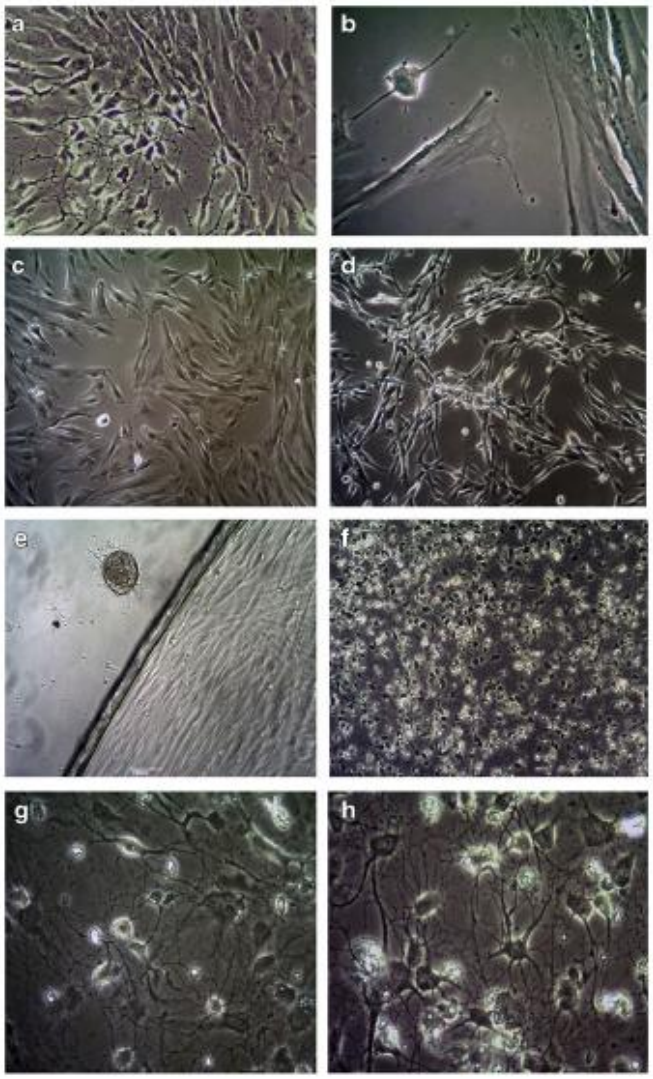

Fig. 2. Morphology of control and differentiating SHEDs. a Subpopulations of neuron-like cells in SHEDs grown under standard conditions; b morphological appearance of neuron-like cell; c SHEDs cultivated in the presence of 10 % FBS; d SHEDs in serumfree medium (15 % Stempro MSC SFM, Invitrogen) after 5 days of culturing; e formation of neurosphere-like spheroids in SHED cultures under neurogenic conditions (Neurobasal A supplemented with B27, plus 20 ng/ml bFGF and 20 ng/ml EGF). Bar=100 μm; f–h SHEDs after 14 days in neural induction medium (Neurobasal A supplemented with B27, plus 1.5 mM dibutyryl cAMP, 10 ng/ml NGF, 10 ng/ml GDNF and 10 ng/ml BDNF). Phase contrast, original magnifications ×200 (a, c, d–f); ×400 (b, g, h).

Therefore, we established a new protocol for the adaptation of SHEDs to serum-free neurogenic conditions (Fig. 1). For serum-free conditions, we used a commercial supplement, which was developed specifically for MSCs (Stempro MSC SFM Invitrogen, Carlsbad, CA, USA). To achieve a gradual adaptation of SHEDs to the serum-free environment and removal of residual intra- and extracellular components of FBS, we cultivated cells in SFM medium for 7–10 days. Under new culture conditions, SHEDs started to proliferate faster and acquired some morphological changes: reduced in size, spindle-shaped cells started to cluster into bundles (Fig. 2d). It has previously been shown that most FBS contaminations are removed from the MSC after 2 days (22). During the adaptation period, cells have divided several times, therefore, we think that FBS contaminants were efficiently eliminated.

Next, we subjected cells to the neurogenic medium composed of Neurobasal A (Invitrogen) medium supplemented with 1 × B27 (Invitrogen) and growth factors (20 ng/ml bFGF and 20 ng/ml EGF, Biological Industries). This medium was well tolerated, and the SHEDs proliferated rapidly and formed monolayers but did not display a

distinct morphological phenotype at this stage. However, after 1 week of culture, hollow cavities appeared in some monolayers, possibly indicating that adhesion between neighbouring cells exceeded monolayer–substrate adhesion force. Importantly, the same effect was observed for monolayers grown on glass coverslips and plastic plates. At this point, there was also a formation of neurosphere-like, round-shaped spheroids with a diameter of approximately 100 μm (Fig. 2e), suggesting that the changes in SHED cultures may reflect a predisposition of cells towards a neurogenic fate. Interestingly, quantitative real-time PCR analysis revealed significant expression of nestin gene in undifferentiated SHEDs and adipose tissue derived MSCs (ADSCs) (Fig. 3), while at the protein level nestin was detected only in SHED cultures (Fig. 4). Adaptation to the serum-free environment also resulted in increased expression of nestin (Fig. 3).This is in agreement with our assumption that adaptation of SHEDs to the serum-free environment could help to reset a programme of neural specification. We also found significant expression of neuronal βIII-tubulin gene in undifferentiated SHEDs, by contrast in ADSCs expression of βIII-tubulin was almost undetectable (Fig. 3). This finding highlights the difference between these two MSC types and also supports our hypothesis, that SHEDs may be more suitable for neural differentiation, than their counterparts from mesodermal tissues.

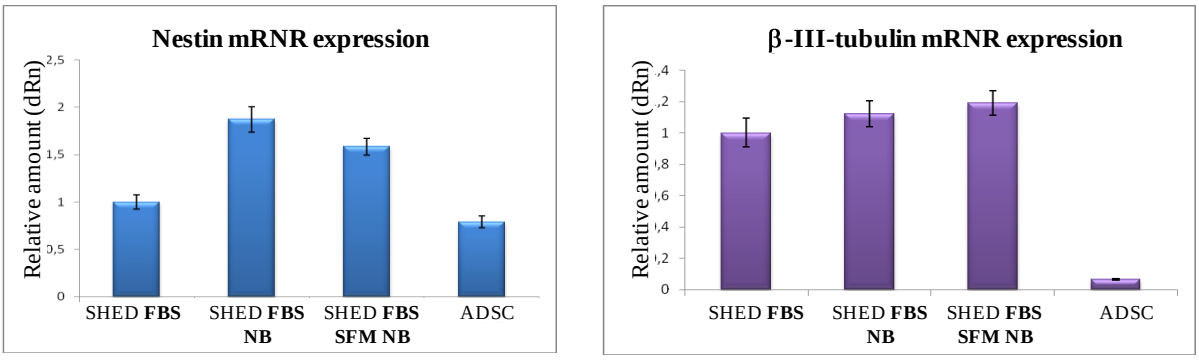

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Fig. 3. Nestin and β-III-tubulin gene expression profiles in SHED and ADSC. Quantitative gene expression differences were evaluated by performing real-time PCR (see methods). A – nestin mRNR expression; B – β-III-tubulin mRNR expression. SHED FBS – cells cultured under standart conditions; SHED FBS NB – cells in specialized neural medium; SHED FBS SFM NB – cells in specialized neural medium with adaptation to serum-free environment.

To induce neural differentiation of SHED cells, we adopted with some modifications a protocol previously used for neural differentiation of ESC-derived neural crest cells (21). After an overnight culture of cells, we observed stellate or bipolar round-shaped neuron-like cells, which appeared to develop on top of the cell monolayer (Fig. 2f–h, Suppl. 1). These cells formed small clusters and were distributed all over the monolayer.

Importantly, adaptation to the serum-free environment significantly accelerated neural differentiation of SHEDs, which occurred already during the first week of culture. During the second and third weeks of differentiation neuron-like cells formed long winding and interconnected neurite-like processes, possibly reflecting neuronal

maturation. We did not observe a significant cell loss during the whole period of differentiation.

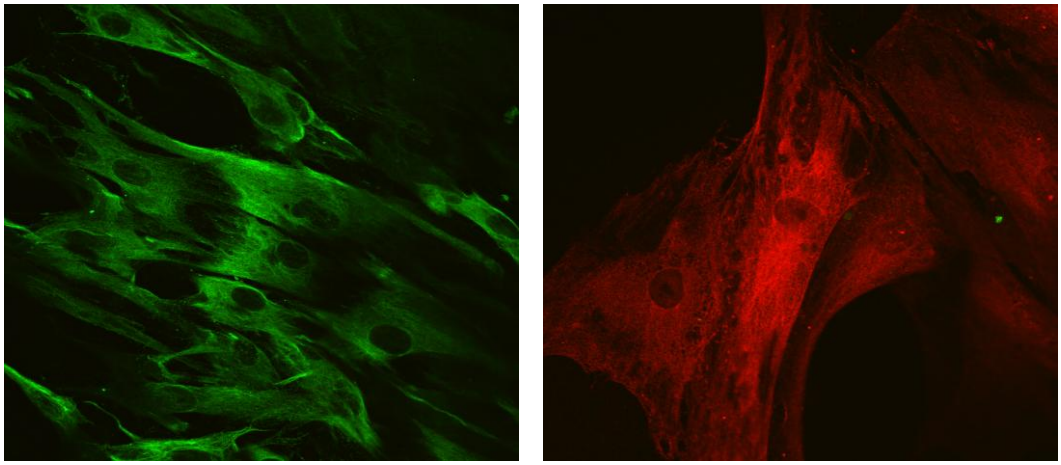

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Fig. 4. Neural progenitor specific and mesenchymal specific marker expression in SHED and ADSC. A – confocal microscopy. Nestin expression in SHED cells cultivated under standart culture conditions was determined using monoclonal antibody against nestin and visualised using secondary antibodies conjugated with Fluor® 488 (see methods). B – confocal microscopy. Adipose tissue derived stem cells (ADSCs) were cultivated under standart culture conditions. Nestin expression in ADSCs was determined using monoclonal antibody against nestin and visualised using secondary antibodies conjugated with Fluor® 488. Vimentin expression in ADSCs was determined using polyclonal antibody against vimentin and visualised using secondary antibodies conjugated with Alexa Fluor® 594.

Immunocytochemistry of differentiated SHEDs

The protein expression patterns of neuronal and glial markers were determined by immunocytochemical analyses. We found that differentiating SHEDs represented heterogeneous cultures composed of cells expressing both neuronal and glial markers. Most cells were positive for the intermediate neuronal marker βIII-tubulin, and some displayed accumulations of MBP in large deposits and possibly in cellular structures seen in Schwann cells (Fig. 5a–c). We also detected formation of MBP-positive processes that engaged with neuron-like cells and started to ensheath them. Importantly, the MBP-positive structures were negative for the neuronal marker βIII-tubulin (Fig. 5c).

Most cells expressed the Brn3a transcription factor, which is a key regulator of differentiating peripheral sensory neurons (23) (Fig. 6a). In addition, differentiating SHEDs were positive to intermediate filament peripherin that is expressed at high levels in PNS (Fig. 6b). Interestingly, the peripherin staining pattern was displayed as non-filamentous particles corroborating as peripherin particles and squiggles are induced during the early development of nerve cells (24).

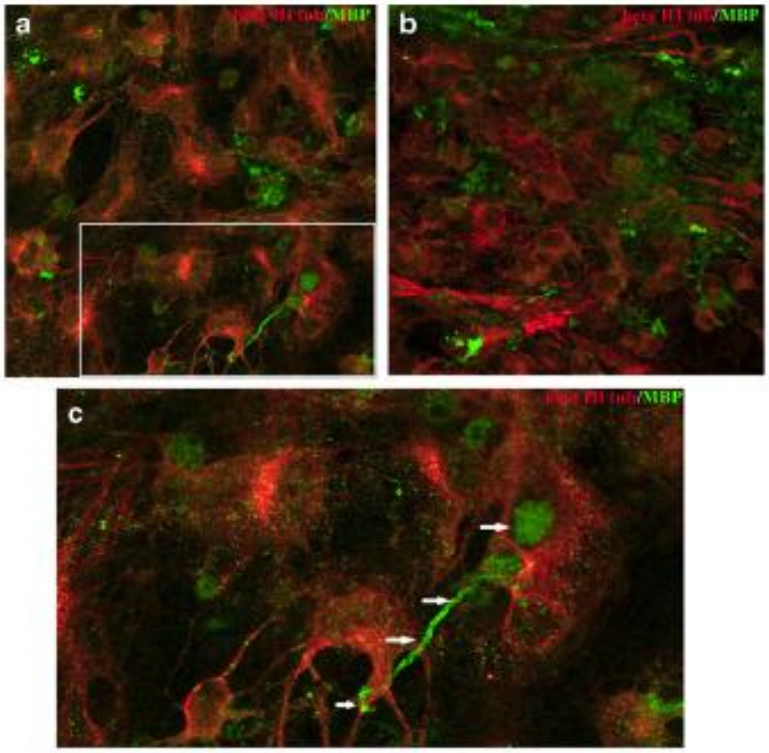

Fig. 3. Expression of neuronal and glial markers in differentiating SHEDs. a–c Immunocytochemical staining of differentiating SHEDs with antibodies against βIII-tubulin (red) and myelin basic protein (MBP, green). c Magnified region from a; arrows indicate the cellular structure similar to the Schwann cell and the MBP-positive process engaging with neuron-like cell.

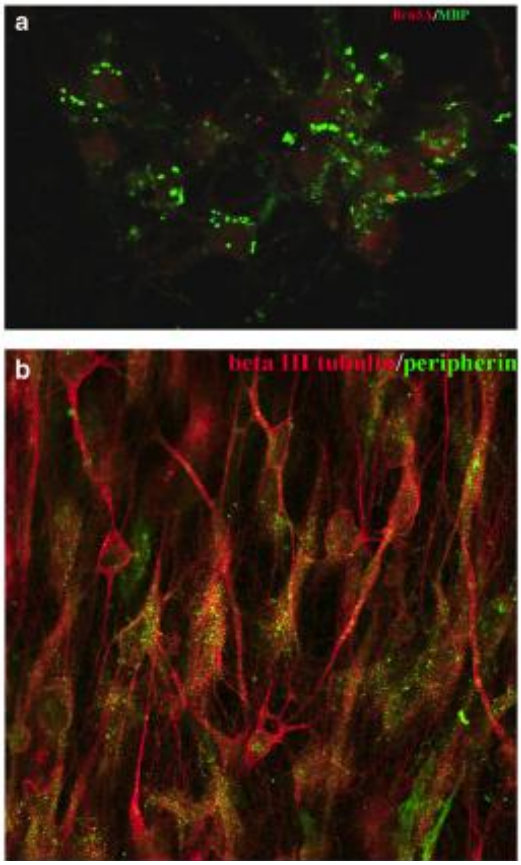

Fig. 6. Markers of peripheral sensory neurons in differentiating SHEDs. Immunocytochemical staining of differentiating SHEDs with antibodies against Brn3A (red) and myelin basic protein (MBP, green) (a). b B-βIII-tubulin (red) and peripherin (green).

Gene Expression Studies of Differentiated SHEDs

We used the RT2 profiler PCR arrays from SABiosciences to analyse panels of genes important for neurogenesis, neural stem cells and the cAMP/Ca2+ signalling pathways (full set of genes – Fig. 7a, b; some important genes – Fig. 8). In total, we assessed 168 genes in undifferentiated and differentiated SHEDs, showing that the expression of 110 genes was changed more than twofold, where 90 were upregulated and 20 downregulated. Among them, 41 were upregulated and four were downregulated more than tenfold.

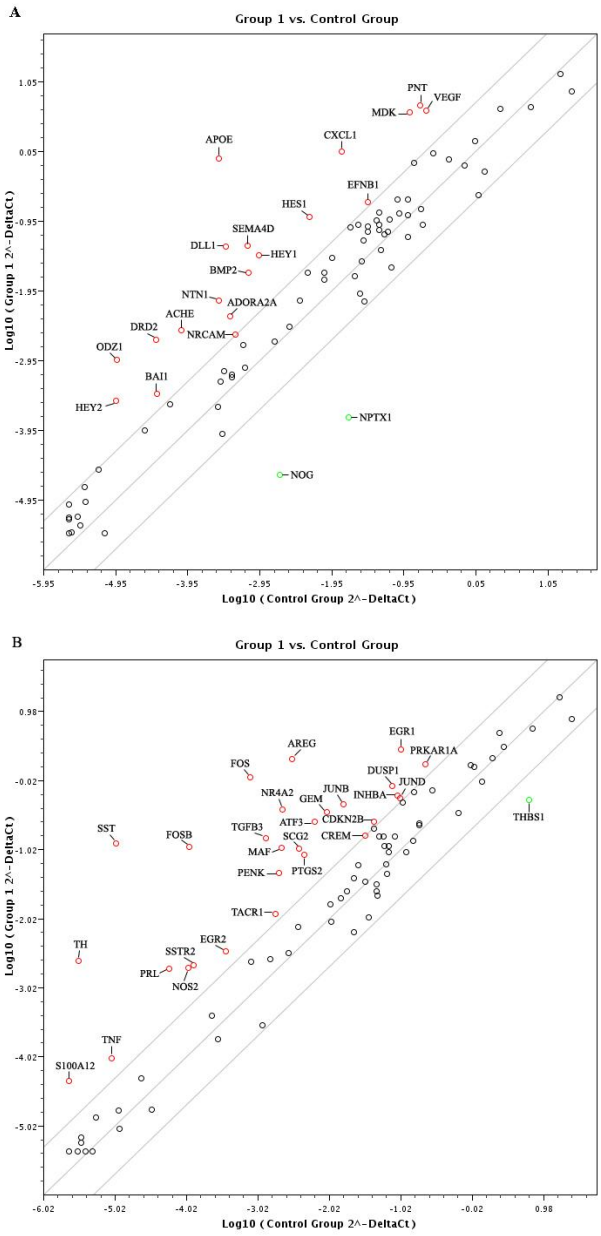

Fig. 7. Neurogenesis, neural stem cell and cAMP/Ca2+ signalling PCR arrays revealed up- and downregulated genes during neural differentiation of SHEDs. The scatter plot compares the normalized expression of genes on the arrays: a — Neurogenesis and Neural Stem Cell array (PAHS-404A); b — Human cAMP/Ca2+ PathwayFinder array (PAHS-066A), between control and differentiation groups by plotting against one another to visualize large gene expression changes. The central line indicates unchanged gene expression; the boundaries limit gene expression changes by fivefold. Red dots upregulated genes, green dots downregulated genes ($p<0.05$). Gene expression levels were analysed using RT2 Profiler PCR Array Data Analysis software (version 3.5, QIAGEN)

Importantly, the expression of some genes regulating neuromediator synthesis and degradation was significantly increased in the differentiated SHEDs (tyrosine hydroxylase, 792-fold; acetylcholinesterase, 33-fold). We also detected strong induction of genes coding the ALK tyrosine kinase ligands secreted by proliferating sympathetic neurons (midkine, 30-fold; pleiotrophin, 27-fold) (25). Differentiated SHEDs were highly enriched in transcripts of neuropeptides that are important for neuronal and endocrine cell-to-cell communications (somatostatin, 11,792-fold; somatostatin receptor 2, 17-fold; proenkephalin, 23-fold and prolactin, 33-fold). In addition, there was a strong upregulation (27-fold) of secretogranin 2.

FOS, FOSB, JUNB and JUND were strongly upregulated (1,450-fold, 998-fold, 29-fold and sixfold, respectively). These participate in the formation of the transcription factor complex AP-1 and are important regulators of cell proliferation and differentiation.

We detected a strong upregulation of cyclic AMPdependent transcription factor ATF-3, CAMP-responsive element

modulator and CAMP-responsive element binding protein 1 (41-fold, fivefold and threefold, respectively). We found that cyclin-dependent kinase inhibitor 2B (p15) was upregulated by sixfold. p15 functions as a cell growth regulator, which prevents the activation of CDK kinases; it is an effector of TGF β-induced growth arrest in several cell types (26). Adenosine A2a receptor was upregulated by 11-fold in differentiated SHEDs. This receptor participates in the downstream activation of adenylyl cyclase, elevates intracellular cAMP levels and is highly expressed in basal ganglia and vasculature (27). We also detected induction of the gene coding for dopamine receptor 2 (55-fold). Expression of NR4A2 gene (also known as Nurr1) was upregulated by 174-fold. Nurr1 transcription factor is critical for the development and maintenance of dopaminergic neurons (28). The expression of ephrin-B1 gene which codes type I membrane protein and ligand of Eph-related receptor tyrosine kinases was upregulated by sixfold.

Gene	Up/down	Folds	Gene	Up/down	Folds
Acetylcholinesterase	↑	++	JUND	↑	+
Adenosine A2a receptor	↑	++	MAF	↑	++
Amphiregulin	↑	+++	Midkine	↑	++
Apolipoprotein E	↑	+++	Netrin 1	↑	++
ATF-3	↑	++	Neuronal pentraxin 1	↓	+++
BMP2	↑	++	Noggin	↓	++
Cyclin-dependent kinase inhibitor 2B (p15)	↑	+	Notch 2	↑	+
CREM	↑	+	NR4A2 (Nurr1)	↑	+++
CREB1	↑	+	ODZ1 (teneurin 1)	↑	++
DLL1	↑	+++	Pleiotrophin	↑	++
Dopamine receptor 2	↑	++	Proenkephalin	↑	++
Early growth response 1 (Krox24)	↑	++	Prolactin	↑	++
Early growth response 2 (Krox20)	↑	+	Reticulon 4 (Nogo)	↓	+
Ephrin-B1	↑	+	Secretogranin 2	↑	++
FOS	↑	+++	Semaphorin-4D	↑	++
FOSB	↑	+++	Somatostatin receptor 2	↑	++
HEY1	↑	++	Somatostatin	↑	+++
HEY2	↑	++	STAT3	↑	+
HES1	↑	++	TGF β-3	↑	+++
JUNB	↑	++	Tyrosine hydroxylase	↑	+++

Fig. 8. Gene expression changes in SHED cells during neural differentiation. Real time PCR was performed using MX3005P® (Stratagene). PCR array for Neurogenesis and Neural Stem Cells (PAHS-404A) (SABiosciences, A QIAGEN company) was used. Data were analysed using RT^2 Profiler PCR Array Data Analysis software (version 3.5, QIAGEN). „+" – reflects gene expression up/down-regulation more than 2 folds in differentiated SHED cells compared with undifferentiated; „+ +" – more than 10 folds; „+ + +" – more than 100 folds.

Significant changes were also found for a variety of genes important in neuronal physiology, as for apolipoprotein E (2905-fold) assumed to play an important role during axonal regeneration after lesion (29). Importantly, this protein has been associated with astrocytic glia of the central nervous system and with non-myelinating glia of the peripheral nervous system (30). There was a significant downregulation (110-fold) of the neuronal pentraxin 1 gene expression in differentiated SHEDs.NPTX 1 protein is an important regulator of neuronal apoptosis and mitochondrial transport in healthy neurons (31). Expression of the ODZ1 gene (also known as teneurin 1) was strongly upregulated (99-fold). Teneurins are transmembrane proteins important for neurite outgrowth and axon guidance, target recognition and synaptogenesis (32). Incidentally, another important regulator of axon guidance and cell migration during development, netrin 1, was upregulated 27-fold. Expression of reticulon 4 (also known as Nogo) was inhibited (threefold). Nogo protein has been identified as amajor inhibitor of neurite outgrowth and neuroregeneration (33). Semaphorin-4D gene expression was upregulated by 66-fold. Semaphorin-4D plays a role in axonal growth cone guidance in the developing central nervous system and is an important regulator of dendrite and axon branching and morphogenesis (34). Expression of the gene coding MAF transcription factor was also upregulated (51-fold). Recent study has demonstrated that the MAF gene plays a crucial role during touch receptor development from mechanosensory neurons (35). Importantly, we also detected significant upregulation of both early growth response 1 (Krox24) and early growth response 2 (Krox20) transcription factors (28-fold and ninefold, respectively). These genes are crucial regulators of myelinating and non-myelinating Schwann cells (36).

For genes regulating major developmental signalling pathways, there was upregulation of BMP signalling components in differentiated SHEDs (TGF β-3, 113-fold; BMP2, 26-fold) but a significant downregulation of BMP antagonist Noggin (83-fold).

Another growth factor being strongly induced in differentiating SHED cells was amphiregulin (684-fold). Amphiregulin is related to EGF and TGFα and has a strong effect on the proliferation of astrocytes, Schwann cells and neural stemcells (37).

Importantly, components of the Notch signalling pathway were also strongly upregulated (Notch 2, 4-fold; DLL1, 129-fold; HES1, 24-fold; HEY1, 34-fold; HEY2, 27-fold).

3D microcarrier cell culture affects proteomic signature of exosomes derived from SHEDs

Microcarrier technology is an efficient method for scaling-up cell production in small volumes, while promoting improved cellular phenotypes in a three-dimensional environment. The main advantage of this system is the possibility to cultivate up to 100 times more cells in the same amount of medium. In addition, these systems could be

potentialy used as a factories for the small scale production of different therapeutic factors. We used BioLevitator™ (Hamilton), a commercially available three-dimensional culturing platform and alginate microcarrier cell culture system (Global Cell Solutions) for the propagation of SHEDs. Exosomes were purified by ultracentrifugation from SHEDs cultivated under two conditions: standart two-dimensional culture flasks, or from SHEDs grown on the laminin-coated microcarriers in bioreactor.

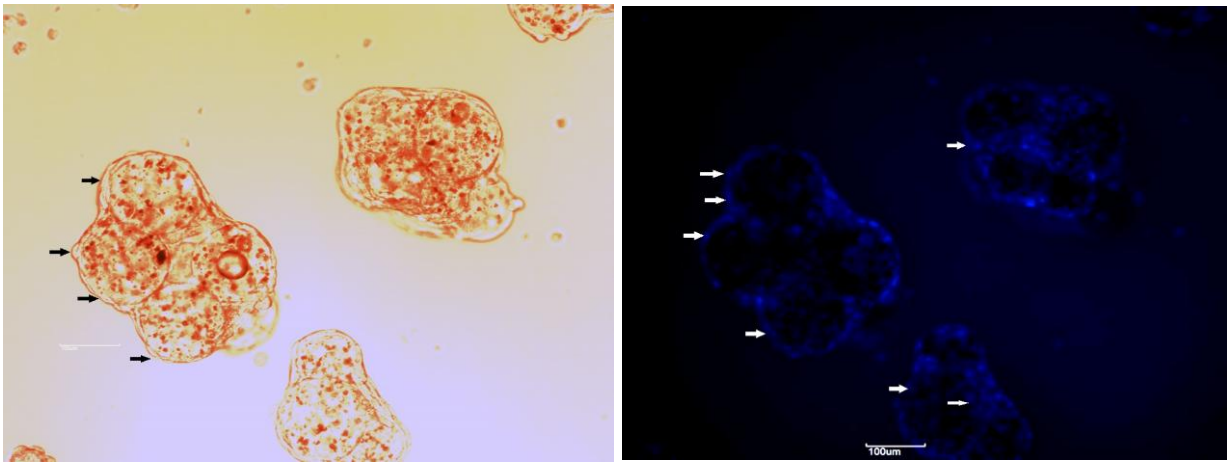

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Fig. 6. SHED culture on microcarries in bioreactor. SHED cells were cultured using bioreactor Biolevitator (Hamilton) on laminin-covered microcarriers (Global Cell Solutions) in serum-free medium MSC Nutristem XF Medium (Biological Industries). A-optical microscopy, phase contrast, original magnification x400; B-the same field of view using fluorescence microscopy, cell DNA was stained with Hoechst 33342 (excitation 350 nm/emission 460 nm), original magnicifation x400. Arrows show some cells and their nuclei.

In both cases cells were grown in serum- and xeno- free medium (MSC NutriStem XF, Biological Industries). For proteomic studies liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) analysis was carried out on an EASY-nLC (Thermo Fisher Scientific) connected to a Velos Pro-Orbitrap Elite hybrid mass spectrometer with nano electrospray ion source (Thermo Fisher Scientific). In total we identified 80 proteins in exosomes from standart SHED cultures and 60 proteins in exosomes from microcarrier cultures (Fig. 6, Suppl. 2 and Suppl. 3). The majority of the identified proteins are included in the vesiclepedia database. Importantly, only 28 proteins were common between exosomes from different preparations. These findings indicate, that 3D microcarrier cell culture have a profound impact on the proteomic composition and possibly physiological properties of exosomes.

Exosomes derived from SHEDs grown on the laminin-coated three-dimensional alginate microcarriers suppress 6-OHDA-induced apoptosis in human dopaminergic neurons

SHEDs have unique neurogenic properties, which could be potentially exploited for therapeutic use. The importance of paracrine SHED signalling for the neuroregeneration has been recognized, but the exact mechanisms behind these effects are presently unknown. Here we investigated the neuroprotective potential of exosomes

and microvesicles derived from SHEDs on human dopaminergic neurons during oxidative stress induced by 6-OHDA.

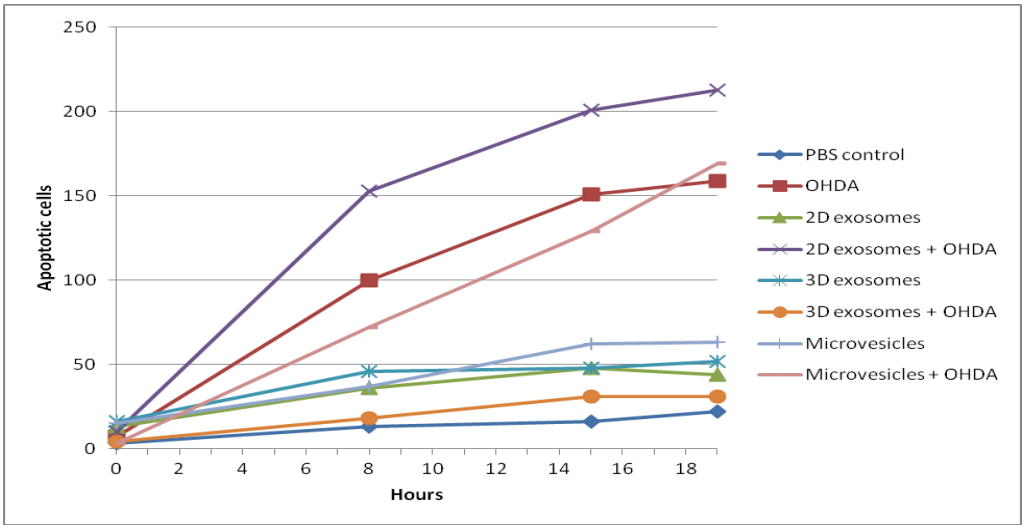

Fig. 7. Apoptosis of dopaminergic neurons after different treatment. Differentiated ReNcells were treated with 100μM 6-OHDA for 2 hours. Thereafter medium was replaced with fresh medium respectively with: exosomes isolated from SHEDs cultured under standart conditions (2D exosomes + OHDA), exosomes isolated from SHEDs cultured on microcarriers in bioreactor (3D exosomes + OHDA) or microvesicles. PBS was used as control. All vesicular fractions were added to 6-OHDA untreated cells as well. For visualization of apoptotic cells CellEvent™ Caspase-3/7 green apoptosis detection reagent was used (see „Methods“). Apoptotic cells were counted using ImageMaster 2D Platinum 7.0 software.

Exposure of dopaminergic neurons to the 6-OHDA induced rapid apoptotic response (Fig. 7 and 8, Suppl. 6). Interestingly, combination of 6-OHDA with exosomes derived from SHEDs grown under standard conditions resulted in even more dramatic apoptotic response (Fig. 7 and 8, Suppl. 7). In this case number of apoptotic cells was about 50 % larger than in single 6-OHDA-treated neuronal cultures, although the dynamics of apoptotic response was similar in both conditions. By contrast, exosomes from standard SHED cultures alone did not induce significant apoptosis suggesting that they may act as „6-OHDA sensitizers'' of dopaminergic neurons. We also checked whether microvesicles derived from SHEDs grown on the laminin-coated three-dimensional alginate microcarriers did suppress 6-OHDA-induced apoptosis in dopaminergic neurons. We detected only a slight suppression during initial phase of incubation with 6-OHDA, while at the later phases numbers of apoptotic cells were comparable with 6-OHDA-treated cultures (Fig. 7 and 8, Suppl. 8). By contrast, exosomal preparations derived from the same supernatants of SHEDs grown on the laminin-coated microcarriers had dramatic effect on the apoptotic response of 6-OHDA-treated dopaminergic neurons (Fig. 7 and 8, Suppl. 9). These exosomes suppressed 6-OHDA-induced apoptosis approximately by 80 % during the whole culture period. Incidentally, the numbers of apoptotic cells were in the same range as in control cultures (Fig. 7 and 8).

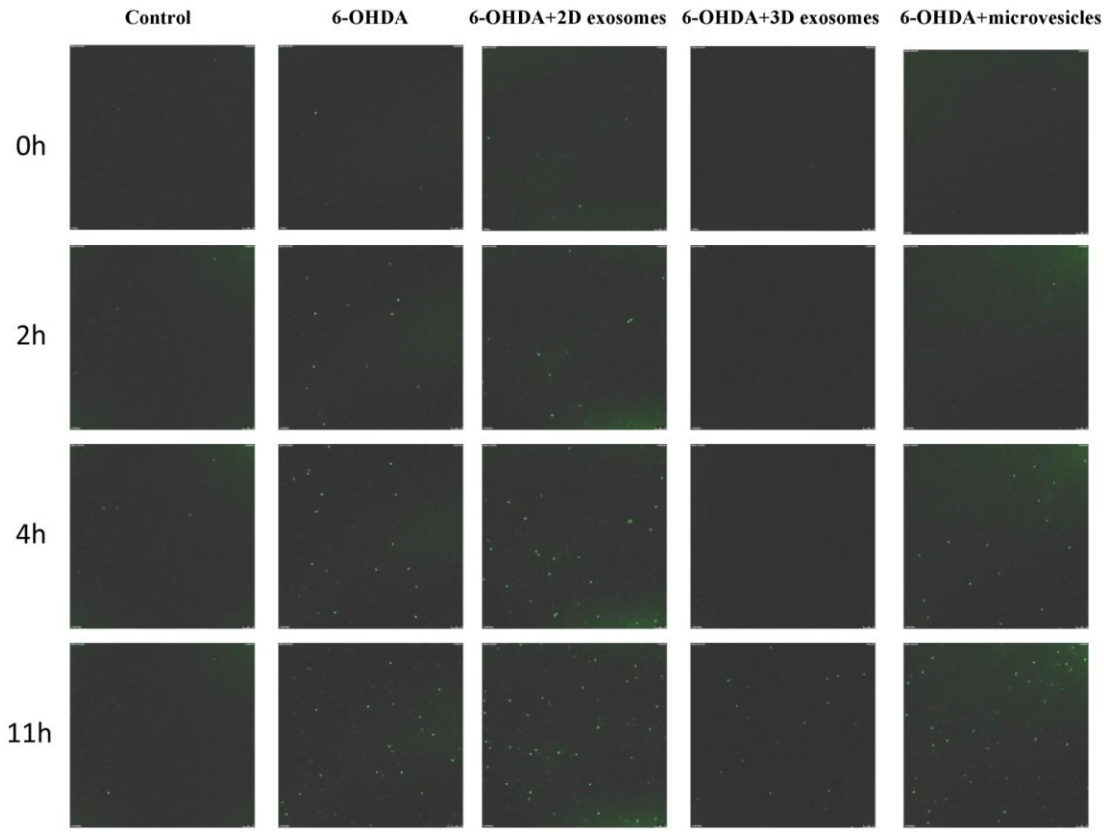

Fig. 8. Influence of exosomes and microvesicles on 6-OHDA induced dopamiergic neurons apoptosis. Apoptotic cells in culture at the beginning of documentation and after 2, 4 and 11 hours. Cells were treated with 100 μM 6-OHDA and respectively with: PBS (control), exosomes from SHEDs cultured under standart conditions (6-OHDA+2D exosomes), exosomes from SHEDs cultured on microcarriers in bioreactor (6-OHDA+3D egzosomos) and microvesicles (6-OHDA+microvesicles). Apoptotic cells stained with fluorogenic substrate CellEvent Caspase3/7 shown as green spots.

Thus, our results demonstrate that exosomes derived from SHEDs grown on the laminin-coated microcarriers display neuroprotective properties in 6-OHDA-treated human dopaminergic neurons.

Conclusions

1. Stem cells derived from human exfoliated deciduous teeth (SHEDs) display characteristic properties of neural progenitor cells (expression of βIII tubulin and nestin genes, expression of nestin protein).

2. Immunocytochemical and gene expression analyses revealed that SHEDs treated according to our new differentiation protocol gave rise to mixed neuronal/glial cell cultures.

3. Comparative proteomic analysis of exosomes derived from SHEDs grown under standard conditions and on laminin-coated microcarriers in bioreactor revealed significant differences. In total we identified 80 proteins in exosomes from standart SHED cultures and 60 proteins in exosomes from microcarrier cultures and only 28 proteins were common between exosomes from different preparations. These findings indicate, that microcarrier cell culture have a profound impact on the proteomic composition of exosomes.

4. Exosomes derived from SHEDs grown under standard conditions increase sensitivity of human dopaminergic neurons to the 6-hydroxydopamine (6-OHDA).

5. Exosomes, but not microvesicles derived from SHEDs grown on laminin-coated microcarriers in bioreactor suppress 6-OHDA-induced apoptosis in human dopaminergic neurons.

List of publications

A. Jarmalavičiūtė, V. Tunaitis, E. Strainienė, R. Aldonytė, A. Ramanavičius, A. Venalis, K.E. Magnusson, A. Pivoriūnas. A New Experimental Model for Neuronal and Glial Differentiation Using Stem Cells Derived from Human Exfoliated Deciduous Teeth. *Journal of Molecular Neuroscience* (2013) 51:307–317.

A. Jarmalavičiūtė, V. Tunaitis, U. Pivoraitė, A. Venalis, A. Pivoriūnas. Exosomes from dental pulp stem cells rescue human dopaminergic neurons from 6-hydroxy-dopamine-induced apoptosis. *Cytotherapy.* Accepted for publication.

Conference reports

A. Pivoriūnas, **A. Jarmalavičiūtė**, V. Tunaitis, M. Turkina, KE Magnusson. „Fetal bovine serum proteins associate with molecular chaperone GRP78 in stromal cells derived from the dental pulp of human exfoliated deciduous teeth“. 36th FEBS Congress of the Biochemistry for Tomorrows Medicine, 2011 June 25-30, Torino, Italy.

A. Pivoriūnas, **A. Jarmalavičiūtė**, V. Tunaitis. „Development of *in vitro* neural differentiation model using adult human stem cells“. XII Conference of Lithuanian Biochemical Society „Biochemijos studijoms Lietuvoje – 50 metų“, 2012 June 28-30, Tolieja, Lithuania.

A. Jarmalavičiūtė, V. Tunaitis, A. Pivoriūnas. „Development of *in vitro* neural differentiation model using human dental pulp stem cells“. 1st International Conference on Stem Cells, 2012 September 6-11, Chania, Crete, Greece.

A. Pivoriūnas, V. Tunaitis, **A. Jarmalavičiūtė**, A. Venalis. „The influence of hypoxia and heat shock on the cytokine/chemokine secretion profiles in mesenchymal stromal cells derived from human exfoliated deciduous teeth“. Stem cells in translation. ISSCR meeting, 2013 September 15-18, Florence, Italy.

U. Pivoraitė, **A. Jarmalavičiūtė**, V.Tunaitis, A. Pivoriūnas. „Automated 3D microcarrier cell culture of human mesenchymal stem cells“. Tarptautinė tarpdisciplininė konferencija “Nanotechnology: Research and Developement“, 2014 May 15-16, Vilnius, Lithuania.

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Reziumė

Kuriant efektyvias terapijos priemones sunkių neurologinių būklių gydymui būtina gerai suprasti žmogaus nervinių ląstelių funkcionavimo molekulinius mechanizmus. Šių uždavinių sprendimui yra reikalingi adekvatūs eksperimentiniai modeliai, kurių pagalba *in vitro* sąlygomis būtų galima tirti žmogaus nervinių ląstelių augimą, diferenciaciją ir išgyvenimą reguliuojančius mechanizmus, modeliuoti patologinius procesus, atlikti farmakologines studijas ir kt. Šiems tikslams naudojamos įvairių tipų žmogaus kamieninės ląstelės, vykdant jų nervinę diferenciaciją *in vitro* sąlygomis. Pirmoje šio darbo dalyje sukūrėmė naują nervinės diferenciacijos *in vitro* modelį, panaudodami kamienines ląsteles, išskirtas iš pieninio danties pulpos (SHED).

Mūsų sukurtas protokolas susideda iš kelių etapų – iš pradžių ląstelės išsėjamos įprastomis sąlygomis terpėje su serumu. Kuomet ląstelės prikimba prie auginimo indų paviršiaus ir pradeda dalytis (po 1-2 dienų), jos pervedamos į terpę be serumo. Šiam tikslui naudojome komercinį serumo pakaitalą (SFM). Mūsų duomenys rodo, kad ląstelės šią terpę be serumo toleravo gerai, dalijosi, šiek tiek susiaurėjo bei įgavo verpstės formą. Po 7-10 dienų ląstelės buvo pervedamos į specializuotą nervinių ląstelių auginimo terpę su augimo faktoriais EGF bei FGF2. Šiame etape ląstelės suaugo į monosluoksnius, kuriuose vėliau pradėjo formuotis ertmės ir sferiniai ląstelių dariniai. Kadangi sferogeniškumas yra būdingas nervinių ląstelių bruožas, papildomai tyrėmė SHED sferogeniškumą, panaudodami mažai lipnaus plastiko indus. Nustatėme, kad SHED pasižymi geromis sferogeninėmis savybėmis. To priežastimi gali būti SHED ląstelių neuroektoderminė kilmė, kadangi, yra žinoma, kad SHED ląstelės, kaip ir neuronai, kyla iš nervinės keteros ląstelių. Manome, kad šių dviejų etapų metu, kuomet ląstelės buvo augintos be serumo, jos išsivalė nuo serumo komponentų, galinčių trikdyti SHED ląstelių nervinę specifikaciją. Galutiniame etape vykdėme nervinės diferenciacijos indukciją, panaudodami tą pačią specializuotą nervinių ląstelių auginimo terpę ir augimo faktorius dbcAMP, NGF, GDNF bei CNTF.

Jau sekančią dieną po nervinės indukcijos kultūrose atsirado pavienės ląstelės su neuronams būdingais morfologiniais pokyčiais – nedideliu išgaubtu ląstelės kūnu ir smulkiomis besišakojančiomis ataugomis. Tęsiant kultivavimą šiomis sąlygomis, tokių ląstelių atsirado vis daugiau, ilgainiui jos pradėjo formuoti tarpusavyje tinklus, o vietomis sudarė gangijines struktūras primenančius darinius. Paraleliai diferencijavę SHED ląsteles pagal tokį patį protokolą, tik praleidę ląstelių adaptavimo prie terpės be serumo stadiją, nustatėme, kad SHED adaptavimas prie terpės be serumo didina nervinės diferenciacijos efektyvumą.

Nervinės diferenciacijos efektyvumą vertinome panaudodami imunocitochemijos metodą. Iš pradžių tyrėme nerviniams pirmtakams būdingo žymens – nestino baltymo ekspresiją nediferencijavusiose SHED ląstelėse. Kontroliniams tyrimams naudojome iš riebalinio audinio išskirtas mezenchimines kamienines ląsteles. Nustatėme, kad nediferencijavusios SHED ląstelės gausiai ekspresuoja nerviniams pirmtakams būdingą nestino baltymą. Tuo tarpu iš riebalinio audinio išskirtose mezenchiminėse kamieninėse ląstelėse nestino baltymo imunocitochemiškai aptikti nepavyko. Įdomu tai, kad atlikus realaus laiko polimerazinę grandininę reakciją, nustatėme beveik vienodus kiekius nestino transkriptų tiek SHED, tiek iš riebalinio audinio išskirtose ląstelėse. Tuo tarpu neuronams būdingo β-III-tubulino transkriptus detektavome tik SHED ląstelėse.

Imunocitochemiškai tirdami pagal mūsų sukurtą protokolą diferencijavusių SHED ląstelių kultūras, nustatėme nervinėms ląstelėms būdingų baltymų raišką: mielino bazinio baltymo (MBP), β-III-tubulino, sinaptofizino, periferino, sensoriniams neuronams būdingo transkripcijos faktoriaus Brn3a, glijos ląstelėms būdingo GFAP, bei S100 baltymo raišką. Taip pat alikome plataus mąsto genų raiškos tyrimus diferencijavusiose SHED ląstelėse. Iš viso įvertinome 168 genų raiškos pokyčius nediferencijuotose ir diferencijuotose SHED ląstelėse. Nustatėme, kad 110 genų raiška pakito daugiau nei du kartus: 90 buvo padidėjusi, 20 – sumažėjusi. Be to, iš jų, 41 geno raiška padidėjo, o 4 sumažėjo daugiau nei 10 kartų. Apibendrinus, mūsų duomenys rodo, kad panaudojus šį protokolą SHED ląstelės diferencijuoja neuronų ir glijos kryptimis, t.y. formuojasi mišrios ląstelių kultūros, sudarytos iš neuronų ir glijos elementų.

Antroje disertacinio darbo dalyje tyrėme SHED produkuojamų egzosomų poveikį 6-hidroksidopamino (6-OHDA) sukeltai dopaminerginių neuronų žūčiai. SHED ląsteles auginome dvejopai – įprastiniu būdu auginimo induose bei bioreaktoriuje ant lamininu dengtų mikronešiklių. Egzosomų išskyrimui panaudojome diferencinio centrifugavimo metodiką. Tyrėme, kaip skirtingomis sąlygomis kultivuojamų SHED produkuojamos egzosomos veikia 6-OHDA sukeltą dopaminerginių neuronų apoptozę. 6-OHDA yra specifiškai dopaminerginius neuronus veikiantis neurotoksinas, naudojamas modeliuojant Parkinsono ligą. Šiame darbe, naudojome komercinę imortalizuotą žmogaus nervinių kamieninių ląstelių liniją ReNcell VM. Šios ląstelės geba diferencijuoti į pilnavertiškus ir funkciškai aktyvius dopaminerginius neuronus.

Tyrimo metu diferencijavome ReNcell VM ląsteles į dopaminerginius neuronus, panaudoję specializuotą nervinių ląstelių auginimo terpę bei augimo faktorius dbcAMP, BDNF, GDNF, TGF–β3 ir askorbo rūgštį. Diferencijavusias ląsteles 2 val. veikėme 100μM 6-OHDA, tuomet ląstelėms terpė buvo pakeičiama į šviežią terpę su tais pačias minėtais augimo faktoriais bei papildomai su egzosomomis, išskirtomis iš įprastai auginamų SHED, egzosomomis, išskirtomis iš bioreaktoriuje ant mikronešiklių auginamų SHED, arba mikrovezikulėmis. Į kontrolinius šulinėlius buvo pilama PBS. Tuomet į visus tiriamus šulinėlius buvo įdedama fluorogeninio 3 ir 7 kaspazių dažo ir gyvų ląstelių vizualizavimas buvo vykdomas panaudojant konfokalinį mikroskopą Leica SP8.

Nustatėme, kad egzosomos, išskirtos iš bioreaktoriuje ant mikronešiklių auginamų SHED, 6-OHDA sukeltą dopaminerginių neuronų žūtį slopina. Tuo tarpu įprastomis sąlygomis augintų SHED sekretuojamos egzosomos didina žmogaus dopaminerginių neuronų jautrumą 6-OHDA. Mikrovezikulės ryškaus poveikio 6-OHDA sukeltai dopaminerginių neuronų žūčiai neturėjo. Taigi, antroje disertacinio darbo dalyje, mes pateikiame naujus duomenis, rodančius, kad SHED ląstelių produkuojamos egzosomos pasižymi neuroprotekcinėmis savybėmis. Šie rezultatai potencialiai gali būti panaudoti naujų terapinių strategijų, skirtų Parkinsono ligos gydymui, kūrimui.

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