VILNIUS UNIVERSITY
STATE RESEARCH INSTITUTE
CENTRE FOR INNOVATIVE MEDICINE

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ANALYSIS OF *IN VITRO* FUNCTIONS OF MESENCHYMAL STEM CELLS ISOLATED FROM DIFFERENT HUMAN TISSUES

Summary of doctoral dissertation
Biomedical sciences, biology (01B);
immunology, serology, transplantation (B500)

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VILNIAUS UNIVERSITETAS
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Virginijus Tunaitis

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Introduction

Human mesenchymal stem cells (MSC) have attracted a great deal of interest for their potential use in regenerative medicine and tissue engineering. MSC are being explored to regenerate damaged tissue and treat inflammation, resulting from cardiovascular disease and myocardial infarction, brain and spinal cord injury, stroke, diabetes, cartilage and bone injury, Crohn's disease and graft versus host disease. Nevertheless, all known therapy protocols require large amounts of MSCs, which can be obtained only by *in vitro* expansion. Therefore safe and effective protocols allowing production of large homogenic populations in a considerably short period of time are needed. One of the most important methodological problems is associated with the use of animal-derived components in the cell culture medium. Typically, 10-20 % of fetal calf serum (FCS) is added to the media for MSC culture. FCS contains a number of components that are poorly characterized and present at variable concentrations in different lots. More importantly, FCS is a potential source of contamination by prions, mycoplasms and viruses, and may give zoonoses Human allogeneic serum (HS) has been used for MSC expansion in several studies, but results are controversial. Growth medium with FCS was more effective than one with HS in stimulating proliferation of bone marrow-derived MSCs. Serum-free medium should have some important benefits compared to media supplemented with FCS or HS. First, the exact composition of serum-free medium is known, at least to the manufacturer. This eliminates the problem of lot-to-lot variability and therefore helps to compare study outcomes. Second, these sera are free from undefined animal components, therefore the risk of contamination, for instance by prions, viruses, mycoplasms, or other infectious agents, is minimized. However, more studies are needed to establish a gold standard serum-free medium for MSCs. In this setting, artificial serum substitutes such as Ultroser G may represent a useful alternative. The stable composition of artificial serum substitute should ensure good reproducibility. Ultroser G has been successfully used as serum substitute for expansion of bone marrow-derived MSC (BM-MSC). In the present study we compared the effects of different supplements on MSCs derived from adipose tissue.

Human dental pulp derived from exfoliated deciduous teeth has been described as a promising alternative source of multipotent stem cells, referred as stem cells from human exfoliated decidous teeth (SHED). While these cells share certain similarities with MSCs isolated from other tissues, basically they are still poorly characterized. Their similarities with MSC-like cells derived from bone marrow, adipose tissue, or other sources include typical fibroblastoid morphology, immunophenotype, and to some extent differentiation

potential. Our initial observation suggests that SHEDs demonstrate higher *in vitro* proliferation rates than stromal MSC-like cells derived from other sources, for example adipose tissue. However, the characterization process is far from complete. Proteomic technology allows systematic large scale qualitative and quantitative mapping of the whole proteome, which ideally represents the entire complement of proteins in a given organism at a given time. Such studies could not only be useful for the identification of specific markers, but also reveal typical proteomic patterns of MSCs during proliferation, differentiation, senescence, or other experimental conditions.

The mechanism by which MSC-like cells promote tissue repair is unknown. Some evidence indicates, that transplanted MSCs create a specific microenvironment (specific combinations of growth factors, chemotactic molecules and so on) that enhance the regeneration of injured tissue, rather than differentiate into specialized cells. It is possible that local physiological and/or pathological stimuli from tissues could induce recruitment, activation and migration of MSCs from the perivascular space to the site of injury. Inflammatory output is partially determined by the local balance of proteases and antiproteases. In attempt to elucidate some new mechanisms of MSC activity we have investigated whether a proinflamatory protein alpha1 – antitrypsin (AAT) could modulate SHED activity differently. Thus, we designed this study, where AAT exposure-related intracellular events were elucidated and functional cell changes identified. This was probably the first report addressing the *in vitro* interaction between tissue-residing stem cells and major human antiprotease AAT.

Results of the present study extend our understanding of processes in MSCs during cultivation *in vitro* and explain some mechanisms responsible for the functionality of these cells.

The aim of dissertation work

The main aim of the current research was to elucidate the influence of different serum substitutes to the proliferation, differentiation, expression of cell surface markers, and total protein expression of mesenchymal stem cells derived from human adipose tissue, and to determine the features of mesenchymal stem cell populations from an exfoliated deciduous tooth and their response to the multifunctional proinflammatory protein alpha1-antitrypsin.

The objectives of this work were as follows:

1. To analyze the effect of fetal calf serum, allogeneic human serum, and synthetic serum substitute on the proliferation, differentiation, and cell surface markers expression of human adipose tissue-derived mesenchymal stem cells.
2. To ascertain the influence of different serum substitutes to the transcription of genes related to adipogenic and osteogenic differentiation (*PPARγ*, *Msx2*, *osteopontin*) in the proliferating mesenchymal stem cells derived from human adipose tissue.
3. To establish and characterize the total expression of the proteins in human adipose tissue-derived mesenchymal stem cells cultivated in growth media with different serum substitutes.
4. To determine the proliferation, differentiation, morphological features, cell surface markers expression, the ability to form colonies, and the proteomic map of abundantly expressed proteins of primary culture of exfoliated deciduous tooth derived mesenchymal stem cells and this culture derived single cell clones.
5. To analyze the influence subphysiological, physiological and inflammatory concentrations of human alpha-1-antitrypsin on the proliferation and motility of mesenchymal stem cells derived from exfoliated deciduous tooth.

Scientific novelty

In this study the significance of different serum additives (fetal calf serum (FCS), pretested fetal calf serum (FCS-Sp), allogeneic human serum (HS), and synthetic serum substitute Ultroser G (U) on the morphology, adipogenic and osteogenic differentiation potential and the expression of characteristic surface antigens of human adipose tissue-derived (AT-MSC) were elucidated. Our results indicate that AT-MSCs cultivated in the presence of FCS and HS display similar growth, differentiation, immunophenotypic and proteomic properties, while synthetic serum substitute Ultroser G induces more profound changes in the physiology of AT-MSCs.

Up to now there was no information available about the transcription level of genes related to adipogenic and osteogenic diferrentiation (*PPARγ*, *Msx2*, *osteopontin*) in the proliferating mesenchymal stem cells. Using real-time PCR, we have found a significant increase in *PPARγ* and *Msx2* gene transcription in proliferating AT-MSCs cultivated with synthetic serum substitute Ultroser G in comparison to FCS.

Analysis of cell surface markers showed that AT-MSC cultivated in growth media with FCS, FCS-Sp, or HS displayed varying levels of CD146, while expression of CD146 antigen in AT-MSCs cultivated in the presence of Ultroser G was not detected.

In previous others studies the effect of different serum supplements on the expression of CD146 was shown in bone marrow derived-MSCs, but not adipose tissue-derived MSCs.

We found that AT-MSCs cultivated in the presence of different serum had similar global proteomic expression patterns, but the comparison of some identified proteins revealed that Ultroser G induces more significant expression changes than other serum substitutes.

In the present study, we used a proteomic approach that allowed us to compare protein expression signatures between a primary cell culture and her daughter clones. As a result, we for the first time established a map of abundantly expressed proteins in MSC-like cells derived from the dental pulp of human exfoliated deciduous teeth (SHEDs).The pattern of some highly expressed proteins in SHEDs was found similar to the already studied proteomic profile of other tissues-derived MSCs and for the first time indicated the common origin of these cells.

We have also analyzed the influence of human alpha-1-antitrypsin on the proliferation and motility of SHEDs. This is the first report addressing the *in vitro* interaction between tissue-residing stem cells and major human antiprotease alpha-1-antitrypsin.

The defensive statements

1. Serum substitute present in cell culture medium may exert certain regulatory properties on mesenchymal stem cells functions.
2. Morphology, functions and proteomic profile of mesenchymal stem – like cells from exfoliated deciduous tooth are related to mesenchymal stem cells derived from other tissues.
3. A major human antiprotease, alpha1-antitrypsin, under certain conditions is able to stimulate proliferation and motility of mesenchymal stem cells.

Material and methods

Isolation and cultivation of human mesenchymal stem cells. Adipose tissue was obtained during plastic surgery, after obtaining informed patient consent according to the guidelines set by Lithuanian Bioethics committee. The adipose tissue was excised from either breast or abdominal tissue of two 26 and 40 years old female donors and designated as AT-MSC1 and AT-MSC2. 20 g pieces of adipose tissue were washed extensively with sterile phosphate-buffered saline (PBS), divided into two parts, cut into smaller pieces in a solution of the latter consisted of 0.2% collagenase type I (Biochrom) in DMEM (Biochrom) with 1% BSA (Applichem), 100 U/ml penicillin and 100 μg/ml streptomycin (Biochrom) and incubated for 45 min at +37ºC. Centrifuged, the supernatant discarded and the stromal vascular fraction (SVF) resuspended in erythrocyte lysis buffer (160 mM NH_4Cl) for 20 min. Then the cell suspension was divided into 4 equal parts and plated into low glucose DMEM (Biochrom) medium with 2.0 mM L-Glutamine (Biochrom), 100 U/ml penicillin and 100 μg/ml streptomycin and supplemented with one of four different serums: 10 % Fetal calf serum (FCS) (HyClone), AT-

MSC-FCS; 10 % FCS MSC qualified (Invitrogen), AT-MSC-FCS-Sp; 10 % human allogeneic serum (Lonza), AT-MSC-HS; 2 % Ultroser G (Pall BioSepra), AT-MSCU.

Cells were harvested from an exfoliated deciduous tooth of a child (female, 7 years old, Caucasian), for which parents had signed informed consent. The material collection procedure was approved by the Lithuanian Bioethics committee. 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 (HyClone). Pulp tissue was scraped out and placed in 3 U/ml of type I Collagenase (Biochrom) dissolved in L-15 for 1 h at 37°C. After digestion the cell suspension was diluted in PBS and centrifuged at 250 g for 5 min. The supernatant was discarded, cells resuspended in low glucose (1000 mg/ml) Dulbecco's modified Eagle medium (DMEM) (HyClone) supplemented with 10% fetal calf serum and antibiotics, and plated in 12.5-cm2 flask.

Cultures were maintained at 37ºC in a humidified atmosphere containing 5% CO2 with medium change routinely twice a week.

Cell proliferation assay. Cell proliferation was tested during a 4-day course. Cells were seeded in a 96-well plate at different densities and cultured for 4 days. Cell proliferation calculated from the number of cells counted every day with hemocytometer or with XTT cell proliferation kit. XTT cell proliferation kit was used according to manufacturer's instructions (Biological Industries).

Differentiation assays. For differentiation experiments cells were seeded at a density $5x10^2/cm^2$ in 6-well (35 mm diameter) culture dishes and grown until the cultures reached subconfluence. Then they were treated with osteogenic-induction medium prepared according manufacturer's instructions (StemCell Technologies). Cell cultures were treated with osteogenesis-inducing medium for 2-3 weeks. For the assessment of osteogenic differentiation cell monolayers were washed with PBS, fixed for 20 min with 4 % paraformaldehyde at room temperature, washed with deionized water and stained with a 2 % Alizarin Red S (Sigma) solution, or by staining of hydroxyapatite crystals (von Kossa) with 1% silver nitrate (Sigma). Control cultures without the differentiation stimuli were stained in the same manner.

For the induction of adipogenic differentiation cells were seeded at a density $5x10^2/cm^2$ in 6-well (35 mm diameter) culture dishes until cultures reached subconfluence and treated with adipogenesis--induction medium prepared according manufacturer's instructions (StemCell Technologies). The medium was changed twice a week, for 3 weeks. Intracellular accumulation of lipids was visualized using Oil Red O reagent (Sigma)..

Flow cytometry. For the FACS analyses cells were trypsinized with a 0.25 % trypsin/1 mM EDTA solution, suspended in 10% FCS media, washed twice with ice cold phosphate buffered saline (PBS) and incubated on ice for 30 min with the following antibodies CD34- FITC, CD45-FITC, CD105-APC (Invitrogen), CD14-PE (Santa Cruz Biotechnology), CD73-PE, CD146-PE (BD Pharmingen), CD90-FITC (Millipore). The cells where then washed twice with PBS, resuspended and used for FACS analyses using FACSAria flow cytometer (BD Biosciences) and 10000 events for each staining were analyzed using BD FACSDiva software (version 6.1).

RNA extraction, cDNA synthesis and real-time PCR. Total RNA was isolated from proliferating cells at passage 3 using Triazol (Invitrogen) according the manufacturer's instructions. 3 μg of RNA extract was converted into firststrand cDNA using the Revert Aid M-MuLV Reverse transcriptase kit (Fermentas). For first-strand cDNA synthesis we used oligo(dT) primers in a 20 μl reaction mixture. Real-time polymerase chain reaction was performed using the MX3005P® instrument and MxPro software (version 4) (Stratagene, La Jolla, CA). The expression levels of *PPARγ*, *Msx2*, and *osteopontin* were analyzed using Maxima SYBR Green qPCR Master mix (Fermentas). The 0.5 μl of cDNA samples and 0.1 μM of primers were used per 25 μl of reaction volume. An initial 10 min denaturation step at 95°C was followed by 45 cycles of 20s denaturation at 95°C, 40s annealing at 55°C and 40 s elongation at 72°C. Fluorescence values of the qPCR products were corrected with fluorescence

signals of the passive reference dye (ROX). RT-PCR reaction mixture, lacking cDNA, and reaction mix without template were used as a negative controls. For normalization of cDNA levels, the housekeeping gene β-actin was used. PCR products were analysed by melting analysis. All results were analysed with MxPro software according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Generated by MxPro software upper and lower limit error bars reflect the deviation in the replicates and include the imprecision of the normalizers. Groups were compared by one-way analysis of variance (ANOVA) followed by Bonferroni multiple comparisons test (GraphPad InStat Software). Differences were considered significant when $p<0.05$.

2-D Gel electrophoresis (2 DE). For preparation of total protein extracts, cells were washed twice with cold PBS, and lysed in lysis buffer (0.01 M Tris-HCl, pH 7.4, 1 mM EDTA, 8 M urea, 0.05M DTT, 10% (v/v) glycerol, 5% (v/v) NP40, 0.2% Pharmalyte, pH range 3−10 (General Electrics,) and 2 mM PMSF) for 5 min; then lysates were centrifuged at 11000 g for 15 min at 4ºC. The supernatants were collected and stored at -80º C until analysed. The first dimension of isoelectric focusing was performed with a 2DE Multiphor unit (Amersham Biosciences) by steps of increased voltage up to ~70 kVh. The focusing Immobiline DryStrips were incubated for 15 min at room temperature in electrophoresis buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% (w/v) glycerol, 2% (w/v) SDS) with 1% (w/v) DTT, followed by 15 min in electrophoresis buffer with 2% (w/v) iodoacetamide. The second dimension was performed in a gradient gel (8–18% ExcelGel SDS polyacrylamide). The 2DE gels were silver-stained (Shevchenko *et al.*, 1996), spots selected by visual inspection and excised by scalpel.

In-gel tryptic digestion and MALDI-TOF MS. Areas of the gel that were deemed to be of interest were cut out and subjected to overnight in-gel tryptic digestion. In brief, the gel slices were dehydrated with 50% acetonitrile and then dried completely using a centrifugal evaporator (DNA Mini, Eppendorf). The tryptic peptides were subsequently extracted from the gel slices as follows. Any extraneous solution remaining after the digestion was removed and placed in a fresh tube. The gel slices were first subjected to an aqueous extraction and then to organic extraction with 5% trifluoracetic acid in 50% acetonitrile shaking occasionally. For matrixassisted laser desorption/ionization (MALDI)-time of flight (TOF) analysis, the peptides were redissolved in 3 μL of 30% acetonitrile and 0.01% trifluoracetic acid and were then prepared with a matrix (α-cyano-4-hydroxycinnamic acid) on the target plate. The analysis was performed on a Voyager MALDI-TOF MS (Perseptive Biosystems Inc.) Data processing of the spectra was performed with Data ExplorerTM Version 4.0 (Applied Biosystems).

Treatment of cells with alpha1-antitrypsin. For all AAT employing studies, cells were cultured in serum free medium for at least 12 h prior to and during the experiments. AAT (human) as a commercially available product Prolastin® (Bayer Corporation) was used.

Cell motility assay. After cells were grown in a control or AAT-containing (0.5, 1 and 5 mg/ml) medium for 12 h they were then harvested and added to Boyden chamber wells (Neuroprobe). Invasion assays were performed using 8-μm pore membranes coated with 50 μg/ml Matrigel (BD Biosciences) that was gelled on the membrane at 37°C for 1 h prior to chamber assembly. SHEDs were added to the upper compartment, while the lower compartment contained 10% fetal bovine serum in DMEM as a source of chemoattractants. Migration was presented as an average of cells on the lower surface of the membrane in three representative fields of each well. Here SHEDs were cultured in the presence of different AAT concentrations after creating artificial wounds in cell monolayers. Cells were allowed to migrate and cover the scraped area for 12 h, after which images were taken.

Gelatinase activity assays. The culture medium was collected and analyzed on 10% Tris-glycine gel containing 0.1% gelatin incorporated as a substrate to identify proteins with gelatinolytic activity. Samples were separated on the gel, renaturated, developed overnight at 37°C and stained with Coomassie Blue. Areas of gelatinase activity showed up as clear bands. The EnzCheck Gelatinase Assay Kit (Molecular Probes) was used for *in situ* zymography

according to the manufacturer's protocol. Control and AAT-treated cells were incubated with DQ-gelatin-FITC for 30 min at 37°C, washed and observed under a fluorescent microscope. The DQ-gelatin substrate used for the assay is heavily quenched with fluorescein and exhibits fluorescence only after cleavage.

Immunocytochemistry. Control and AAT treated cell monolayers were washed, fixed in 4% paraformaldehyde for 20 min, permeabilized with 0.1% Triton for 10 min and stained for AAT using anti-AAT primary antibody (Dako). Secondary fluorescence conjugated antibody was applied following extensive washes. Images were obtained with confocal microscopy, as described later, or with a Nikon fluorescence microscope Eclipse TE 2000–2004 and Nikon digital camera (Nikon Instruments) and analyzed with NisElements BR software (Nikon Instruments.). All sections were counterstained with 0.5 μm DAPI for 10 min, mounted in antifading Fluorescent Mounting Medium (Dako).

Confocal microscopy. Cells were stained for internal AAT and examined using confocal imaging with a two photon BioRad Radiance 2000 microscope (Carl Zeiss). Image acquisition settings were identical for all experiments. The images shown are representative of at least three experiments.

Cell lysis and Western blot analysis. In preparation of total cell lysates, cells were washed twice with cold PBS, pH 7.3 and lysed in RIPA buffer. Then benzonase (Merck) was added to the final concentration of 2.5 units/ml and cell lysates were homogenized through a 21 G needle. Samples were centrifuged at 14,000 rpm for 30 min at 4°C. Supernatants were aliquoted and kept at -20°C until analyzed. Protein concentrations were measured by the Bio-Rad DC protein assay (Bio-Rad)Cell lysates were diluted in sample buffer and loaded on a 10% SDS-PAGE gel, subjected to electrophoresis and then blotted onto a polyvinylidene fluoride membrane. The membrane was probed with an anti-PKD total (Santa Cruz Biotechnology) and phosphorylated (at Ser916 and Ser744/748), anti-PKB (Akt) total and phosphorylated (at Thr308 and Ser473) and anti-ERK total and phosphorylated antibodies at 1:1000 dilution (Cell Signaling Technology). AAT in lysates was detected using anti-AAT primary antibody (Dako) at 1:2500 dilution. Bands were visualized by incubation with appropriate horseradish peroxidase-conjugated secondary antibodies and chemiluminescence substrate (Pierce).

Statistics. Results are shown as mean ± standard deviation. The differences in the means of experimental results were analyzed for their statistical significance by two-sided t-test and one-way analysis of variance followed by individual comparisons (ANOVA), where p less than 0.05 was regarded as significant.

Results and Discussion

Effects of different serum on morfology, proliferation, differentiation, and immunophenotype of adipose tissue-derived mesenchymal stem cells. In this study we analyzed responses of MSCs to different growth supplements commonly in use. MSCs were isolated from breast or abdominal adipose tissue and plated into DMEM supplemented with one of four different serums: fetal calf serum (AT-MSC-FCS), pretested fetal calf serum (AT-MSC-FCS-Sp), human allogeneic serum (AT-MSC-HS), or syntetic serum substitute Ultroser G (AT-MSC-U).

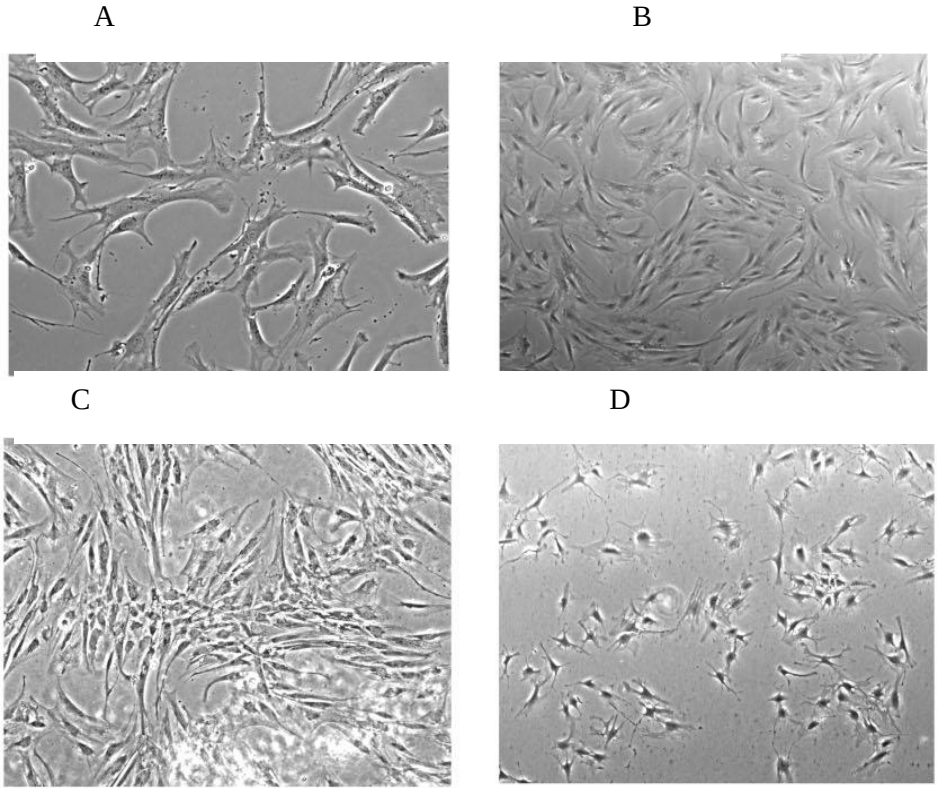

Figure 1. Morphology of AT-MSCs cultivated with different serum supplements. AT-MSCs cultivated in the presence of: **A**-fetal calf serum (FCS); **B**-pretested (FCS-Sp); **C**-allogeneic human serum (HS) and **D**-synthetic serum supplement Ultroser G. Phase-contrast, magnification 100x.

We observed remarkable differences in the morphology of cells cultivated in the media with different serums (Figure 1). The culture of AT-MSC-U consisted of very small spindle-shaped fibroblastoid cells, while AT-MSC-HS, AT-MSC-FCS-Sp and AT-MSC-FCS cells phenotypically were larger and flatter with longer outgrowths.

There were significant differences in growth rates between AT-MSC cultures cultivated with different serum supplements. Typically, AT-MSC1/2-U doubled on average within about 25-27 hours, while it occured for AT-MSC-HS, AT-MSC-FCS-Sp and AT-MSC-FCS in a range of 40-50 hours. Thus, cultivation of AT-MSCs in a presence of serum supplement Ultroser G resulted in a twofold increase in growth rate, compared to cells grown with animal or human allogeneic serums. Importantly, we observed similar effects in two cell lines isolated from different donors. The differentiation studies revealed high adipogenic and osteogenic potentials of all cells tested (Figure 2).

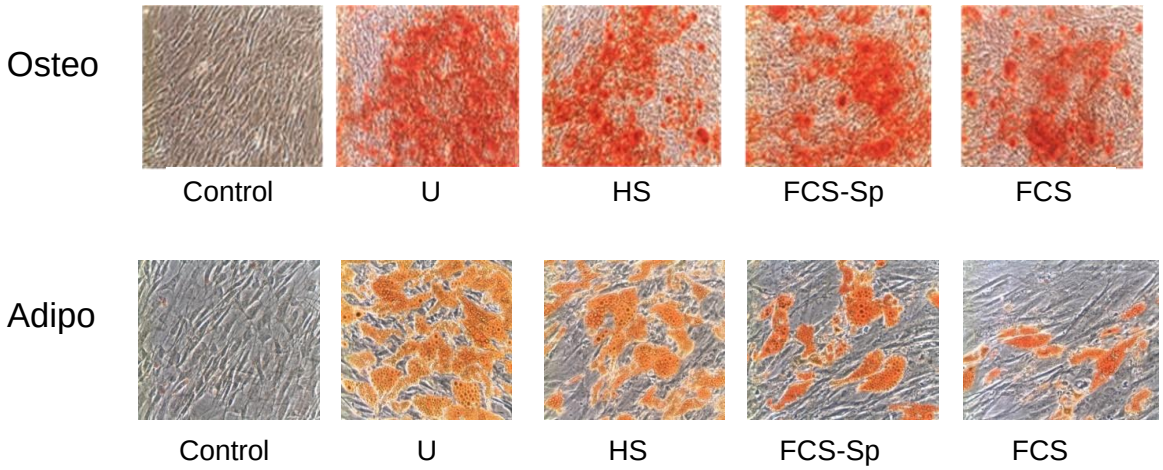

Figure 2. **Adipogenic and osteogenic differentiation of AT-MSCs cultivated with different serum supplements.** Osteogenic (Osteo) and adipogenic (Adipo) differentiation was evaluated in cells cultured with medium promoting differentiation for 3 weeks and evidenced by formation characteristic calcium deposits.stained with Alizarin Red S or lipid vacuoles stained using Oil Red O staining. Control cultures without differentiation stimuli were stained in the same manner. AT-MSCs cultivated in the presence of fetal calf serum (FCS); pretested FCS (FCS-Sp); allogeneic human serum (HS) and synthetic serum supplement Ultroser G (U). Phase-contrast, magnification 100x.

However, the highest adipogenic potential was demonstrated by the two AT-MSC-U lines. FACS analysis showed that all cells, expressed characteristic antigens of MSC-like cells, including CD73, CD90 and CD105 but not the hematopoietic markers CD14, CD34 and CD45. Interestingly, we did not detect expression of CD146 antigen in AT-MSCs cultivated in a presence of Ultroser G, in contrast to AT-MSC-FCS, AT-MSC-FCS-Sp and AT-MSC-HS which displayed varying levels of CD146 (Table 1).

Table 1. Flow cytometric analysis of AT-MSCs cultivated with different serum supplements

	CD14	**CD34**	**CD45**	**CD73**	**CD90**	**CD105**	**CD146**
AT-MSC1-FCS	1.9	0.3	5.3	99.8	98.4	100	46.2
AT-MSC1-FCS Sp	0.4	1.6	0.9	93	99	99.9	52.8
AT-MSC1-HS	1.7	1.5	9.9	96.9	90.1	100	13.5
AT-MSC1-U	3.0	0.3	2.0	98.4	98	99.9	0.5
AT-MSC2-FCS	0.7	0.6	0.6	97.2	99.7	97.9	17.7
AT-MSC2-FCS Sp	2	5.2	0.1	99.9	100	99.7	13.5
AT-MSC2-HS	0.2	0.9	0.2	99.7	99.8	98.6	5.8
AT-MSC2-U	0.2	0.8	0.0	97.2	99.9	98.4	0.2

Interestingly, the CD146 expression varied significantly between the donors; 46 % of AT-MSC1-FCS and only 17.7 % of AT-MSC2- FCS cells were positive for CD146. The mechanism of CD146 deprivation in AT-MSC-U is not clear. One possibility is that occurs a selection of CD146 negative clones in the presence of Ultroser G, or, that components of serum substitute downregulate expression of CD146 in AT-MSC-U.

Expression of PPARγ, Msx2 and osteopontin genes in AT-MSCs cultivated with different serum supplements. We used real time PCR to compare expression levels of some genes important for adipo- and osteogenesis in MSCs. Figure 3 represents the influence of growth supplements on the expression of *PPARγ*, *Msx2* and *osteopontin* genes in either line of proliferating MSCs (3th passage). There were remarkable increase of *PPARγ* gene expression levels in AT-MSC1-U and AT-MSC2-U cells, by 81- and 28-fold, respectively. Recent studies support the role of PPARγ as master regulator of adipogenesis; thus, heterozygous PPARγ+/- mice had pronounced bone phenotype with increased bone density and decreased adiposity (Wan *et al.*, 2007). These data suggest that osteo- and adipogenesis are regulated by reciprocal mechanism and that PPARγ acts as molecular switch of adipogenic differentiation in MSCs. Cultivation of MSCs with the serum substitute Ultroser G also resulted in significant increase in the *Msx2* expression by 4- and 5.8- fold in AT-MSC1-U and AT-MSC2-U cells, respectively. Msx2 is a homeodomain transcription factor, which plays an important role in osteogenesis. Mice deficient in Msx2 manifest reduced bone formation associated with decrease in osteoblast numbers (Satokata *et al.*, 2000). Thus, the presence of high levels of *Msx2* and *PPARγ* transcripts in AT-MSC2-U cells correllates with high osteogenic and adipogenic differentiation potential. This is an unexpected finding, because PPARγ and Msx2 have reciprocal roles during adipogenic and osteogenic differentiation (Ichida *et al.*, 2004).

By contrast, AT-MSC1-U cells displayed lowest expression of *osteopontin*, while we detected the highest expression levels of *osteopontin* gene in AT-MSC1-FCS and AT-MSC2-HS cells (Figure 3 A, B). Osteopontin is an extracellular matrix glycoprotein playing an important role in bone remodeling, immune regulation and chemotaxis. Its synthesis is promoted by key transcriptional regulators (such as Runx2) during osteogenic differentiation of MSCs (Rangaswami *et al.*, 2006). When confluent cultures were cultivated in the presence of different serums and compared to control cultures used in differentiation experiments and grown in the presence of FCS (Figure 3C), we observed similar tendencies of gene expression as in proliferating cells.

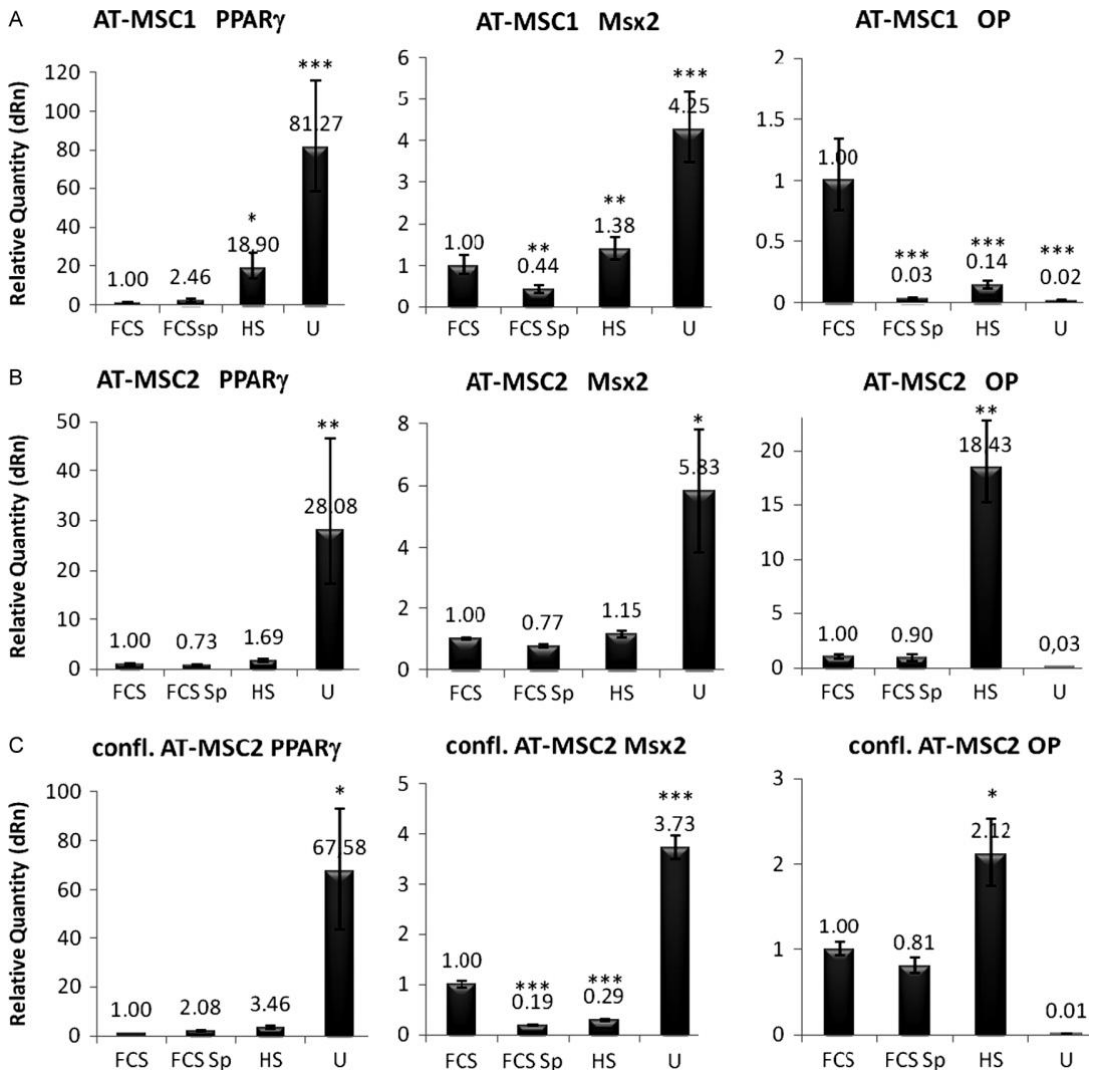

Figure 3. The effects of different serum supplements on the expression of *PPARγ* , *Msx2* and *osteopontin* in proliferating AT-MSC cells. Gene expression levels were analysed in adipose tissue-derived proliferating AT-MSC1 (A) and AT-MSC2 (B) cell lines, and fromconfluent culture of AT-MSC2s (C). Cells were cultivated with FCS, pretested fetal FCS specialized forMSCs (FCS Sp), allogeneic human serum (HS) and synthetic serum supplement Ultroser G (U). The relative abundances of mRNAs were normalized to the amount of *β-actin*. Relative expression of genes is presented as fold difference to genes expressed in AT-MSCs cultivated in the presence of FCS (calibrator). Upper and lower error limits are based on variation in the replicates and including the imprecision of the normalizers. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ with respect to FCS.

We next investigated changes of *PPARγ*, *Msx2* and *osteopontin* expression during osteogenic and adipogenic differentiation, specifically after 3 weeks of cultivation under adipogenic or osteogenic conditions (Figure 4). Adipogenic differentiation resulted in minor changes of *PPARγ* expression in AT-MSC2-U cells, while in AT-MSC2-FCS, AT-MSC2-FCS-Sp and AT-MSC2-HS there was a considerable increase. We thus speculate that proliferating AT-MSC2-U cells reached some sort of expression „limit“ which could not be exceeded even in the presence of adipogenic inducers.

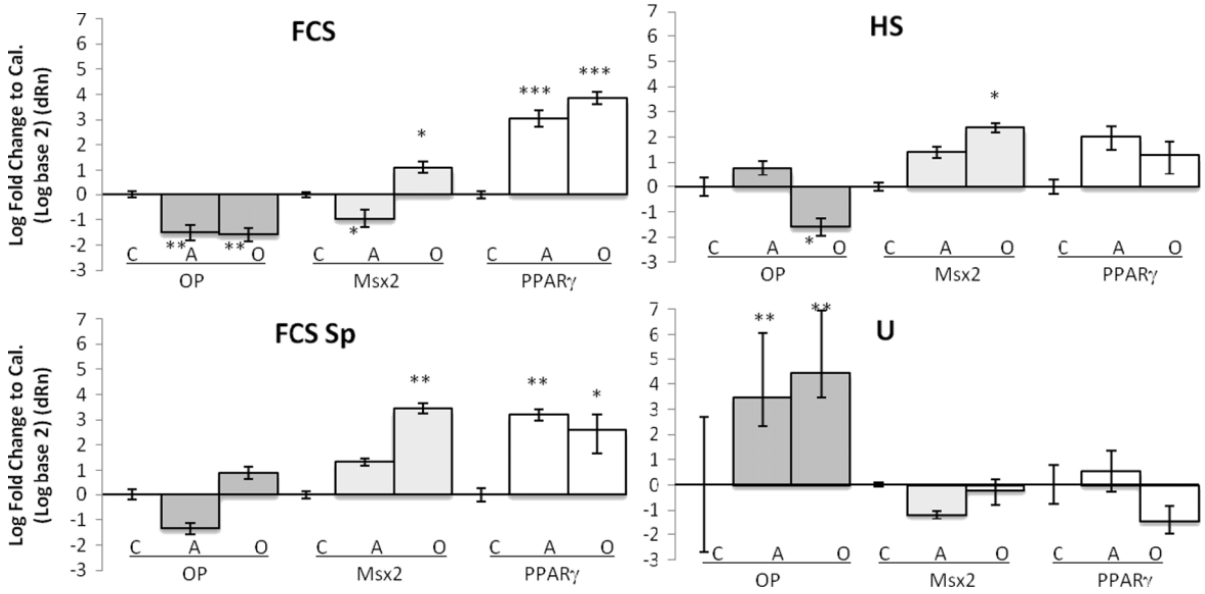

Figure 4. **The effects of different serum supplements on the expression of *PPARγ*, *Msx2*, and *osteopontin* in differentiating AT-MSC cells.** Gene expression levels were analysed in adipose tissue-derived AT-MSC2 on the 21 day of adipogenic (A) or osteogenic (O) differentiation. Cells were cultivated with FCS, pretested fetal FCS specialized for MSC (FCS Sp), allogeneic human serum (HS) and synthetic serum supplement Ultroser G (U). Data are normalized to β-actin and displayed as fold difference in gene expression relative to a reference control (C) on a 2-log scale with upper and lower error limits based on variation in the replicates and include the imprecision of the normalizers. * $p<0.05$, ** $p<0.01$, and *** $p<0.001$ respect to control.

Osteogenic differentiation increased expression of *Msx2* transcripts in AT-MSC2-FCS, AT-MSC2-FCS-Sp and AT-MSC2-HS, while in AT-MSC2-U cells, levels of *Msx2* remained relatively unchanged. Thus, AT-MSC2-U cells demonstrate similar tendencies of *Msx2* expression to that of *PPARγ*-high expression levels in proliferating cells with minor changes after induction of differentiation. The differentiation studies revealed that AT-MSC-U cells also displayed high osteogenic potential. Thus, the presence of high levels of *Msx2* and *PPARγ* transcripts in AT-MSC2-U cells correllates with high osteogenic and adipogenic differentiation potential. The highest increase in *osteopontin* gene expression was detected in AT-MSC2-U cells during both osteogenic and adipogenic differentiation, while there was decreased expression during osteogenic differentiation of AT-MSC2-FCS and AT-MSC2-HS cells.

Proteomic analysis of AT-MSCs grown under different culture conditions. The proteomic approach allowed us to further characterize the impact of different serums on AT-MSCs. Total proteins from AT-MSC1-FCS, AT-MSC1-FCS-Sp, ATMSC1-HS, AT-MSC1-U and AT-MSC2-FCS, AT-MSC2-FCS-Sp, AT-MSC2-HS, AT-MSC2-U cells were isolated and fractionated with two dimensional electrophoresis (2 DE). Some differentially expressed spots were excised and subjected to in-gel tryptic digestion and identification by mass spectrometry analysis (MALDI-TOF-MS) and human protein database searching. The positions of all proteins identified on the 2 DE gels were in the

expected range of their theoretical isoelectric points and molecular sizes. In total we identified 22 and 48 distinct proteins in AT-MSC1 and AT-MSC2 cells, respectively (Table 2 and Table 3). We thus compared proteomic expression profiles of cells extracted from the same tissue sample and cultivated in different mediums until the 3th passage. We found very similar global proteomic expression patterns of MSCs cultivated in a presence of different serums. Importantly, a high degree of similarity was observed only between cells of the same origin (AT-MSC1, or AT-MSC2). This likely reflects variations between individual donors (age, genetic background) and may be also that fat tissue was extracted from different anatomical locations. Therefore, we compared the effects of different serums on AT-MSCs of the same origin. The identified proteins are represented in Tables 2-3. Proteins identified by mass spectrometry analysis were designated as "+", those failing identification marked as "±", those absent on the corresponding gel areas as "-". Identification of highly expressed proteins in AT-MSC1s revealed some important differences.

Thus, we identified with high level of confidence protein prostaglandin F2 receptor (PGF2α) negative regulator (PTGFRN) in AT-MSC1-FCS, AT-MSC1-FCS-Sp and ATMSC1- HS cells, but not in AT-MSC1-U cells. PGF2α regulates numerous important physiological functions, including adipogenesis. The fact, that we did not identify PTGFRN protein in AT-MSC1-Us, may indicate that PGF2α-FP signalling pathway is amplified in these cells.

Table 2. Identified proteins of AT-MSC1 cells cultivated with different serum suplements

Protein name	Ultro SerG	HS	FCS-Sp	FCS	Protein name	Ultro SerG	HS	FCS-Sp	FCS
Ubiquitin-like modifier-activating enzyme 1 (UBA1)	+	+	+	+	Vimentin (VIME)	+	+	+	+
Leucine rich PPR motif. (LRPPRC)	-	+	-	-	α-Enolase (ENO1)	+	+	+	+
Glucose regulated protein GRP94 (HSP90B1)	-	+	-	+	Tubulin- β (TUBB4Q)	+	+	+	+
Heat shock protein 90 (HSP90AB1)	+	-	+	+	Serpin β9 (SERPINB9)	±	+	+	-
Prostaglandin F2 receptor reg (PTGFRN)	-	+	+	+	β actin-like protein 2 (ACTBL2)	+	+	+	+
Axin-1 (AXIN1)	-	+	+	+	Actin, cytoplasmic (ACTB)	+	+	+	+
Heat shock protein 70-8 (HSPA8)	+	+	+	+	Annexin A2 (ANXA2)	+	+	+	+
Pyruvate kinase isozymes M1/M2 (PKM2)	+	-	-	+	Torsin 1A (TOR1A)	-	+	+	-
Gamma Taxilin (TXLNG)	±	+	+	-	GAPDH	+	+	+	+
Tioredoxin domain-cont. protein 2 (TXNDC2)	+	+	+	+	14-3-3 protein zeta delta (YWHAZ)	+	+	+	+
Tubulin α (TUA1B)	+	+	+	+	Phosphoglycerate mutase 1 (PGAM 1)	+	+	+	+

Neither did we identify axin-1 protein in AT-MSC1-U cells in contrast to AT-MSC1-FCS, AT-MSC1-FCS-Sp and AT-MSC1-HS cells. Axin is an intracellular inhibitor of canonical Wnt signalling and it facilitates phosphorylation and degradation of β-catenin by GSK3-β and CK1-α (Logan and Nusse, 2004). Therefore, it is tempting to speculate that some of the effects observed in ATMSC1- U cells, i. e. increased proliferation and high osteogenic potential could be a consequence of amplified Wnt signalling pathway. Remarkably, we did identify protein plastin-3 in AT-MSC2-FCS, AT-MSC2-FCS-Sp and AT-MSC2-HS cells, but not in AT-MSC2-U cells. We speculate, that this could be related to the different shape of AT-MSC-U.

Table 3. Identified proteins of AT-MSC2 cells cultivated with different serum supplements

Protein name	Ultro SerG	HS	FCS-Sp	FCS	Protein name	Ultro SerG	HS	FCS-Sp	FCS
E3 Uiquitin-protein ligase (RE1A)	+	+	+	+	Septin-2 (SEPT2)	+	+	+	+
Alpha-actinin-4 (ACTN4)	+	+	+	+	Perilipin-2 (PLIN2)	+	+	+	+
Transportin-3 (TNPO3)	+	+	+	+	Alpha-enolase (ENOA)	+	+	+	+
Methionine synthase (METH)	-	+	±	-	AP-4 complex subunit mu-1 (AP4M1)	-	+	+	+
78 kDa glucose-regulated protein (GRP78)	+	+	+	+	UDP-glucose 6-dehydrogenase (UGDH)	-	+	+	+
Heat shock cognate 71 kDa protein (HSP7C)	+	+	+	+	Fascin (FSCN1)	-	+	±	±
Plastin-3 (PLST)	-	+	+	+	Aspartyl-tRNA synthetase, cytoplamic (SYDC)	-	+	+	+
Serum albumin (ALBU)	-	+	-	-	Tryptophanyl-tRNA synthetase, cytoplasmic (SYWC)	-	+	±	+
Serine/threonine-protein kinase (TBK1)	-	+	+	-	Vimentin (VIME)	+	+	+	+
Lamin-A/C (LMNA)	±	+	+	+	Tubulin beta chain (TBB5)	±	+	+	+
Protein disulfide-isomerase (PDIA1)	+	+	+	+	Tubulin alpha-1C chain (TBA1C)	-	+	+	+
Vimentin (VIME)	+	+	+	+	Ataxin-3 (ATX3)	+	+	+	+
Actin, cytoplasmic 1 (ACTB)	+	+	+	+	Annexin A6 (ANXA6)	±	±	+	+
Actin, cytoplasmic 2 (ACTG)	±	+	±	±	78 kDa glucose-regulated protein (GRP78)	-	±	±	+
Annexin A5 (ANXA5)	+	+	+	+	Serine/threonine-protein kinase (TBK1)	-	±	+	+
Heat shock protein beta-1 (HSPB1)	+	+	+	+	Annexin A2 (NXA2)	+	+	+	+
Triosephosphate isomerase (TPIS)	+	+	+	+	Developmentally-regulated GTP-binding protein 2 (DRG2)	±	+	+	+
Phosphoglycerate mutase 1 (PGAM1)	+	+	+	+	Peroxiredoxin-6 (PRDX6)	-	±	±	+

In summary, we show that MSCs cultivated in the presence of different serums display similar proteomic expression patterns. Nevertheless, identification of highly expressed proteins revealed several important differences, mostly in the cells cultivated with synthetic serum supplement Ultroser G. Thus, fetal calf and human allogeneic serums have similar impact on growth, differentiation, immunophenotype and proteomic profiles of MSCs. Considering safety issues, however, human allogeneic serum has obvious advantage, validating the use of this supplement for MSC *in vitro* expansion protocols. By contrast, artificial serum substitute induced more profound changes in MSCs physiology indicating the need for further fundamental studies before its introduction into clinical practice.

Characterization of MSC-like stem cells derived from the dental pulp of human exfoliated deciduous teeth (SHEDs). The primary culture of MSC-like stem cells derived from the dental pulp of human exfoliated deciduous teeth consisted of small spindle-shaped fibroblastoid cells, which subsequently formed large dense colonies. The SHEDs retained ability to form colonies only until third passage. However, the colony-forming efficiecy (CFE) assay that was performed at the first passage revealed high CFE index (16%). The ability to form colonies, allowed to derive single cell clones from primary SHED culture. For the establishment of clonal cell strains, we used limiting dilution method. We identified 16 wells containing single stromal cell colonies, and 2 of them (clones C2 and F5) were expanded for further analysis. These cells were propagated for 5–12 passages to generate sufficient number of cells for experiments. Both clones displayed typical fibroblastoid morphology, though with some differences, especially at the early passages (Fig. 5).

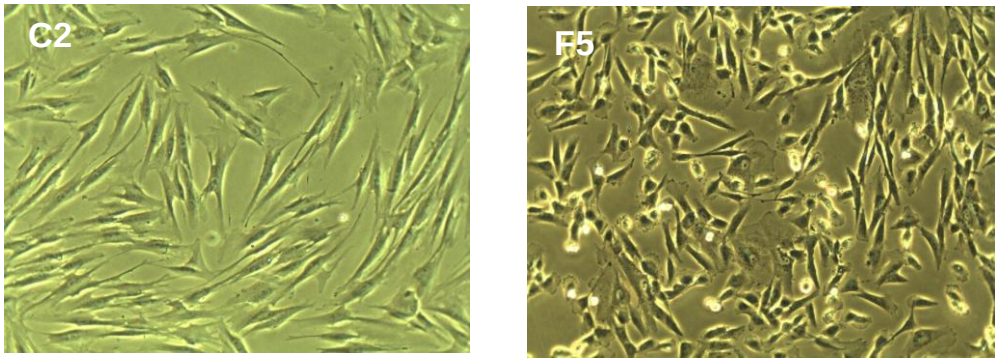

Figure 5. Morphology SHEDs clonal strains C2 and F5. Phase-contrast, magnification 100x.

This apparent heterogeneity was evident until the fifth passage. The primary SHEDs culture and both clones displayed similar growth rates with population doubling time of about 24 h. The SHED cells and both clones cultured under osteogenic conditions started to proliferate faster and formed multilayers within the first week. After 2-3 weeks,

staining with silver nitrate (von Kossa) revealed characteristic patterns of mineralized matrix. However no signs of differentiation were observed after 4 weeks of SHEDs culture and C2 clone in adipogenic induction medium. In contrast, clone F5 displayed specific signs of adipogenic differentiation. Flow cytometry revealed that these cells expressed characteristic antigens of MSC cells, including CD73, CD90, CD105, CD146, and did not express hematopoietic markers CD14, CD34, and CD45. Interestingly, we also found that expression of CD146 antigen in both clones was considerably weaker than in the parental cell line (27.1% in C2 and 37.8% in F5 to 66.3% in primary SHEDs), while at the same time all cells expressed similar levels (>96%) of CD90, CD73, and CD105. We speculate that proportion of CD146-positive cells may reflect level of culture senescence. We will address this issue in our future studies.

Identification of proteins expressed in SHEDs and C2 and F5 clones. In the present study, we used a proteomic approach that allowed us to compare protein expression signatures between a primary cell culture and her daughter clones. As a result, we for the first time established a map of abundantly expressed proteins in MSC-like cells derived from the dental pulp of human exfoliated deciduous teeth (SHEDs). Our goal was to create a reliable list of proteins that are typically expressed in SHEDs and their daughter clones and then to compare this list with known proteomic profiles of MSC-like cells derived from other sources The protein spot patterns visualized on 2-DE gels were very reproducible, and approximately 150–300 protein spots were identified on each gel. Some differentially expressed spots were excised and subjected to in-gel tryptic digestion and identifi cation by mass spectrometry analysis (MALDI-TOF-MS) and human protein database searching. The identified proteins are presented in Table 4. All identified proteins were analyzed and quantifiably compared by using computer-assisted methods. Therefore, for the identification, we chose highly expressed proteins. Importantly, remarkably high depth of coverage was achieved a lowest sequence coverage of 27%. Thus, all proteins were identifi ed with high level of confidence. The largest group comprised proteins that belong to the cellular cytoskeleton. The protein vimentin was identified with high confidence in primary stromal cell culture and both clones. Importantly, MSC-like cells derived from different tissues (bone marrow, umbilical cord blood, adipose tissue) express high levels of vimentin (Park *et al.*, 2007). We also identified fibronectin 1 protein, which was uniquely expressed in the F5 clone. It plays a fundamental role in the organization and composition of the extracellular matrix and cell-matrix adhesion sites. Interestingly, when differential gene expression in MSCs derived from bone marrow, umbilical cord blood, and adipose tissue was determined in relation to fibroblasts, significant up-regulation of fibronectin 1 was

detected, suggesting an importance for MSCs (Wagner *et al*., 2005); up-regulation of fibronectin expression may be a prerequisite for the differentiation of MSC-like cells.

Table 4. Identified proteins of SHEDs and C2 and F5 clones

	Av. vol. ratio (fold change)					
	Cytoplasm			*Nucleus*		
Protein name	C2 SHED	F5 SHED	C2 F5	C2 SHED	F5 SHED	C2 F5
Actin, beta (ACTB)	1.30	1.10	1.18	−1.14	−1.39	1.22
Actin, gamma (ACTG1-	1.12	−1.12	1.00	−1.97	−2.42	1.23
Actinin, alpha 4				−1.21	−2.53	2.10
Annexin II, isoform 2 (ANX2)	1.24	1.12	1.11	−2.31	−1.92	−1.21
Chaperonin containing subunit 3				−2.00	−2.87	1.44
Fibronectin 1						
Fibronectin 1, is. CRA_						
Glucose-regulated prot GRP78 (HSPA5, Bip)	1.57	1.02	1.55			
Heat shock 70-kDa prot isoform 1 (HSPA8 or	1.38	−1.11	−1.24	−1.28	−1.93	1.50
Heterogeneous nuclear ribonucleoprotein K (HNRPK)				−1.25	−2.24	1.78
Lamin B1				−1.38	−22.91	16.63
Nucleoporin NYD-SP7 (or TSGA10)				−1.50	−1.14	−1.32
Paf/RNA polymerase I complex component				−4.88	−14.90	3.06
Pyruvate kinase 3, isof (PK3)	1.07	1.95	−1.82	−1.44	−1.49	1.04
Ribosimal protein PO (RPLPO)				−1.46	−1.46	1.00
Septin 2 (SEPT2)				−2.85	−3.90	1.37
TBC1 domain family, m	1.16	1.58	−1.36			
Triose phosphate isom (TPI1)	1.72	−1.23	−1.41			
Tubulin, beta (TUBB)				−1.91	−1.49	−1.28
Vimentin (VIM)				−1.34	−2.62	1.95

In summary, the present identification of highly expressed proteins in SHEDs revealed proteomic profile that is very similar to that of MSC cells derived from other tissues. This similarity demonstrates that SHEDs may be identical, to MSC cells. Accordingly, our analysis revealed the proteomic profile of subpopulation, which became dominant during the culture of the clonal cell strain. Moreover, the heterogenization within a culture seemed very similar between parental population and her daughter clones. In conclusion, our study for the first time presents a map of highly expressed proteins in SHEDs.

Effects of major human antiprotease alpha1-antitrypsin on the motility and proliferation of stem cells from human exfoliated deciduous teeth. The mechanisms of MSCs motility within the tissues *in vivo* remain enigmatic. Therefore we analysed ability to induce migration processes of MSC-like SHEDs *in vitro* when exposed to the

multifunctional proinflammatory protein alpha1-antitrypsin (AAT). The interaction of SHEDs and AAT has never been studied before. This novel and interesting notion opens further possibilities to explore the turnover of externally applied proteins in *ex vivo* cultured progenitor cells. Physiologic and inflammatory doses of AAT slightly but significantly upregulated the SHEDs proliferation rate as assessed by the XTT assay (Fig. 6).

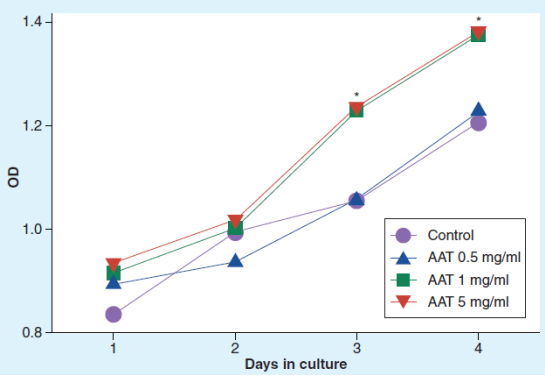

Figure 6. Cell proliferation changes upon AAT exposure. Mesenchymal stem cells were exposed to different concentrations of AAT (0.5, 1 and 5 mg/ml) during the whole week. Control cells remained in serum-free cell culture medium. The statistically significant variations versus controls on a certain day in culture are labeled with *, which denotes $p < 0.05$, $n = 3$ for each group; OD: Optical density.

A statistically significant increase in growth was detected on days 3 and 4, in cells incubated with physiological and supraphysiological doses of AAT (1 and 5 mg/ml), as compared with control cells. Interestingly, subphysiological did not induced proliferation of cells. We found an increased cell motility when AAT was present at the physiological (i.e., 1 mg/ml) and higher concentration (Fig. 7). Figure 7A reflects the mean number of transmigrated cells in three separate experiments, where AAT was applied to the cells 12 h prior to the test; representative micro-and macro-pictures of the membrane are presented in Figure 7B. There was a significant increase in the ability of the cells to invade when AAT was present in the environment. In parallel, we assessed the migratory properties of SHEDs, where increasing doses of AAT potentiated their migration and coverage of the scraped area (Figure 7C).We found an increased ability of SHEDs to migrate *in vitro* when exposed to physiological and supraphysiological doses of AAT (1 and 5 mg/ml). We hypothesize that native AAT may exert a regeneration-supportive action when applied on MSC-like cells. The migratory ability of MSC-like cells might be partially dependant on their MMP profile, especially when migration through extracellular matrix is involved.

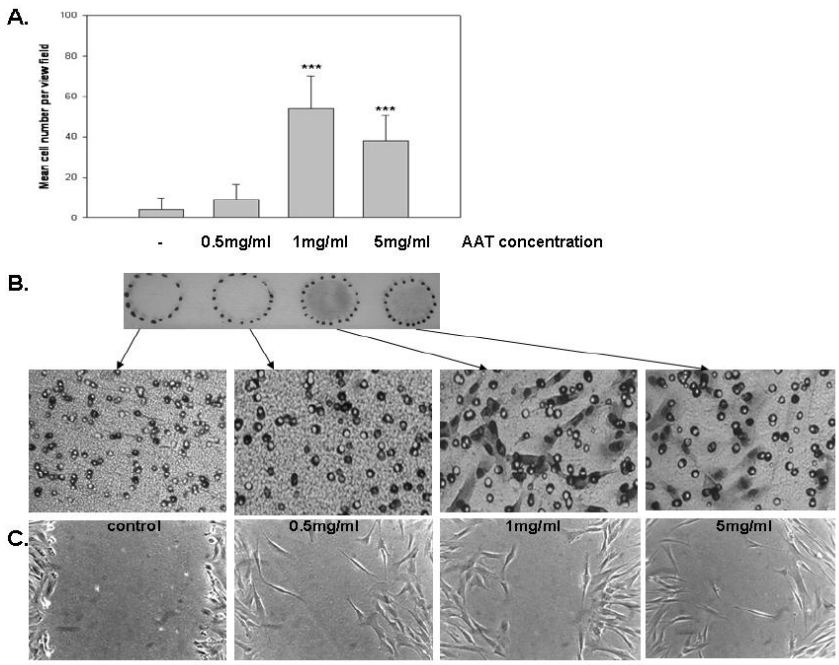

Figure 7. Modulation of mesenchymal stromal cell motility by alpha1-antitrypsin. (A) The graph represents the mean number of transmigrated cells in three representative fields of view for each experimental point. The results of all three experiments and a mean value of transmigrated cells is presented ± standard deviation.. *** denotes $p < 0.001$. **(B)** Representative macro- and microimages (×100 magnification) of membranes are presented. **(C)** Confluent cells were cultured in the presence of different concentrations of AAT (0.5, 1 and 5 mg/ml) for 12 h. An artificial wound was created immediately before adding AAT by scraping the cell monolayer with a pipette tip. Magnification ×100.

Activity of secreted MMPs was measured in the cell culture medium, while cell-associated MMP activity was detected by *in situ* zymography. Qualitatively, the amounts of active, secreted gelatinases were clearly increased in AAT-treated cell culture medium, but remained almost constant *in situ*. The molecular weight (~65 kDa) of the band (Figure 8A) suggests that it might be MMP-2 (also known as gelatinase A), a type IV collagenase, that specifically cleaves type IV collagen, the major structural component of the basement membrane. MMP-2 is a known tool used by stem cells on their homing trip. Our data indicates that a putative mechanism involved in AAT-induced SHED motility might be associated with the increase in gelatinolytic capacity. Combining results from both assays allowed us to conclude that AAT exposure in all doses used increased MMP activity in SHEDs. Such increased ability to cleave extracelullar matrix might be one of the basic mechanisms that helps SHEDs migrate and invade tissues more effectively. Our data thus demonstrates a relationship between AAT presence in the cellular environment and increased stem cell motility.

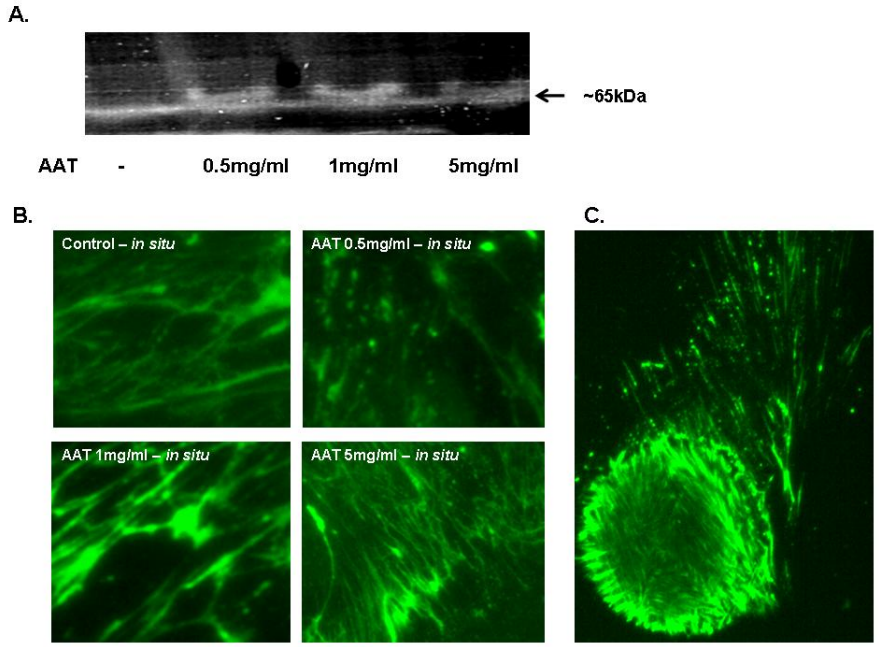

Figure 8. Gelatinolytic activity of mesenchymal stromal cells. (A) The zymography gel displays gelatinase activity in cell culture medium from AAT-treated and control cells. Medium was collected and subjected to PAGE electrophoresis in a gel containing 0.1% of gelatin. After incubation in renaturing and development buffer, bands were visualized with Coomassie Blue. Clear bands represent the areas of gelatinase activity. **(B)** Local distribution of active gelatinases as assessed by *in situ* zymography. Cells were cultured in the presence of AAT for 12 h. Cells were thoroughly washed again and images were taken at ×200 magnification. Fluorescent areas represent the accumulation of active gelatinases. **(C)** *In situ* zymography of a single cell immediately after plating at ×400 magnification.

SHEDs after the AAT application to the cell culture medium demonstrated the activation of several important signaling networks in the cell, specifically PKD, Akt and ERK. Cells exposed to increasing doses of AAT during the first 20 min of interaction exhibited increased and dose-dependent amounts of phosphorylated PKD (at Ser916 and Ser744/748), Akt (at Thr308 and Ser473) and ERK compared with untreated controls (Fig. 9). Akt signaling represents a central node that integrates diverse signals from the extracellular environment and contributes to the activation of various cellular processes, including survival, proliferation, growth, metabolism and angiogenesis (Toker, 2008). We have found that unstimulated SHEDs exhibit basal levels of Thr308 and Ser473 phosphorylation, indicating that the Akt pathway is constitutively active. This may, in part, explain the high-proliferation potential of these cells. AAT has induced robust dose-independent phosphorylation of Akt on both sites, Thr308 and Ser473, suggesting a possible mechanism for improved survival and proliferation.

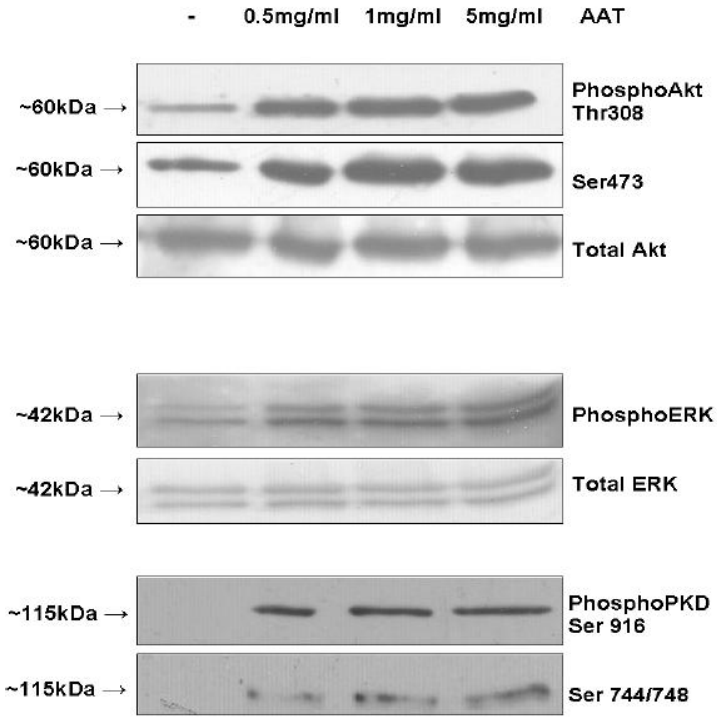

Figure 9. Alpha1-antitrypsin exposure-induced signaling events. Total and phosphorylated levels of protein kinases, PKD, Akt and ERK assessed by western blot. Samples were subjected to electrophoresis, blotted, and the membrane was probed with an anti-PKD phosphorylated (at Ser916 and Ser744/748), anti-PKB (Akt) total and phosphorylated (at Thr308 and Ser473) and anti-ERK total and phosphorylated antibodies. Bands were visualized by incubation with appropriate horseradish peroxidase-conjugated secondary antibodies and chemiluminescence substrate. AAT: alpha1-antitrypsin.

Activation of ERK 1/2 is involved in many cellular responses, including cell motility, proliferation and survival (Raman *et al.*, 2007). We demonstrate that SHEDs express basal levels of constitutively active ERK 1/2 and that AAT activates the ERK 1/2 signaling pathway in SHEDs (Fig. 9). Importantly, activation of ERK 1/2 has been directly linked with increased proliferation of human dermal fibroblasts in response to treatment with AAT.

PKD is a member of calmodulin-dependent kinase superfamily and requires diacylglycerol (DAG) for activation. PKD positively modulates cell proliferation, motility, invasion and adhesion (Rozengurt *et al.*, 2005). Recent studies also indicate that PKD plays an important role in the initiation of specific differentiation programs. Our data indicate that AAT induces PKD activation in SHEDs (Figure 2). To our knowledge, this is the first report describing the role of AAT in activation of PKD. In contrast to Akt and ERK 1/2, no basal activity of PKD was observed in proliferating SHEDs. Importantly, AAT treatment resulted in the phosphorylation of Ser744/748 and Ser916. The latter is important for the maintenance of conformation and influences the duration of kinase activation by DAG-independent mechanisms. Interestingly, phosphorylation of PKD on Ser916, but not on Ser744/748, was necessary for the bone morphogenetic protein 2-induced osteoblastic cell differentiation of bone marrow derived-MSCs, suggesting that the PKC-independent signaling pathway may be involved (Lemonnier *et al.*, 2004). Our results demonstrate that AAT activates several important signaling

networks in SHEDs. These networks may be responsible for the increased proliferation, motility and invasion of SHEDs in response to AAT.

In summary, we present the first set of data on how a major human serum antiprotease AAT modulates properties of MSC-like cells of dental pulp origin, specifically suggesting that AAT can increase MSC proliferation and motility. This is very relevant, for instance, in the context of lung pathophysiology in chronic obstructive pulmonary disease, where tissue restoration is decreased and destruction is increased. Both of these processes may be partially influenced by AAT. When deficient, AAT does not sufficiently counteract neutrophil proteases and does not stimulate resting MSCs. In light of our findings, it is tempting to propose that AAT supplementation in severely AAT-deficient individuals might not only provide a necessary protease shield, but also stimulate an intrinsic regeneration process

Conclusions

1. Human adipose tissue derived mesenchymal stem cells cultured in the presence of synthetic serum substitute Ultroser G displayed higher proliferative and differentiative activity than cells grown with serum of natural origin.
2. Synthetic serum substitute Ultroser G diminishes expression of CD146 on the mesenchymal stem cells derived from adipose tissue.
3. In contrast to serum of animal and human origin, synthetic serum substitute Ultroser G activates expresion of *PPARγ*, and *Msx2* genes and repress expression of *osteopontin* gene in proliferating adipose tissue-derived mesenchymal stem cells.
4. Analysis of total proteins in human adipose tissue derived mesenchymal stem cells revealed higher amount of serum specific proteins in cells cultivated in growth media with fetal calf serum and allogeneic human serum, while synthetic serum substitute did not induced expression of specific proteins.
5. Stem cells from human exfoliated deciduous teeth displayed mesenchymal stem cell-like proteomic pattern and cell surface markers expression.
6. Cloning experiments revealed heterogenicity of primary population of stem cells from human exfoliated deciduous teeth, comprising at least three types of subpopulations.
7. Physiological and inflammatory concentrations of human alpha-1-antitrypsin (1mg/ml and 5mg/ml) increase the proliferation and motility of mesenchymal stem cells derived from exfoliated deciduous tooth.

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1. Pivoriūnas A, Surovas A, Borutinskaitė V, Matuzevičius D, Treigytė G, Savickienė J, **Tunaitis V**, Aldonytė R, Jarmalavičiūtė A, Suriakaitė K, Liutkevičius E, Venalis A, Navakauskas D, Navakauskienė R, Magnusson K-E. Proteomic analysis of stromal cells derived from the dental pulp of human exfoliated deciduous teeth *Stem Cells and Development,* 2010 Jul;19(7):1081-93.

2. Aldonyte R, **Tunaitis V**, Surovas A, Suriakaite K, Jarmalaviciute A, Magnusson K-E, Pivoriunas A. Effects of major human antiprotease alpha-1-antitrypsin on the motility and proliferation of stromal stem cells from human exfoliated deciduous teeth. *Regenerative Medicine,* 2010 Jul;5(4):633-43.

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1. Pivoriūnas A, Surovas A, **Tunaitis V**, Treigytė G, Borutinskaitė V, Ivanovas E, Savickienė J, Magnusson K-E, Navakauskienė R. Different functional properties and proteomic signatures are displayed by single colony-derived strains from human stromal cells of dental pulp origin. *33rd FEBS Congress June 28-July 3, 2008, Athens, Greece.*

2. Pivoriūnas A, Surovas A, Aldonytė R, Suriakaitė K, **Tunaitis V**, Navakauskienė R, Borutinskaitė V, Magnusson K-E. Proteomic profiling of primary and secondary clones of dental pulp-derived human stromal cells. *22nd IGB MEETING CNR - Department of Life Sciences"Stem Cells: From Molecular Physiology to Therapeutic Applications": Capri, 10 - 13 October 2009.*

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2001-2002 Linkopings universitet, Department of Medical Microbiology, Sweden (12 month), purpose: Localization analysis of protein kinase D in the cell. (Stipendium of *Maria Curie* fondation)

SKIRTINGŲ SUAUGUSIO ŽMOGAUS AUDINIŲ MEZENCHIMINIŲ KAMIENINIŲ LĄSTELIŲ FUNKCIONAVIMO MECHANIZMŲ TYRIMAI

ĮVADAS

Suaugusio organizmo mezenchiminių kamieninių ląstelių (MKL) terapija yra viena iš perspektyviausių sričių šiuolaikinėje medicinoje. Tyrimai ir klinikiniai bandymai parodė, kad MKL gali būti sėkmingai panaudotos pažeistų audinių regeneracijai ir uždegiminio proceso slopinimui. Šiuo metu vykdoma virš šimto klinikinių bandymų, grindžiamų MKL panaudojimu. Vis dėlto lauktas terapinis mezenchiminių kamieninių ląstelių efektas dar nepasiektas. Šiuo metu nėra aiškus gydomąjį poveikį turinčių ląstelių likimas terapijos metu, nepilnai išnagrinėti MKL dauginimosi, diferencijavimosi į skirtingų tipų audinius, migracijos mechanizmai, nežinoma visų sekretuojamų molekulių sudėtis. Kadangi apie 85 % klinikinių tyrimų atliekama su laboratorijoje padaugintomis ląstelių kultūromis, ypatingą svarbą MKL savybėms turi tinkamų auginimo sąlygų parinkimas. Sėkmingai padauginti mezenchimines kamienines ląsteles iki terapijai pakankamo kiekio trukdo ne tik žinių apie ląstelių biologiją trūkumas, bet ir MKL padauginimo protokolų įvairovė, susijusi su sunkiai standartizuojama auginimo terpės sudėtimi, skirtingomis MKL donoro organizmo savybėmis ir nevienodomis auginimo sąlygomis (nevienodas auginimo indų plastikas, persėjimų dažnumas, ląstelių tankis ir kt.). Siekiant surasti tinkamą MKL padauginimo būdą, kuris ne tik nepablogintų, bet galbūt ir pagerintų jau nustatytas terapijai svarbias savybes, būtina nustatyti, kaip ir kokiu būdu auginimo sąlygų pakeitimas veikia mezenchiminių kamieninių ląstelių funkcijas.

Viena iš svarbiausių ir heterogeniškiausių auginimo terpės sudėtinių dalių yra serumas. Ląstelių padauginimui šiuo metu dažniausiai yra naudojama auginimo terpė su fetaliniu veršelio serumu (FBS). Kadangi tai yra gyvulinės kilmės komponentas, jo naudojimas terapijai skirtų MKL auginimui kelia potencialų pavojų, susijusį su tarprūšiniu žinomų ir dar nenustatytų ligų pernešimu ir imuninio atsako prieš FBS baltymus sukėlimu. Dėl šios priežasties daugėja tyrimų, kuriuose, kaip alternatyvą fetaliniam veršelio serumui, rekomenduojama naudoti žmogaus serumą ar beseruminę auginimo terpę. Keičiant auginimo terpės sudėtį siekiama kompleksiškai įvertinti ląstelėje atsirandančius pokyčius, kurie gali būti svarbūs MKL funkcijoms. Naujausiuose skirtingų laboratorijų atliktuose darbuose yra lyginamas serumų poveikis mezenchiminių kamieninių ląstelių proliferacijai, diferenciacijai ir imunosupresijai. Pirminiai duomenys parodė, kad FBS naudojimui puiki alternatyva gali būti terpė su žmogaus serumu. Nežiūrint to, šiame serume tirtų mezenchiminių kamieninių ląstelių ir augintų terpėje su

FBS savybės nebuvo identiškos. Iki šiol nežinoma, kas nulemia šiuos skirtumus ir kiek jie gali būti svarbūs terapiniam MKL panaudojimui.

Kita auginamų mezenchiminių kamieninių ląstelių savybė yra populiacijos heterogeniškumas. MKL savybėmis dažniausiai pasižymi tik tam tikra dalis ląstelių, o kita populiacijos dalis gali turėti skirtingas savybes. Jau seniai žinoma, kad retai išsėtos kaulų čiulpų MKL formuoja kolonijas, sudarytas iš skirtingu greičiu augančių ląstelių. Šių subpopuliacijų savybės skiriasi tiek gebėjimu diferencijuotis, tiek paviršiaus žymenų ekspresija bei proteomine visuma. Daugelyje laboratorijų atlikti kaulų čiulpų MKL populiacijos tyrimai padėjo geriau suprasti ląstelių savybių pokyčius ir ilgo jų kultivavimo galimybes. Taigi populiacijos sudėtis – svarbi MKL charakteristika. Mūsų atliktų MKL populiacijų tyrimų objektas buvo kito potencialaus MKL šaltinio – pieninio danties pulpos kilmės mezenchiminės kamieninės ląstelės (PD-MKL). Iki šiol dar nebuvo atlikta šio MKL šaltinio populiacijos analizė, taigi ir jos priskyrimas mezenchiminių kamieninių ląstelių grupei rėmėsi tik proliferacijos ir diferenciacijos duomenimis. Palyginamieji PD-MKL kultūros ir jos klonų kultūros proteomos tyrimai, taip pat paviršiaus žymenų, diferenciacijos ir proliferacijos analizė parodė šio šaltinio ląstelių savybes ir jų panašumą į jau gerai apibūdintas kitų audinių mezenchimines kamienines ląsteles.

Terapijai ypatingai svarbi mezenchiminių kamieninių ląstelių savybė yra jų migravimas į pažeistą vietą. Vienas iš svarbiausių uždegiminės aplinkos komponentų yra neutrofilų gaminamos serino proteinazės, kurios remodeliuoja ekstraląstelinį matriksą, taip pat reguliuoja daugelio uždegimui svarbių citokinų ir jų receptorių aktyvumą. Natūralus neutrofilų gaminamų serino proteinazių inhibitorius yra antiproteinazė alfa1-antitripsinas (AAT), kurio koncentracijos padidėjimas yra susijęs su uždegimine organizmo būsena. Tačiau iki šiol dar nebuvo jokių AAT poveikio mezenchiminėms kamieninėms ląstelėms tyrimų.

Mezenchiminių kamieninių ląstelių tyrimai paprastai yra skirti šių ląstelių panaudojimui terapijoje. Kartu jie leidžia geriau suprasti fundamentinius organizmo funkcionavimo principus ir pavienių ląstelių veiklos reguliavimą. Šiame darbe atlikti auginimo terpės serumo poveikio riebalinės kilmės MKL tyrimai ir pieninio danties pulpos MKL populiacijos ir funkcinių savybių analizė leis geriau suprasti MKL padauginimo *in vitro* galimybes ir šių ląstelių funkcionavimo mechanizmus.

Darbo tikslas

Įvertinti ląstelių padauginimui naudojamos auginimo terpės serumo įtaką riebalinio audinio mezenchiminių kamieninių ląstelių augimo greičiui, paviršiaus žymenims, diferenciacijai ir baltymų ekspresijai bei ištirti pieninio danties pulpos mezenchiminių kamieninių ląstelių populiacijos savybes ir jų atsaką į uždegimui būdingą alfa1-antitripsiną.

Darbo uždaviniai

1. Nustatyti terpėje su fetaliniu veršelio serumu, alogeniniu žmogaus serumu ir sintetiniu serumo pakaitalu augintų žmogaus riebalinio audinio mezenchiminių kamieninių ląstelių paviršiaus žymenų ekspresijos, augimo greičio ir gebėjimo diferencijuotis skirtumus.
2. Nustatyti skirtingų rūšių serumų įtaką su adipogenine ir osteogenine diferenciacijomis susijusių genų (*PPARγ*, *Msx2*, *osteopontino*) transkripcijai riebalinio audinio mezenchiminėse kamieninėse ląstelėse.
3. Įvertinti ir palyginti baltymų ekspresijos visumą riebalinio audinio mezenchiminėse kamieninėse ląstelėse, augintose skirtingais serumais praturtintose terpėse.
4. Ištirti iš žmogaus pieninio danties pulpos išskirtų mezenchiminių kamieninių ląstelių ir iš šios kultūros kilusių klonų morfologijos, proliferacijos, diferenciacijos, paviršiaus žymenų ekspresijos bei baltymų ekspresijos ypatumus.
5. Nustatyti žmogaus alfa1-antitripsino subfiziologinės, fiziologinės ir uždegiminės koncentracijų poveikį pieninio danties pulpos mezenchiminių kamieninių ląstelių augimo greičiui ir migracijai.

Mokslinis naujumas

Ištirtas auginimo terpės įvairių rūšių serumų (fetalinio veršelio serumo (FBS), žmogaus alogeninio serumo (ŽS), sintetinio serumo pakaitalo Ultroser G) poveikis riebalinio audinio mezenchiminių kamieninių ląstelių (R-MKL) auginimo greičiui ir diferenciacijai. Nustatyta, kad R-MKL, augintos su sintetiniu serumo pakaitalu, pasižymėjo didesniu augimo greičiu ir stipresne diferenciacija, nei ląstelės, augintos terpėje su FBS ir ŽS.

Iki šiol dar nebuvo informacijos apie bazinę *Msx2*, *PPARγ* ir *osteopontino* genų ekspresiją skirtingos sudėties terpėse auginamose nediferencijuotose mezenchiminėse kamieninėse ląstelėse. Gauti rezultatai parodė, kad auginimo terpės serumo sukelti R-

MKL gebėjimo diferencijuoti skirtumai yra tiesiogiai susiję su tirtų *Msx2*, *PPARγ* ir *osteopontino* genų transkripcijos lygiu proliferuojančiose ląstelėse.

Riebalinio audinio mezenchiminių kamieninių ląstelių paviršiaus žymenų ekspresijos tyrimai atskleidė CD146 žymens ekspresijos priklausomybę nuo serumo sudėties. Iki šiol ši savybė buvo pastebėta tik iš kaulų čiulpų išskirtose MKL, o riebalinio audinio mezenchiminėse kamieninėse ląstelėse dar nebuvo tyrinėta. Analizuojant tirtų serumų įtaką CD146 ekspresijai nustatyta, kad R-MKL populiacija, auginta terpėje su FBS, turėjo daugiau CD146 teigiamų ląstelių nei terpėje su HS. Terpėje su sintetiniu serumo pakaitalu Ultroser G augintos R-MKL CD146 neekspresavo.

Ląstelių kultūrose, auginamose terpėse su skirtingais serumais, yra ekspresuojamas skirtingos sudėties baltymų rinkinys. Atlikus proteominius tyrimus buvo nustatyta skirtingomis sąlygomis auginamų R-MKL ekspresuojamų baltymų visuma. Analizuojant baltymų ekspresijos tyrimo rezultatus, daugiausiai skirtumų nustatėme terpėje su sintetiniu serumo pakaitalu Ultroser G augintose ląstelėse. Šis rezultatas patvirtino šiame tyrime gautus diferenciacijos genų transkripcijos ir paviršiaus žymenų ekspresijos duomenis, rodančius ypatingą sintetinio serumo poveikį R-MKL.

Tiriant MKL populiacijos sudėtį, mūsų grupė pirmoji atliko iš žmogaus pieninio danties pulpos išskirtų mezenchiminių kamieninių ląstelių (PD-MKL) klonų kompleksinę analizę. Išanalizavus PD-MKL klonų morfologiją, augimo greitį ir gebėjimą diferencijuoti, buvo išskirtos trijų tipų populiaciją sudarančių ląstelių grupės. Pirmą kartą atlikti PD-MKL kiekybiniai baltymų ekspresijos tyrimai atskleidė tipinius mezenchiminių kamieninių ląstelių baltymų profilius ir parodė atskirų klonų ekspresuojamų baltymų sudėtį.

Tiriant ląstelių migravimo mechanizmus, iki šiol dar nebuvo naudotas alfa1-antitripsino (AAT) poveikio tyrimas nei pieninio danties pulpos MKL, nei kito audinio mezenchiminėms kamieninėms ląstelėms. Nustatėme, kad uždegiminės AAT koncentracijos aktyvina pieninio danties pulpos mezenchiminių kamieninių ląstelių augimą ir migraciją.

Ginamieji teiginiai

1. Serumo kilmė ir sudėtis yra kritiškai svarbūs reguliuojant žmogaus riebalinio audinio mezenchiminių kamieninių ląstelių funkcines savybes.
2. Iš žmogaus pieninio danties pulpos galima išskirti ląstelių populiacijas, kurios pagal savo morfologiją, funkcines savybes bei ekspresuojamų baltymų sudėtį yra gimininges kitų audinių mezenchiminėms kamieninėms ląstelėms.

3. Alfa1-antitripsino baltymas stimuliuoja mezenchiminių kamieninių ląstelių augimą ir migraciją *in vitro*, todėl gali veikti, kaip organizmo audinių regeneraciją reguliuojantis veiksnys.

Tyrimų metodikos

Tiriant iš skirtingų žmogaus audinių išskirtas mezenchimines kamienines ląsteles buvo atlikti kompleksiniai ląstelių tyrimai. Mezenchiminių kamieninių ląstelių apibūdinimui ir jų funkcijų tyrimui analizavome ląstelių morfologiją, proliferacijos greitį, gebėjimą diferencijuotis, klonogeninį ir migracinį aktyvumą, paviršiaus žymenų ekspresiją (tėkmės citometrija). Tiriant viduląstelinius pokyčius atlikome kiekybinį genų transkripcijos įvertinimą (realaus laiko PGR), bendrą ekspresuojamų baltymų proteominę analizę (2-DE, masių spektometrija), proteazinio aktyvumo tyrimą, viduląstelinių signalinių kelių analizę (imunoblotas).

Rezultatų apibendrinimas

Šiame darbe buvo kompleksiškai tirta *in vitro* kultivuojamų ląstelių funkcijų priklausomybė nuo vienos svarbiausių ir heterogeniškiausių auginimo terpės sudėtinių dalių – serumo. Nagrinėta MKL populiacijos sudėtis ir atlikta jos klonų analizė bei MKL migracinės savybės, naudojant *in vitro* migravimo į uždegiminį židinį modelį. Be to, analizuoti MKL migracijoje dalyvaujantys signaliniai keliai, tiriant universalių viduląstelinio signalo perdavimo molekulių aktyvinimą.

Saugus mezenchiminių kamieninių ląstelių padauginimas iki pakankamo terapijai kiekio yra vienas iš svarbiausių uždavinių. Kompleksiniuose terpės serumo poveikio MKL tyrimuose naudojome iš riebalinio audinio išskirtas ląsteles, kurios nuo pat išskyrimo buvo augintos tik vienos rūšies serume. Šio tyrimo modelio unikalumas pasižymėjo tuo, kad buvo lyginamos to paties donoro ląstelės, kurių auginimas skyrėsi tik vienu komponentu – serumu. Darbe naudoti keturių rūšių serumai ar jo pakaitalai: pritaikytas MKL auginimui fetalinis veršelio serumas (FBS-Sp), atsitiktinai parinktas fetalinis veršelio serumas (FBS), alogeninis žmogaus serumas (ŽS) ir sintetinis serumo pakaitalas (Ultroser G).

Charakterizuojant MKL stebėti morfologiniai skirtumai rodė, kad terpėje su FBS-Sp ir FBS ląstelės buvo didesnės. Ląstelės terpėje su FBS rodė morfologinius ankstyvo senėjimo požymius. Su Ultroser G serumo pakaitalu augintos ląstelės pasižymėjo didžiausiu greičiu ir stipriausia adipogenine ir osteogenine diferenciacija. Skirtingos sudėties terpėse augintų MKL tėkmės citometrijos tyrimų rezultatai, naudojant MKL būdingus paviršiaus žymenis (CD14, CD34, CD45, CD73, CD90 ir CD105), buvo

panašūs. Paviršiaus žymenų tyrimai parodė, kad, jei ląstelė auginama Ultroser G serume, jos paviršiuje CD146 neaptinkamas. Vertinant populiacijos padvigubėjimo greičio ir diferenciacijos charakteristikas patraukliausios buvo terpėje su Ultroser G augintos ląstelės. Tačiau diferenciacijos metu ekspresuojamų genų mRNR kiekybiniai tyrimai realaus laiko PGR metodu parodė, kad sintetinis serumo pakaitalas gali aktyvinti atitinkamų osteogeninės diferenciacijos (*Msx2*) ir adipogeninės diferenciacijos (*PPARγ*) žymenų ekspresiją. Kito vėlyvos osteogeninės diferenciacijos žymens – *osteopontino* – analizė parodė, kad bazinė jo transkripcija proliferuojančiose Ultroser G ląstelėse buvo labai silpna ir, lyginant su kitomis ląstelėmis, augintomis natūralios kilmės serumuose, diferenciacijos metu yra aiškiai padidėjusi. Tyrimas parodė, kad ląsteles auginant terpėje su FBS-Sp, FBS ar ŽS, genų *Msx2*, *PPARγ* ir *osteopontino* genų bazinė transkripcija ir diferenciacijos indukuoti transkripcijos pokyčiai iš esmės atitiko literatūroje publikuojamus rezultatus. Visi šie tyrimai buvo atlikti iš dviejų skirtingo amžiaus donorų skirtingos anatominės vietos išskirtų riebalų mezenchiminių kamieninių ląstelių. Kokia rastų skirtumų priežastis ir ar tai gali būti kliūtis naudojant skirtingo tipo ląsteles terapijai, yra ateities tyrimų objektas. Galima spėti, kad sintetiniame serumo pakaitale esantys augimo faktoriai galėjo aktyvinti su diferenciacija susijusius signalinius kelius ir tuo pačiu išlaikyti didelį proliferacijos greitį. Galimos terpėje su Ultroser G augintų ląstelių indukuotos pradinės diferenciacijos būsenos galimybę patvirtina ir dvikryptės elektroforezės duomenys, pagal kuriuos R-MKL-U ląstelėse rasta mažiau ekspresuojamų ir identifikuotų baltymų nei su kitais serumais augintose ląstelėse. Taigi remiantis gautais rezultatais galima teigti, kad naudojant sintetinius serumo pakaitalus būtina patikrinti, ar MKL funkcijos identiškos kaip ir literatūroje aprašytų, dažniausiai terpėje su FBS augintų, kamieninių ląstelių. Mūsų nuomone, siekiant išvengti FBS keliamų problemų (zoonozės, imuninio atsako prieš serumo baltymus), gali būti naudojamas žmogaus serumas – viena iš tinkamiausių ląstelių auginimo terpės serumo alternatyvų.

Kultivuojama MKL populiacija nėra homogeniška. Heterogeniškumą gali sukelti kultūros išskyrimo metu atsiradęs užterštumas kito tipo ląstelėmis, skirtingo diferenciacijos laipsnio kamieninės ląstelės ar hierarchija. Norėdami nustatyti, ar tiriama MKL populiacija yra heterogeniška ir kuo skiriasi atskiri populiacijos klonai, atlikome iš iškritusio pieninio danties pulpos išskirtų mezenchiminių kamieninių ląstelių (PD-MKL) populiacijos individualių klonų išskyrimo ir padauginimo *in vitro* eksperimentus. Sėkmingai išskyrę, padauginę ir charakterizavę individualius MKL klonus, galėjome eksperimentiškai palyginti jų savybes ir įvertinti pirminės MKL populiacijos homogeniškumą. Ištyrę pirminės kultūros ir jos klonų paviršiaus žymenių (CD) ekspresiją, nustatėme MKL būdingą teigiamų ir neigiamų CD rinkinį. Siekdami rasti

MKL populiaciją sudarančių ląstelių skirtumus ir panašumus, atlikome pasirinktų PD-MKL klonų proteominę analizę. Daugelis šio tyrimo metu identifikuotų baltymų sutapo (tarp pirminės ląstelių kultūros PD-MKL ir jos dukterinių klonų F5 ir C2). Tai rodo, kad pirminė ląstelių kultūra ir jos dukteriniai klonai išlaiko pastovų gausiai ekspresuojamų (dažniausiai citoskeleto) baltymų visumą. Analizuodami 2DE gelius taip pat nustatėme, kad ekspresuojantys baltymai sutampa su iš kitų audinių išskirtų MKL būdingais baltymais. Remdamiesi mūsų tyrimų rezultatais darome išvadą, kad pirminė MKL, išskirtų iš pieninio danties pulpos, kultūra yra heterogeniška, t.y. sudaryta iš skirtingomis savybėmis pasižyminčių ląstelių subpopuliacijų. Paviršiaus žymenų ekspresija, ląstelėse ekspresuojamų baltymų visuma ir kitos tirtos savybės yra analogiškos kaip kitų audinių kilmės MKL.

MKL migravimui į uždegiminį židinį yra svarbūs uždegiminės aplinkos komponentai. Neutrofilų gaminamų serino proteinazių natūralus inhibitorius yra antiproteinazė alfa1-antitripsinas (AAT). Uždegimo metu šio baltymo koncentracija sisteminėje kraujotakoje ir pažeidimo židinyje padidėja keletą kartų. Uždegimo poveikį mezenchiminėms kamieninėms ląstelėms atlikome kompleksiškai tirdami AAT įtaką pieninio danties pulpos mezenchiminėms kamieninėms ląstelėms. Nustatėme, kad uždegiminės ATT dozės sustiprina PD-MKL proliferacinį ir migracinį aktyvumą. Eksperimentai panaudojant žaizdos gijimo modelį tai patvirtino. Ląstelėms migruojant audiniuose proteazinis ląstelių aktyvumas yra būtinas ekstraląstelinio matrikso remodeliavimui ir skaidymui. Mūsų duomenys rodo, kad AAT didina želatinazinį PD-MKL aktyvumą. Tolesniame etape ištyrėme, kaip kinta minėtoms ląstelės funkcijoms svarbių signalinių kelių aktyvumas. Mes nustatėme bazinį Akt signalinio kelio aktyvumą proliferuojančiose mezenchiminėse kamieninėse ląstelėse ir šio kelio aktyvinimą nepriklausomai nuo AAT dozės. Rezultatai parodė, kad AAT mezenchiminėse kamieninėse ląstelėse suaktyvina kitą svarbų ląstelės veiklos moduliatorių proteino kinazę D (PKD) ir nuo PKCε priklausomu (Ser744/748 fosforilinimas), ir nepriklausomu (Ser916 fosforilinimas) keliu.

Tikimės, kad šiame disertaciniame darbe pristatyti tyrimai padės vystyti MKL *in vitro* kultivavimo technologijas ir geriau suprasti šių ląstelių funkcionavimo mechanizmus.

IŠVADOS

1. Žmogaus riebalinio audinio mezenchiminės kamieninės ląstelės, augintos terpėje su sintetiniu serumo pakaitalu Ultroser G, pasižymėjo didesniu proliferaciniu ir diferenciaciniu aktyvumu nei ląstelės, augintos su natūralios kilmės serumais.
2. Sintetinis serumo pakaitalas Ultroser G mažina iš riebalinio audinio išskirtų mezenchiminių kamieninių ląstelių paviršiaus žymens CD146 ekspresiją.
3. Skirtingai nuo gyvulinės kilmės ir žmogaus serumų, sintetinis serumo Ultroser G pakaitalas nediferencijuotose riebalinio audinio mezenchiminėse kamieninėse ląstelėse aktyvina su adipogenine ir osteogenine diferenciacijomis susijusių genų *PPARγ*, *Msx2* ekspresiją, tačiau blokuoja *osteopontino* geno ekspresiją.
4. Palyginę riebalinio audinio išskirtų mezenchiminių kamieninių ląstelių, kultivuotų su skirtingais serumais, baltymų ekspresijos visumą, savitų baltymų nustatėme ląstelėse, kultivuotose su natūralios kilmės serumais, o terpė su sintetiniu serumo pakaitalu jokių unikalių baltymų ekspresijos ląstelėse neindukavo.
5. Iš pieninio danties pulpos išskirtos ląstelės pasižymėjo mezenchiminėms kamieninėms ląstelėms būdinga baltymų ir paviršiaus žymenų CD73, CD90, CD105 ir CD146 ekspresija ir turėjo didelį proliferacinį bei klonogeninį aktyvumą.
6. Klonavimo eksperimentai parodė, kad pirminė pieninio danties pulpos mezenchiminių kamieninių ląstelių kultūra yra heterogeniška, sudaryta iš mažiausiai trijų tipų morfologiškai ir funkciškai skirtingų subpopuliacijų.
7. Fiziologinės ir uždegimui būdingos 1mg/ml ir 5mg/ml alfa1-antitripsino baltymo koncentracijos stimuliuoja pieninių dantų pulpos mezenchiminių kamieninių ląstelių proliferaciją ir migraciją.

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- Esu dėkingas visiems disertacijos rengime dalyvavusiems imunologams už vertingas pastabas, redagavimą ir pagalbą.
- Ačiū visiems kolegoms už disertacijos rašymo inicijavimą, vertingas pastabas, palaikymą ir produktyvų bendradarbiavimą.
- Dėkoju visiems buvusiems darbo vadovams už teorines žinias ir praktinius įgūdžius.
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