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GABRIELIS KUNDROTAS

INVESTIGATION OF HUMAN MESENCHYMAL STEM CELL GENETIC CHARACTERISTICS TO ENSURE THERAPY SAFETY

Summary of Doctoral Dissertation
Biomedical Sciences, Biology (01B)

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Scientific supervisor:
Prof. dr. Gražina Slapšytė (Vilnius University, biomedical sciences, biology – 01B)

Scientific consultant:
Dr. Živilė Gudlevičienė (National Cancer Institute, biomedical sciences, medicine – 06B)

The doctoral dissertation will be defended at Vilnius University, Academic Board of Examiners in Biology:
***Chairman*:**
Dr. Mykolas Mauricas (State Research Institute Centre for Innovative Medicine, biomedical sciences, biology – 01B)
***Members*:**
Dr. Eiva Bernotienė (SRIC for Innovative Medicine, biomedical sciences, medicine – 06B)
Dr. Virginija Bukelskienė (Vilnius University, biomedical sciences, biology – 01B)
Dr. Vita Pašukonienė (National Cancer Institute, biomedical sciences, biology – 01B)
Dr. Jolanta Vidugirienė (Promega Corporation, Madison, the USA, physical sciences, biochemistry – 04P)

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VILNIAUS UNIVERSITETAS

GABRIELIS KUNDROTAS

ŽMOGAUS MEZENCHIMINIŲ KAMIENINIŲ LĄSTELIŲ GENETINIŲ SAVYBIŲ TYRIMAS SAUGIAI TERAPIJAI UŽTIKRINTI

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Mokslinė vadovė:
prof. dr. Gražina Slapšytė (Vilniaus universitetas, biomedicinos mokslai, biologija – 01B)

Mokslinė konsultantė:
dr. Živilė Gudlevičienė (Nacionalinis vėžio institutas, biomedicinos mokslai, medicina – 06B)

Disertacija ginama Vilniaus universiteto Biologijos mokslo krypties taryboje:
Pirmininkas:
dr. Mykolas Mauricas (Valstybinis mokslinių tyrimų institutas Inovatyvios medicinos centras; biomedicinos mokslai, biologija – 01B)
Nariai:
dr. Eiva Bernotienė (VMTI Inovatyvios medicinos centras, biomedicinos mokslai, medicina – 06B)
dr. Virginija Bukelskienė (Vilniaus universitetas, biomedicinos mokslai, biologija – 01B)
dr. Vita Pašukonienė (Nacionalinis vėžio institutas; biomedicinos mokslai, biologija – 01B)
dr. Jolanta Vidugirienė (Promega Korporacija, Madisonas, JAV; fiziniai mokslai, biochemija – 04P)

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1. INTRODUCTION

Human mesenchymal stem cells (MSCs) are non-hematopoietic, adherent fibroblast-like cells with ability of self-renewal and potential for multilineage differentiation[1]. MSCs are the major stem cells exploited in clinical trials for their potential in immune regulation and tissue regeneration[2]. MSC therapy holds promise for treating degenerative diseases, cancer and repair of damaged tissues, where limited therapeutic options exist[3]. Despite such tremendous prospects, many unanswered questions remain regarding the impact of manipulations *in vitro* on cell properties and MSC preparation safety for the recipients.

Human bone marrow is the major source of MSCs for clinical use[4]. However, the limited cell numbers obtained from invasive isolation techniques is a disadvantage of using bone marrow[5]. This has led many researchers to investigate alternate sources of MSCs including adipose tissue[6] and umbilical cord[7]. Application of more efficient and less patient frustrating method for MSC isolation from bone marrow is urgently needed.

High quantities of MSCs are needed for clinical applications, thus requiring extensive cell expansion in long-term culture[8]. However, the biological properties of MSCs are likely to be altered by culture conditions[9]. First of all, prolonged *in vitro* expansion triggers MSC senescence[4] which refers to irreversible cell growth arrest[10]. It reduces life span of the cells[11]. Moreover, senescence impairs therapeutic potential of human MSCs[12] and increases the risk of cell neoplastic transformation[13]. MSC senescence is measured by β-galactosidase activity and changes in cell morphology and proliferation kinetics[14,15]. However, there is no golden standard in the measurement of cell ageing and a more specific molecular markers would be necessary in order to grade the level of senescence of human MSC preparations[16].

Literature data on human MSC genomic stability during prolonged *in vitro* expansion is very contradictory. Some authors have shown genomic stability of MSCs during *in vitro* expansion[17,18]. While other researchers have documented the occurrence of karyotypic instability in cultured MSCs[15,19]. It has been admitted that genome instability enables tumor cells to acquire their characteristics[20], therefore the tumorigenic potential of the human MSCs has become the most important concern for clinical use of MSCs[21]. Although some publications reporting malignant transformation of human

MSCs later on have been retracted[22,23], there is still debate concerning the genetic stability of human MSCs and the implication for clinical safety[24,25]. The European Medicines Agency[26] and the U.S. Food and Drug Administration[27] recommend to evaluate the MSC preparations for chromosomal stability before their clinical use.

Human umbilical cords offer an abundant and noninvasive source of MSCs without ethical issues and are emerging as a valuable alternative to bone marrow for producing MSCs[28]. Cryopreservation is the only method for long-term storage of viable tissues used for cellular therapy. However, the freeze-thaw process strongly contributes to cell damage[29] and its impact on umbilical cord MSC genome stability is not clear.

Fetal bovine serum is the most frequently used to supply growth factors to MSC culture medium[30]. However, it carries the risk of contamination with infectious viral/prion agents and the induction of immune response in the final recipient[31]. The solution to this problem would be to expand human MSCs in a well-defined serum-free culture media that support the efficient production of the cells[32].

AIM OF THE STUDY was to investigate the influence of *in vitro* manipulations to the genetic characteristics of human mesenchymal stem cells.

MAIN TASKS OF THE STUDY

1. To develop a protocol for human MSCs isolation from residual bone marrow material and *in vitro* expansion to clinically relevant numbers;
2. To investigate the phenotypic and functional (morphology, immunophenotype, senescence, proliferation potential) and genetic (karyotype, gene expression) properties of bone marrow MSCs at early and late passages;
3. To evaluate the impact of long-term *in vitro* expansion on bone marrow MSC characteristics by comparing the results of MSC analysis at late and early passages;
4. To select potential molecular biomarkers for assessment of bone marrow MSC senescence *in vitro*;
5. To investigate the impact of controlled freeze-thaw process on genomic stability of human umbilical cord tissue MSCs;

6. To study the effect of novel culture medium composition (recombinant protein enriched medium *vs.* standard medium enriched with fetal bovine serum) to the proliferation and gene expression of umbilical cord tissue MSCs.

STATEMENTS TO BE DEFENDED

1. MSCs can be successfully isolated from a small volume of human bone marrow and efficiently expanded *in vitro* to clinically relevant numbers;
2. Long-term *in vitro* cultivation affects the bone marrow MSC morphology, proliferation potential, senescence, chromosomal stability and gene expression;
3. Comparison of selected gene expression of human bone marrow MSCs in early *vs.* late passages could reveal new biomarkers of cell senescence;
4. The controlled freeze-thaw process of human umbilical cord tissue does not affect the chromosomal stability of MSCs;
5. Culture medium supplementation with recombinant proteins has a significant impact on proliferation and gene expression of umbilical cord tissue MSCs.

SCIENTIFIC NOVELTY AND PRACTICAL VALUE OF THE STUDY

1. We developed a new protocol for the MSC isolation from residual human bone marrow material and *in vitro* expansion to clinically relevant numbers. In this way the MSC product could be manufactured without additional bone marrow biopsy;
2. We identified novel 38 potential biomarkers of human bone marrow MSC senescence *in vitro*. These markers provide new knowledge about the biology of MSCs and could form the basis for the development of MSC product quality control;
3. We showed that umbilical cord tissue controlled freezing and thawing do not affect the genomic stability of MSCs. It indicates the safety of cryoconservation for long-term storage of umbilical cord tissue;
4. We showed that culture medium supplementation with recombinant proteins has a significant impact on proliferation and gene expression of umbilical cord tissue MSCs;
5. It is the first study of human MSC genomic stability and senescence in Lithuania.

2. MATERIALS AND METHODS

The approval to conduct the study was issued by Vilnius Regional Biomedical Research Ethics Committee, Lithuania (Permission No 158200-09-381-104).

Umbilical cord tissue MSCs. Samples were provided for karyotype (n=5) and gene expression (n=3) analysis from *The Cell Factory* (Belgium). All cord tissues obtained after written informed consents of the donors for the material's use in R&D experiments.

Bone marrow collection. Specimens were collected from 8 healthy adult donors after obtaining written informed consent at Vilnius University Santariskiu Clinic, Children Hematology and Transplantation Center.

Isolation of MSCs using ficoll gradient density centrifugation. 60 ml of bone marrow was diluted (1:1) with RPMI 1640 medium (Invitrogen) and centrifuged through ficoll gradient (GE Healthcare Bio-Sciences) for 20 min at 300 *g*. The whitish ring of cells was collected and washed with 20 ml DME medium containing 10% of fetal bovine serum (Invitrogen). Mononuclear cells were plated into the 175 cm^2 ventilated flasks (BD Biosciences) in the density $100 \cdot 10^6$/flask and cultivated for 24 hours in the DME medium containing 10% of fetal bovine serum (FBS) at 37 °C with 5% CO_2 and fully humidified atmosphere.

Isolation of MSCs using red blood cell lysis. 6 ml of bone marrow was mixed with erythrocyte lysis buffer (Qiagen) and centrifuged for 5 min at 480 *g*. The cell pellet was resuspended with 5 ml of RPMI 1640 medium (Invitrogen) and washed through centrifugation. The cells were placed into T175/T75 cm^2 tissue culture flask (BD Biosciences) and allowed to adhere for 24 hours in the DME medium containing 10% of FBS (StemCell Technologies) and 1% penicillin/streptomycin (Gibco) at 37 °C with 5% CO_2 and fully humidified atmosphere.

Culture of MSCs. After 24 hours MSCs were subsequently cultivated in MesenCult MSC Basal Medium (Human) containing 10% of MesenCult FBS for Human MSCs (StemCell Technologies) and 1% penicillin/streptomycin. The medium was changed every 3-4 days. The cells were harvested at 80-100% confluence with 0.25% trypsin-EDTA (Invitrogen), subcultured with seeding density 4000/cm^2 into new T175/T75 cm^2 flasks and maintained under the standard conditions.

Cell number and proliferation kinetics. The cell number was determined using a CASY cell counter and analyzer (Roche) and long-term growth kinetics *in vitro* was assessed by determining cumulative population doublings (CPDs). The number of PDs was calculated using the formula: $PD=\log(X_b/X_a)\cdot 3.3$, where X_a represents the initial cell number, X_b represents the cell harvest number, and 3.3 is a coefficient. The cell number was determined using a Bürker counting chamber (LO-Laboroptik GmbH) during MSC isolation protocol development.

Morphology. The cell morphology was determined using inverted phase contrast microscope (Nicon, Japan) and NIS-Elements imaging software. Spread cell area analysis was performed in ImageJ v1.50e image processing tool set. Spread cell area analysis was performed by Jan Krasko (National Cancer Institute).

Cryopreservation and thawing. MSCs were cryopreserved with MSC Freezing Solution (Biological Industries) and stored in -150 ^{o}C freezer for 6 months. Samples were thawed in a water bath at 37 ^{o}C and diluted with culture medium. After centrifugation at 150 *g* for 10 min, MSCs were plated with seeding density 4000/cm^2 into T75 cm^2 flasks and incubated under the standard conditions.

Flow cytometry. MSCs were characterized by flow cytometry using antibodies to CD44, CD73, CD90, and CD105 cell surface markers and using a mixture of antibodies to CD34, CD11b, CD19, CD45, HLA-DR cell surface markers (BD Stemflow™ Human MSC Analysis Kit) according to the manufacturer's protocol. Viability of the MSCs samples was assessed by 5-minute 7-AAD staining. Cytometric measurements were performed on BD LSR II flow cytometer. 10.000 cells per tube were analyzed with FlowJo X software. Flow cytometry was conducted by Jaroslav Denkovskij (State Research Institute Centre for Innovative Medicine) during MSC isolation methodology development and by Jan Krasko (National Cancer Institute) during MSC long-term culture.

Senescence-associated β-galactosidase staining. Senescence of MSCs was studied using Senescence Cells Histochemical Staining Kit (Sigma-Aldrich) according to the manufacturer's protocol. The percentage of senescent cells was calculated using the formula: (number of cells with intracellular blue deposits/ total number of cells)·100%.

Karyotype analysis. Cytogenetic evaluation of MSCs was conducted by G-banding method. Colchicine was added into culture at a final concentration of 0.2 μg/ml

for 4 hours at 37 ^{o}C. MSCs were harvested using trypsin and resuspended in a hypotonic 0.075 mMKCl solution for 30 min at 37 ^{o}C. After centrifugation the cells were fixed with methanol:acetic acid (3:1) solution. After dropping the cell suspension onto microscope slides, these were trypsinized and stained with Giemsa solution (Sigma-Aldrich). Slides were scanned, metaphases were captured and analyzed with Leica CytoVision® platform. Seven to seventeen metaphase spreads were analyzed for chromosome number and structure abnormalities at each established passage.

RNA isolation. Total RNA was isolated from MSCs using miRNeasy Mini Kit (Qiagen) following the manufacturer's instructions. Total RNA concentration and quality were checked using a NanoDrop spectrophotometer (Thermo Scientific) and verified by analysis on an Agilent 2100 Bioanalyzer using RNA 6000 Nano LabChip (Agilent Technologies).

PCR arrays. MSC samples were analyzed using Human Mesenchymal Stem Cell RT² Profiler™ PCR Array and Human Cellular Senescence RT² Profiler™ PCR Array (Qiagen). Template cDNA was synthesized from 800 ng of the total RNA using the RT² First Strand Kit (Qiagen) following manufacturer's protocol. The reaction mix was prepared by mixing cDNA with 2x RT^2 SYBR Green ROX FAST Mastermix (Qiagen) and 20 ml of the cocktail was aliquoted into each well on the PCR array. PCR arrays were performed in Rotor-Gene Q thermocycler (Qiagen), as follows: 95 °C for 10 sec, 40 cycles of 95 °C for 15 sec and 60 °C for 30 sec. Each sample was tested in technical duplicate. The data were analyzed using RT^2 Profiler PCR Array Data Analysis v3.5 software (Qiagen). The relative expression of each target gene was determined with the $\Delta\Delta C_t$ method and normalized to the geometric mean of 5 housekeeping genes. A gene was considered differentially expressed if it showed more than two-fold change and *P* value <0.05 indicated significance.

miRNA PCR array. miRNA levels in MSCs were analyzed with miRNome miScript miRNA PCR Array (Qiagen). Template cDNA was synthesized from 600 ng of the total RNA with miScript II RT Kit using miScript HiSpec Buffer (Qiagen) following manufacturer's protocol. The templates were mixed with RT^2 SYBR Green qPCR Master Mix (Qiagen) and 20 µl aliquoted into each well of PCR arrays. PCR was performed in Rotor-Gene Q thermocycler (Qiagen), as follows: 15 min at 95 °C, 40 cycles of 15 sec at 94 °C, 30 sec at 55 °C and 30 sec at 70 °C. Each sample was tested in

technical duplicate. The miRNA data were analyzed using miScript miRNA PCR Array Data Analysis software (Qiagen). The relative expression of each target miRNA was determined with the $\Delta\Delta C_t$ method and normalized to the geometric mean of 6 small RNAs. A miRNA was considered differentially expressed if it showed more than two-fold change and *P* value <0.05 indicated significance.

Functional gene ontology analysis. Gene Ontology Consortium (http://geneontology.org/) was used for enrichment analysis of specific gene sets.

Data Analysis. Statistical analysis was performed using SPSS software. Results are shown as mean ± standard deviation. The Mann–Whitney U was performed to assess statistical differences between independent groups. The Student's paired t-test was performed to assess statistical differences between dependent groups. Statistical differences were considered significant when *P* value <0.05.

Data Access. Bone marrow MSCPCR array data were deposited into a public database Gene Expression Omnibus under accession number GSE68933.

3. RESULTS AND DISCUSSION

During this study we developed a novel MSC isolation from human bone marrow protocol. Then we investigated the impact of tissue cryopreservation, culture medium composition and long-term *in vitro* expansion on human mesenchymal stem cell genetic characteristics. Study summarizing scheme is shown in Figure 1.

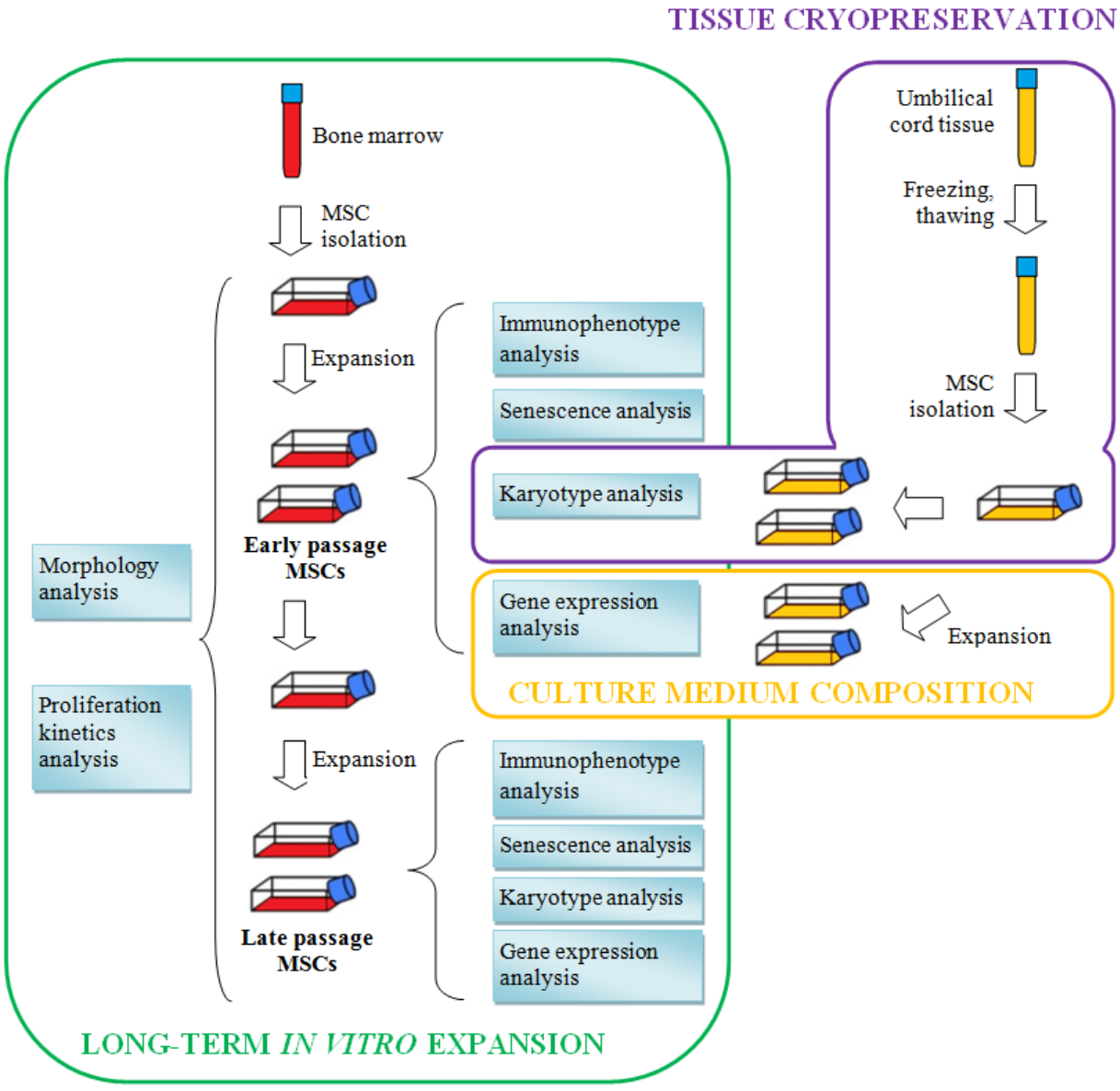

Figure 1. Study summarizing scheme. The impact of tissue cryopreservation, culture medium composition and long-term *in vitro* expansion on human MSC genetic characteristics was investigated during this study.

3.1 Development of the protocol for human mesenchymal stem cell isolation from residual bone marrow material and expansion *in vitro* to clinically relevant numbers

For therapeutical applications currently applied doses are in the range of $1–5 \cdot 10^6$ MSCs/kg body weight. Disadvantage of using human bone marrow (BM) MSCs is the limited cell numbers obtained from invasive isolation techniques[5]. MSCs commonly are isolated from high volume (60 ml) of bone marrow aspirates through density gradient centrifugation[33]. An application of more efficient and less patient frustrating MSC isolation from bone marrow method is urgently needed.

In this study, we applied red blood cell lysis (RBCL) method for MSC isolation from residual material after bone marrow transplantation (4 samples of 6 ml). In the control group the MSCs were isolated from 60 ml (4 samples) of bone marrow by ficoll density gradient centrifugation (FDGC) method. BM-MSCs were expanded in T175 culture flasks up to passage 2 (P2) (Figure 2). The cells were harvested at 100% confluence. The difference between FDGC and RBCL at P0statistically was not significant (P=0.7728). There was no statistically significant difference between two groups at P1 (P=0.1489) and at P2 (P=0.5637). The difference between FDGC at P1 and RBCL at P2 was also not significant (P=1). Figure 2 shows that the number of BM-MSCs isolated by CGF and RBCL methods increases in parallel, so the therapeutic dose of the cells using RBCL method should be achieved within a few days of next passage.

Expanded MSCs usually are identified according the plastic adherence, morphology and expression of surface markers[34,35]. In our study, all BM-MSCs were adhered to the plastic surface and had typical spindle shaped morphology (Figure 3). The expression of CD105, CD73, CD90 (>95%) and loss of CD14, CD34, CD45 and HLADR (<2%) was stated in all samples at P2.

On the basis of these results, we state that RBCL method for MSC isolation from low human bone marrow volume is as efficient as usual FDGC method for MSC isolation from large bone marrow volumes. Moreover, it has several advantages. First of all, there is no need for extra bone marrow biopsy. It is safe for potential donors because the risk of adverse events, such as hemorrhage, fracture at the site of the biopsy and persistent pain, is avoided[36]. Secondly, RBCL is much faster than FDGC because it

requires less manual interventions and manipulations. Finally, RBCL simplifies the standardized and GMP-conformed culture isolation for clinical application of MSCs since all mononuclear cells are isolated and subsequently the composition of heterogeneous MSC mixture is less affected[37].

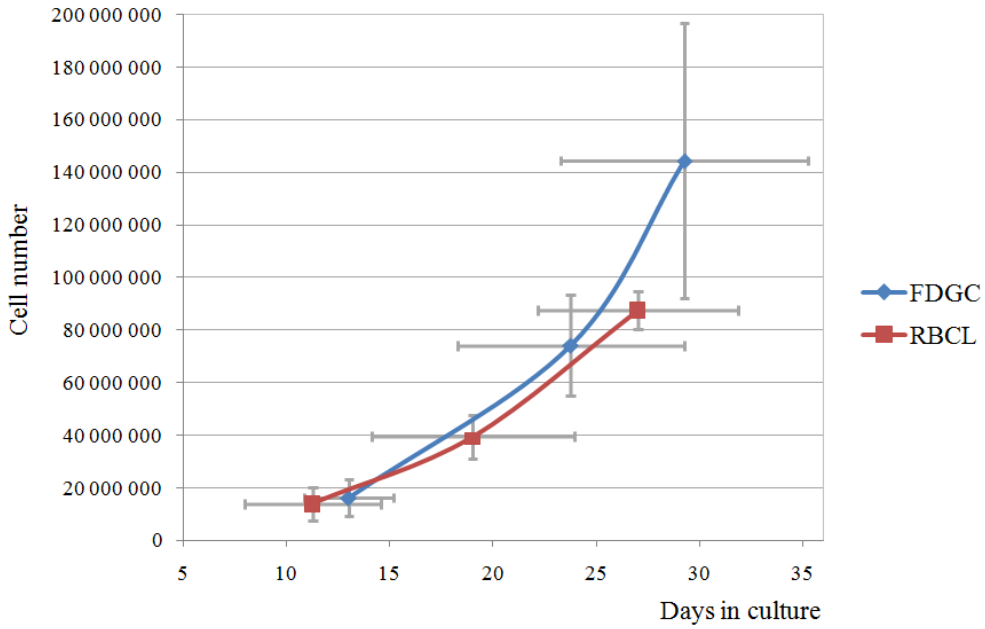

Figure 2. Number of human bone marrow MSCs. Amount of MSCs obtained at different passages (P0, P1, P2) using ficoll density gradient centrifugation (FDGC) and red blood cell lysis (RBCL) methods. Results are expressed as mean values ± SD.

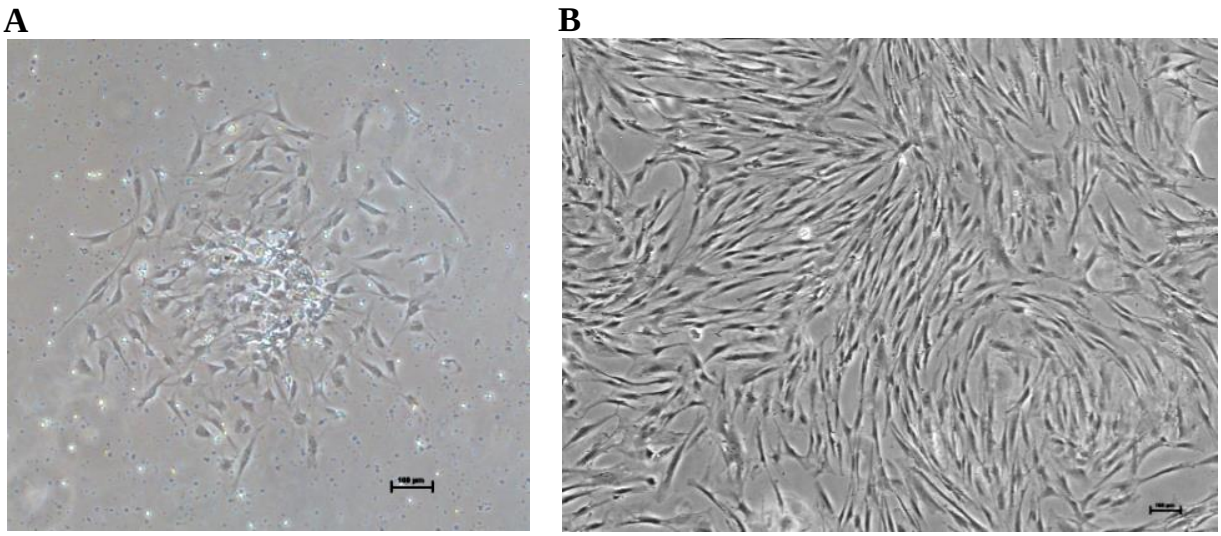

Figure 3. Morphology of human bone marrow MSCs. A – The cells forming a colony 1 day after isolation by RBCL method. **B** – Culture of MSCs with typical spindle shape morphology 11 days after seeding. Original magnitude 100x, scale 100 μm.

3.2 The impact of long-term expansion *in vitro* on MSC characteristics

High quantities of mesenchymal stem cells (MSCs) are needed for clinical applications, thus requiring extensive cell expansion in long-term culture[8]. However, the biological properties of MSCs are likely to be altered by culture conditions[9]. Therefore we expanded human bone marrow MSCs (BM-MSCs) *in vitro* in T75 flasks until passage 6–7 (P6–P7) and assessed the cell proliferative capacity, adherence to plastic, morphology, immunophenotype, senescence status, cytogenetic stability and applied array technology as suggested by The International Society for Cellular Therapy (ISCT)[34], The European Medicine Agency[38,39] and the U.S. Food and Drug Administration[27]. The cells were harvested at 80% confluence. BM-MSCs were cryopreserved at P1 and P3-P4. We categorized BM-MSC lifespan as early passage (P3-P4) and late passage (P6-P7) according to proliferation ability and the percentage of senescence-associated β-galactosidase-positive cells, similarly as proposed before[40].

3.2.1 The impact of long-term expansion *in vitro* on bone marrow MSC phenotypic, functional characteristics and karyotype stability

We showed that after isolation BM-MSCs reached P1 after 21±6 days and P3-P4 after additional 14.00±2.65 days resulting in 8.08±0.74 cumulative population doublings (CPDs) (Figure 4D). The cells at P3-P4 maintained a typical spindle-shaped or fibroblast-like morphology[35] (Figure 4A), were adherent and exhibited immunophenotype (Figure 6A-C) in accordance with ISCT guidelines[34]. Mean viability of BM-MSCs was 94.02±2.92% at early passages. Cytogenetic analysis at P3-P4 revealed a normal diploid karyotype (2n=46). We did not observe any chromosome structure aberrations that would disrupt chromosome integrity, such as deletion, inversion, translocation or ring chromosomes (Figure 5A), similarly as shown earlier[18]. Thus cryopreserved human BM-MSCs at P1 can be expanded within two weeks to clinically relevant yield of cells with unchanged characteristics.

By expanding BM-MSCs using additional three passages (until P6-P7 after cryopreservation at P3/P4) we investigated the possibility to achieve additional clinically relevant amounts of cells. However, in the late passages the BM-MSC growth gradually

decreased and it took 34.67±5.51 more days to complete with 15.08±3.04 CPDs (Figure 4D). The proportion of enlarged cells with flattened morphology also gradually increased, which became obvious P6-P7 (Figure 4B-C). Mean viability of BM-MSCs was 93.47±5.61% at late passages. 2 out of 3 samples at P6-P7 showed changes in surface marker expression while sample #1 remained stable throughout the *in vitro* expansion (Figure 6D-F). These results together with literature data[14,41] indicate that identification of late passage MSCs by using cell surface markers can be complicated. The karyotype of cells at P6-P7 remained stable (Figure 5B). We did not observe any clonal numerical or structural cytogenetic alterations, compatible with data obtained by some other groups[17,18]. After all, slower proliferation and altered morphology indicate possible MSC senescence[42].

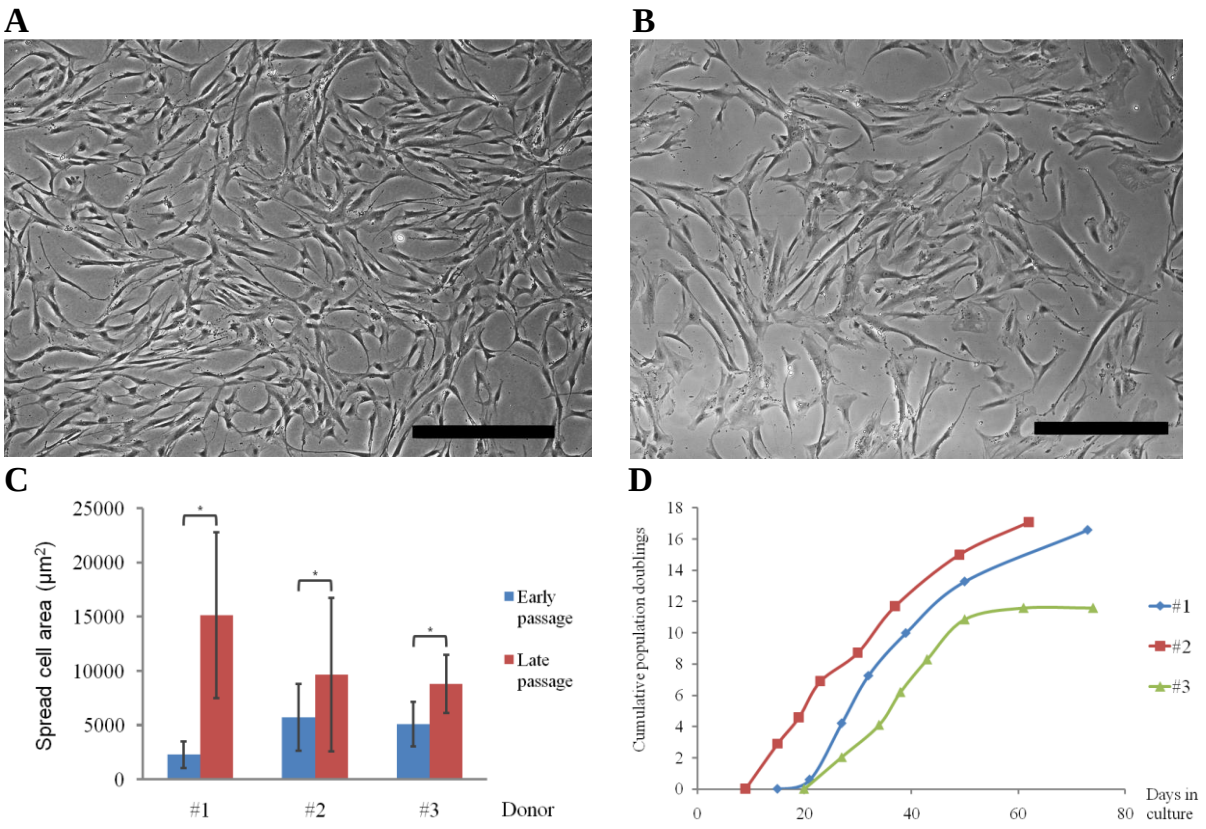

Figure 4. Morphology and proliferation kinetics of human bone marrow MSCs during long-term *in vitro* culture. A - Typical homogeneous population of fibroblast-like cells at P4. **B -** Heterogeneous population including enlarged cells with altered morphology at P7. Original magnification 40x, scale bars represent 500 μm. **C -** Average spread cell area at early (P3-P4) and late (P6-P7) passages of individual samples (**P*<0.05). **D -** Growth kinetics of MSC cultures from 3 donors scored as cumulative population doublings (y-axis) plotted against time in culture (x-axis). Each marker represents a passage.

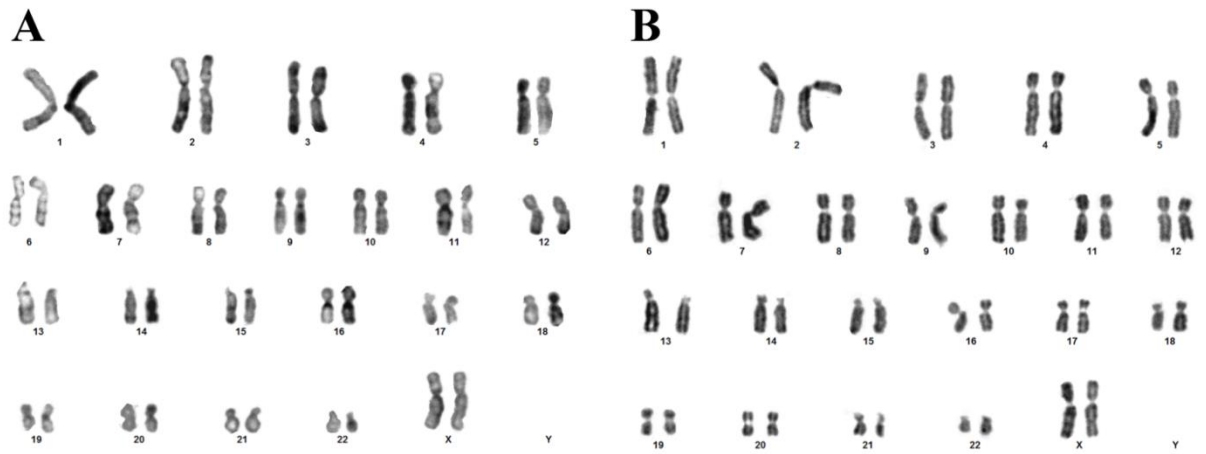

Figure 5. Human bone marrow MSC karyotype during *in vitro* culture. Representative karyograms of human MSC normal diploid karyotype (2n=46, XX) at P3 (**A**) and P6 (**B**). Original magnitude 1000x.

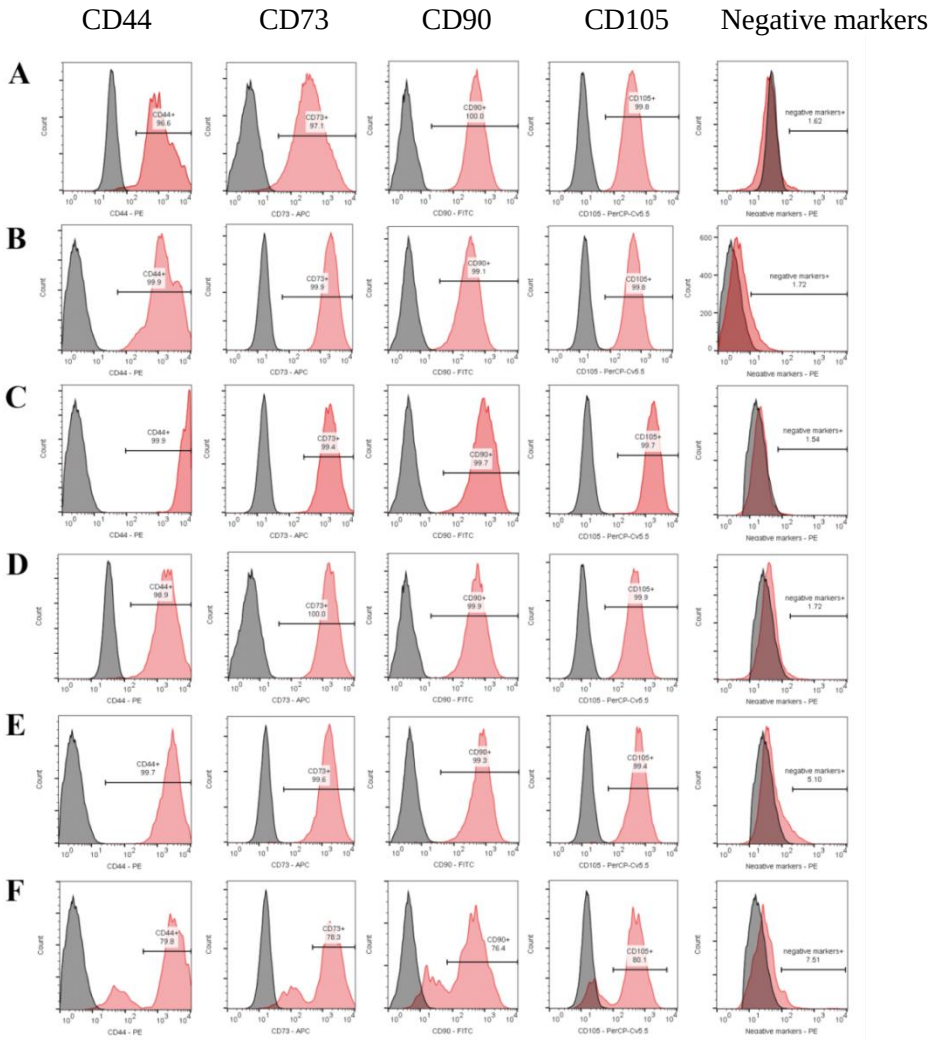

Figure 6. Immunophenotype of human bone marrow MSCs in long term culture. Analysis of samples #1 (**A**), #2 (**B**), #3 (**C**) at the early passages and analysis of samples #1 (**D**), #2 (**E**), #3 (**F**) at the late passages is demonstrated. Histograms on the left (grey) represent isotype controls and histograms on the right (red) represent stained cells. Negative markers: CD34, CD11b, CD19, CD45, HLA-DR.

3.2.2 Bone marrow MSC senescence during long-term *in vitro* expansion and novel MSC senescence biomarkers

The most widely used biomarker for senescent cells is senescence-associated β-galactosidase staining[43]. We determined that 41.97±4.57% (P=0.0043) of the late passage BM-MSCs reached senescence, whereas only 1.59±0.94% of cells stained positively in the early passages (Figure 7A-B).

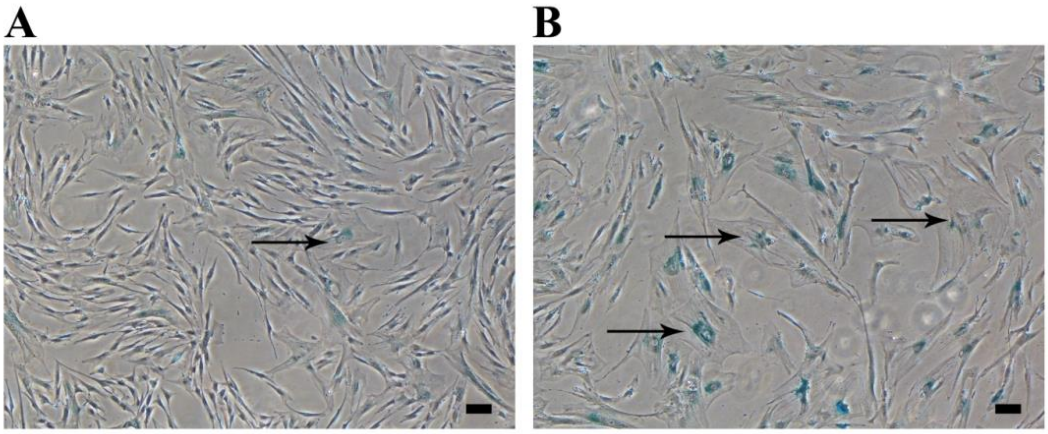

Figure 7. Human bone marrow MSC senescence during *in vitro* culture. Representative images of enlarged senescence-associated β-galactosidase-positive cells with altered morphology (indicated with black arrows) at P4 (**A**) and P7 (**B**). Original magnification 40x, scale bars represent 100 μm.

To date no molecular markers are available which specifically reflect the MSC senescence[44]. Molecular analysis of a suitable panel of genes might provide a powerful tool to track cellular aging of MSCs and thus to assess efficiency and safety of long-term expansion[43]. Therefore we investigated the cells using qPCR arrays consisted of selected key genes involved in maintaining cell stemness and causing cells to lose the ability to divide. From 162 genes, 4 genes were significantly (P<0.05) up-regulated (≥2 fold) and 9 genes were significantly (P<0.05) down-regulated in late passage BM-MSCs when compared with early passage MSCs (Figure 8A-B, Table 1). Functional gene ontology analysis revealed that these genes are associated with biological processes such as cell cycle, metabolism, cell aging and response to stress (Figure 9), which are important causes of cellular senescence[45]. It is known that senescence increases the risk of cell neoplastic transformation[13]. However, none of the genes in our study was linked to cancer associated functions such as polyploidization, transformation or invasion, as was

shown in senescent human MSCs with high levels of aneuploidy[46]. *THBS1*[47], *CCNB1*, *CHEK1*[48], *TBX2* and *PCNA*[49] have been associated with human BM-MSC senescence in culture. For the first time we show that the expression of genes *POU5F1*, *PTPRC*, *ACTA2*, *E2F1*, *E2F3*, *Tbx3*, *PLAU* and *CDC25C* is altered in senescent human BM-MSCs during long-term *in vitro* expansion.

To further evaluate the human BM-MSCs, we measured the expression of selected 420 miRNAs which are epigenetic gene expression key regulators[50] and only recently have emerged as important effectors of senescence[51]. Analysis showed significant (P<0.05) ≥2 fold changes in expression of 33 miRNAs (Figure 8C). Previously, *miR-29b*, *miR-455-3p*[52] and *miR-335*[53] have been linked to human BM-MSC senescence in culture. For the first time we report the link between the change of expression of the rest 30 miRNAs (Table 1) and human BM-MSC senescence during prolonged *in vitro* expansion. Among them expression of *miR-193a* and *miR-200a*, which have been determined to regulate human hematopoietic stem cell niche-defining genes[54], was the most altered in senescent BM-MSCs. Premature senescence phenotype of the hematological patient BM-MSCs only very lately has been revealed[55-57]. We hypothesize that miRNAs identified in our study could be candidates for hematological disease biomarker future research.

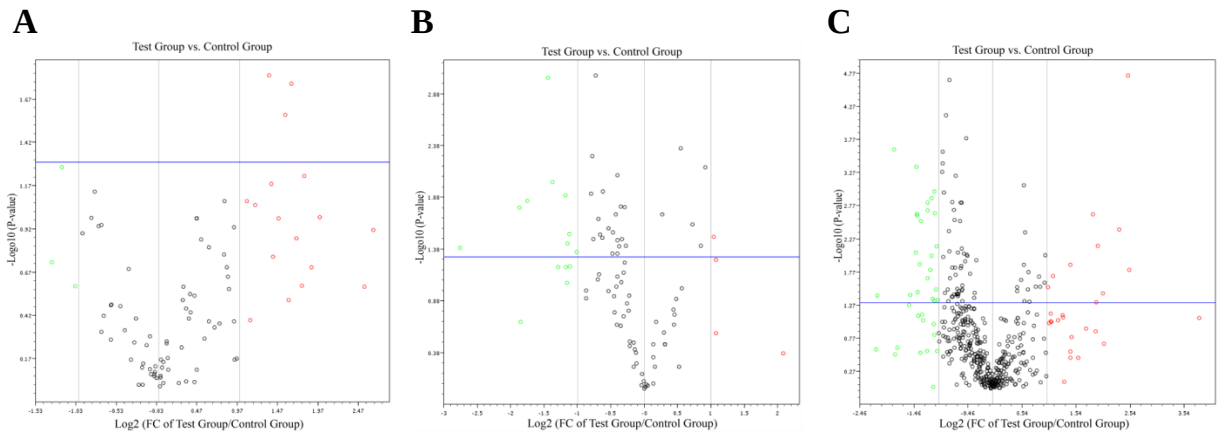

Figure 8. **Volcano plots of human mesenchymal stem cell related gene (A), cellular senescence related gene (B) and miRNA (C) expression changes in late passage (Test Group) versus early passage human bone marrow MSCs (Control Group).** Red dots are the genes whose expression increased more than 2 fold, while green dots are decreased more than 2 fold. Vertical grey side-lines represent fold-change (FC) cutoff (≥2 fold) and horizontal blue line represents P-value cutoff (P<0.05). Genes and miRNAs whose fold expression changes and P-values met the boundaries are listed in the Table 1.

Table 1. Significant fold regulation of genes and miRNAs in late passage versus early passage human bone marrow MSCs

Gene/miRNA Symbol	Fold Regulation*	*P-value*	miRNA Symbol	Fold Regulation*	*P-value*
ACTA2	3.1249	0.017625	*hsa-miR-7-5p*	-2.0189	0.045330
POU5F1	2.9597	0.026534	*hsa-miR-29b-1-5p*	-2.0528	0.006955
PTPRC	2.5796	0.015727	*hsa-miR-15b-3p*	-2.0742	0.031753
THBS1	2.0582	0.031849	*hsa-miR-576-5p*	-2.0967	0.002239
PCNA	-2.0236	0.044944	*hsa-miR-660-5p*	-2.1088	0.001035
E2F1	-2.1738	0.030206	*hsa-miR-25-5p*	-2.1203	0.046255
CDC25C	-2.2195	0.037200	*hsa-miR-29a-5p*	-2.1573	0.043583
TBX3	-2.2662	0.012653	*hsa-miR-224-5p*	-2.1874	0.001306
TBX2	-2.5941	0.009478	*hsa-miR-875-3p*	-2.1947	0.009676
PLAU	-2.7200	0.000930	*hsa-miR-188-3p*	-2.2305	0.015998
CHEK1	-3.3642	0.014370	*hsa-miR-455-5p*	-2.2997	0.001550
CCNB1	-3.6560	0.016672	*hsa-miR-139-5p*	-2.3091	0.001997
E2F3	-6.7673	0.040950	*hsa-miR-106b-3p*	-2.3068	0.021249
hsa-miR-422a	5.7364	0.016040	*hsa-miR-337-3p*	-2.5181	0.002904
hsa-miR-376b-3p	5.6578	0.000018	*hsa-miR-877-5p*	-2.5503	0.012980
hsa-miR-200a-5p	5.0577	0.003929	*hsa-miR-92a-1-5p*	-2.6194	0.034756
hsa-miR-219a-2-3p	4.0963	0.036284	*hsa-miR-218-5p*	-2.6433	0.002247
hsa-miR-639	3.8552	0.006863	*hsa-miR-130b-5p*	-2.6437	0.002407
hsa-miR-223-3p	3.7575	0.049123	*hsa-miR-192-5p*	-2.6692	0.000444
hsa-miR-608	3.6014	0.002329	*hsa-miR-187-3p*	-2.6816	0.008664
hsa-miR-429	2.7041	0.013235	*hsa-miR-200a-3p*	-2.8797	0.038458
has-miR-210-3p	2.1681	0.019652	*hsa-miR-193a-3p*	-3.5535	0.000240
hsa-miR-335-5p	2.0328	0.028915	*hsa-miR-935*	-4.4097	0.038848

*Fold-Regulation represents fold-change results in a biologically meaningful way. Fold-change values greater than one indicate a positive- or an up-regulation, and the fold-regulation is equal to the fold-change. Fold-change values less than one indicate a negative or down-regulation, and the fold-regulation is the negative inverse of the fold-change.

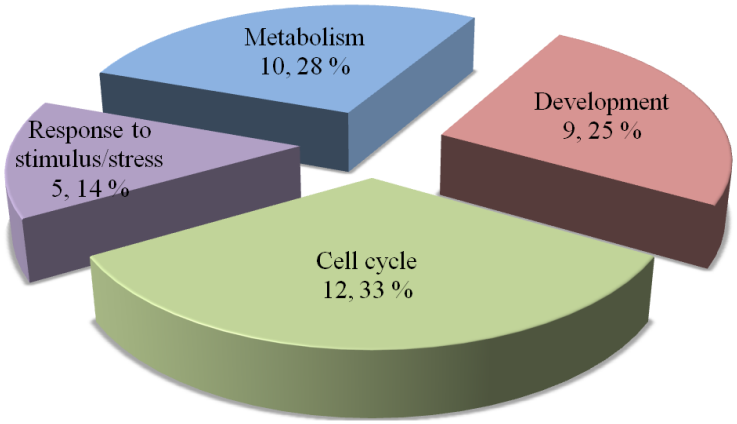

Figure 9. Gene Ontology term enrichment analysis of set of 13 genes with altered expression in late passage human bone marrow MSCs. A set of 13 genes (Table 1) was significantly (P<0.05) assigned to 36 Gene Ontology (GO) terms in the category of "biological process" which were later combined into a higher hierarchy of the cell biological processes. The first number indicates number of GO terms and the second number shows the percentage among all 36 GO terms.

3.3 The impact of tissue freezing and thawing process on the genomic stability of MSCs

Human umbilical cords (UC) offer an abundant and noninvasive source of mesenchymal stem cells (MSCs) without ethical issues[28]. Cryopreservation is the only method for long-term storage of viable cells and tissues for future cellular therapy. However, the freeze-thaw process contributes to cell damage[29] and its impact on UC-MSC genome stability is not clear so far. *Da-Croce et al.* showed that 1 out of 8 UC-MSC samples after slow cooling had a tetraploid karyotype (92, XXYY), while all 9 fresh samples had normal karyotypes[58].

During this study we investigated the karyotype of UC-MSCs (n=5) isolated from frozen-thawed UCs and expanded *in vitro* until P1-P2. Two samples had insufficient number of good quality metaphases. The analysis of remaining 3 samples showed normal diploid karyotype (2n=46, XX). None of structural abnormalities, such as deletion, inversion, translocation or ring chromosomes, were detected (Figure 10). For the first time we showed that human umbilical cord controlled tissue freezing and thawing do not affect the genomic stability of MSCs.

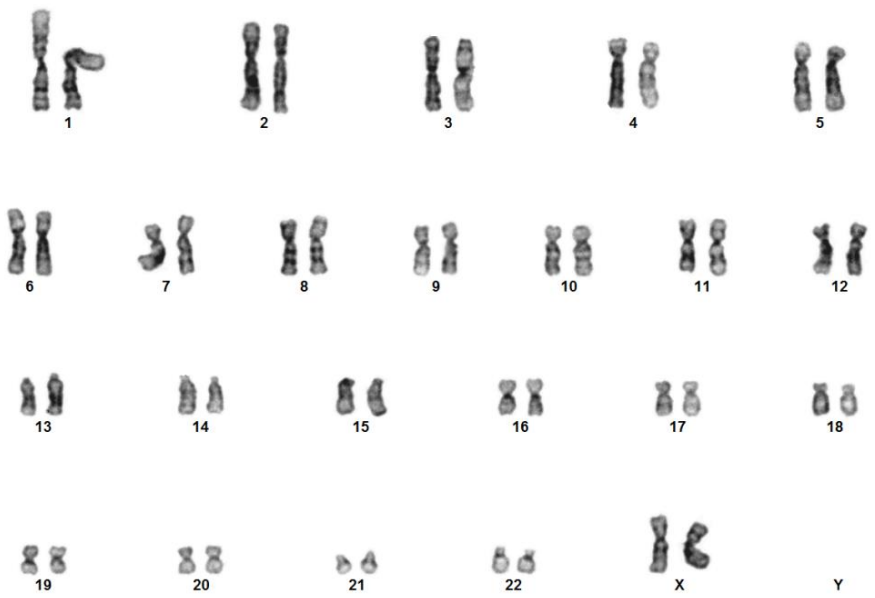

Figure 10. MSC karyotype after the freeze-thaw process of human umbilical cord tissue. Representative karyogram of MSC normal diploid karyotype (2n=46, XX) after freeze-thaw process of human umbilical cord tissue and *in vitro* expansion until P2. Original magnitude 1000x.

3.4 The impact of the culture medium composition on gene expression of MSCs

Fetal bovine serum (FBS) is the most frequently used to supply growth factors to MSC culture medium[30]. However, it carries the risk of contamination with infectious viral/prion agents and the induction of immune response in the final recipient[31]. In this study we investigated the impact of novel culture medium composition (recombinant protein enriched medium *vs.* common DMEM enriched with 10% FBS) on the gene expression of umbilical cord (UC) tissue MSCs. From analyzed 162 genes, 24 genes were significantly (P<0.05) up-regulated (≥2 fold) and 9 genes were significantly down-regulated in UC-MSCs which proliferated 5-10 times faster in medium with recombinant proteins (P2) when compared to UC-MSCs expanded in DMEM containing 10 % FBS (P3) (Figure 11A-B, Table 2). Functional gene ontology analysis revealed that these genes are associated with biological processes such as development, metabolism, response to stimulus and apoptosis (Figure 12). Analysis in the "cellular component" category showed that most genes are linked to secretory granule and extracellular space.

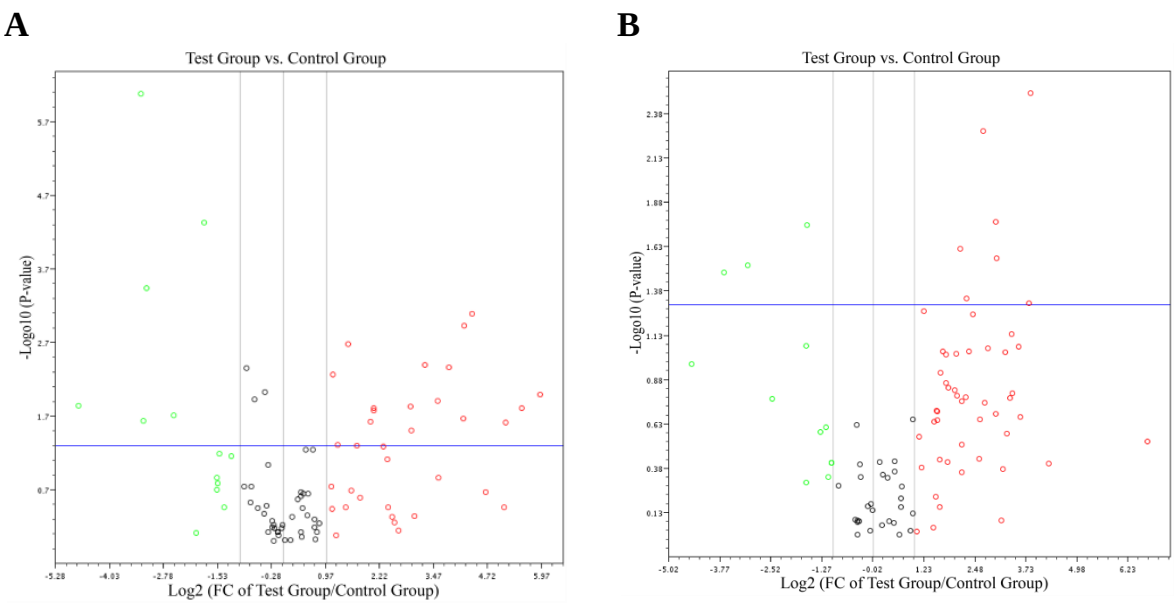

Figure 11. Volcano plots of human mesenchymal stem cell related gene (A) and cellular senescence related gene (B) expression changes in human umbilical cord MSCs expanded in medium with recombinant proteins (Test Group) *versus* the cells expanded in FBS containing medium (Control Group). Red dots are the genes whose expression increased more than 2 fold, while green dots are decreased more than 2 fold. Vertical grey side-lines represent fold-change (FC) cutoff (≥2 fold) and horizontal blue line represents *P*-value cutoff (*P*<0.05). Genes whose fold expression changes and *P*-values met the boundaries are listed in the Table 2.

Table 2. Significant fold regulation of genes in umbilical cord MSCs expanded in medium with recombinant proteins *vs.* the cells expanded in FBS containing medium

Gene symbol	Fold regulation*	*P-value*	Gene symbol	Fold regulation*	*P-value*
NT5E	61,4066	0,010097	*THBS1*	4,3629	0,024300
PTK2	45,6430	0,015555	*SMAD4*	4,2466	0,016861
BGLAP	35,2200	0,024137	*TGFB3*	4,2290	0,015553
NES	20,5679	0,000820	*ANPEP*	4,0455	0,023919
BMP4	18,2310	0,001181	*HDAC1*	2,8134	0,002125
RHOA	17,8311	0,021687	*HAT1*	2,3922	0,049337
CDKN2C	14,3397	0,003202	*CD44*	2,2227	0,005383
CTNNB1	14,1460	0,004367	*IGFBP3*	-3,0967	0,017843
CHEK1	14,0316	0,049187	*VEGFA*	-3,5843	0,000047
NUDT6	11,8624	0,012551	*GDF15*	-5,7985	0,019293
ENG	9,6486	0,003983	*CREG1*	-8,4506	0,030048
CCNA2	8,0982	0,027285	*IL6*	-9,0234	0,000365
CDKN1C	7,9133	0,017071	*JAG1*	-9,4984	0,023224
GDF5	7,7399	0,031158	*ICAM1*	-9,8333	0,000001
HGF	7,6440	0,014940	*CDKN2B*	-12,6734	0,032893
EGR1	6,4499	0,005217	*CSF3*	-26,7910	0,014262
CITED2	4,8387	0,046415			

*Fold-Regulation represents fold-change results in a biologically meaningful way. Fold-change values greater than one indicate a positive- or an up-regulation, and the fold-regulation is equal to the fold-change. Fold-change values less than one indicate a negative or down-regulation, and the fold-regulation is the negative inverse of the fold-change.

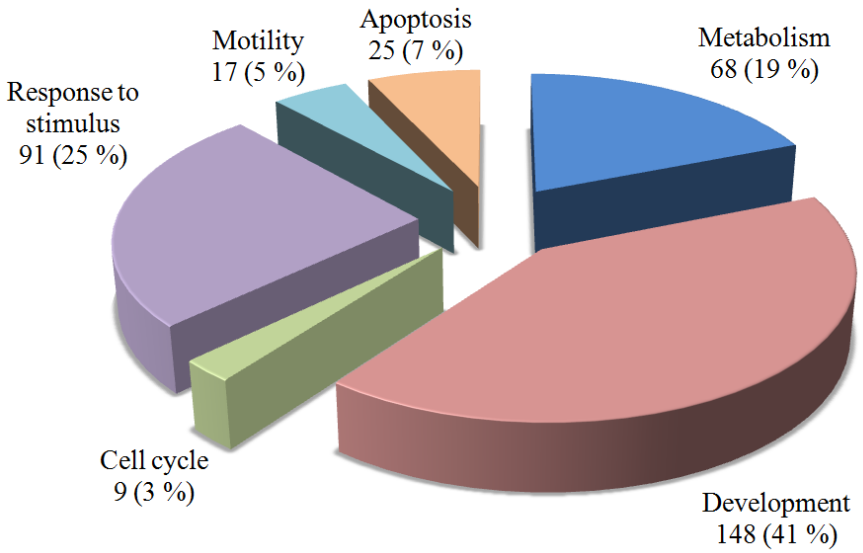

Figure 12. Gene Ontology term enrichment analysis of set of 33 genes with altered expression in umbilical cord MSCs expanded in medium with recombinant proteins when comparing to the cells expanded in FBS containing medium. A set of 33 genes (Table 2) was significantly ($P<0.05$) assigned to 358 Gene Ontology (GO) terms in the category of "biological process" which were later combined into a higher hierarchy of the cell biological processes. The first number indicates number of GO terms and the second number shows the percentage among all 358 GO terms.

4. CONCLUSIONS

1. Human bone marrow is a convenient source of mesenchymal stem cells (MSCs) for clinical applications. MSCs could be successfully isolated from the residual bone marrow transplantation material (6 ml) by erythrocyte lysis and expanded *in vitro* to clinically relevant numbers ($>1 \cdot 10^8$).

2. The bone marrow MSC phenotypic, functional and genetic characteristics (morphology, proliferation kinetics, immunophenotype and karyotype) do not change during short-term *in vitro* expansion (until passages 3-4). The cells do not show the signs of cellular senescence.

3. MSCs maintain chromosomal stability in passages 6-7. Slower proliferation, altered morphology and positive staining for β-galactosidase activity indicate MSC senescence at late passages. The impact of long-term *in vitro* expansion on MSC immunophenotype is donor-dependent.

4. Significant difference ($P<0.05$) in expression of 13 genes, involved in maintaining cell stemness or stopping cell division, and 33 miRNAs was determined in late passage MSCs when compared with early passage cells. Changes in expression of 8 genes and 30 miRNAs were linked to bone marrow MSC senescence during prolonged *in vitro* expansion for the first time.

5. Umbilical cord tissue controlled freezing and thawing process does not affect the genomic stability of MSCs.

6. MSC expansion under GMP conditions significantly changed the expression of genes involved in maintaining cell stemness or stopping cell division. Significant difference ($P<0.05$) in expression of 33 genes was found in umbilical cord tissue MSCs which expanded 5-10 times faster in medium enriched with recombinant proteins when comparing to the cells expanded in standard medium enriched with fetal bovine serum.

5. PRACTICAL RECOMMENDATIONS

1. Human bone marrow mesenchymal stem cell (MSC) isolation and expansion technique developed during this research is faster, cheaper and donor saving when compared to currently used techniques and could be applied at institutions producing an advanced therapy medicinal products.

2. The cellular therapy quality control could be developed on the basis of the results obtained in this study in order to assess the stemness, possible senescence and malignant transformation of *in vitro* manipulated MSCs.

6. PUBLICATIONS AND PRESENTATIONS

Published articles in journals with a citation index of the Institute for Scientific Information database "ISI Web of Science":

1. **Kundrotas G**, Gasperskaja E, Slapsyte G, Gudleviciene Z, Krasko J, Stumbryte A, Liudkeviciene R. Identity, proliferation capacity, genomic stability and novel senescence markers of mesenchymal stem cells isolated from low volume of human bone marrow. Oncotarget (IF=6,36). 2016;7:10788-10802.
2. Gudleviciene Z, **Kundrotas G**, Liudkeviciene R, Rascon J, Jurga M. Quick and effective method of bone marrow mesenchymal stem cell extraction. Open Medicine (IF=0,153). 2015;10:44-49.

Articles published in other peer-reviewed journals:

1. **Kundrotas G.** Surface markers distinguishing mesenchymal stem cells from fibroblasts. Acta Medica Lituanica. 2012;19:75-79.

Oral presentations:

1. Quick and donor saving method for mesenchymal stem cell extraction for clinical use. The 12th Baltic Congress of Laboratory Medicine. 2014 09 19. Riga, Latvia.
2. Investigation of human mesenchymal stem cell genetic features to ensure their safety in clinical practice. Conference of Young Scientists 2012: Biofuture. Perspectives of nature and life sciences. 2012 12 05. Vilnius.
3. Precise controlling of MSC characteristics during *in vitro* cultivation to ensure effective and safe stem cell application in clinical practice for advanced therapy in oncology. The 6th International Forum on Innovative Technologies for Medicine. 2012 12 22. Bialystok, Poland.
4. Investigation of genetic features of mesenchymal stem cells to ensure safe treatment in oncology. The 11th Baltic Congress of Laboratory Medicine. 2012 05 11. Vilnius.

Poster presentations:

1. Development of Quality Control protocol for clinical application of mesenchymal stem cells. **Kundrotas G**, Gudleviciene Z, Slapsyte G, Gasperskaja E. Stem cell research in Lithuania: past, present and future. 2015 08 27. Vilnius.
2. Stemness and genetic stability of human mesenchymal stem cells isolated from ultralow bone marrow volume. **Kundrotas G**, Gudleviciene Z, Gasperskaja E, Slapsyte G. The 6th Baltic Congress of Oncology. 2014 10 3-4. Vilnius.
3. Karyotype analysis of early passage mesenchymal stem cells isolated from frozen umbilical cord tissue. **Kundrotas G**, Gasperskaja E, Slapsyte G, Jurga M. Science at Faculty of Natural Sciences, the 8th scientific conference. 2014 10 03.
4. Quick and donor-saving method for mesenchymal stem cell extraction for biobanking and clinical use. Gudleviciene Z, **Kundrotas G**, Liudkeviciene R. European, the Middle Eastern & African Society of Biopreserving & Biobanking (ESBB) and Biobanking and Biomolecular resources Research Infrastructure (BBMRI) Conference. 2013 10 8-11. Verona, Italy.
5. Precise controlling of MSC characteristics during *in vitro* cultivation to ensure effective and safe stem cell application in clinical practice for advanced therapy in oncology. Gudleviciene Z, **Kundrotas G**, Schveigert D, Slapsyte G. The 6th International Forum on Innovative Technologies for Medicine. 2012 12 21-23. Bialystok, Poland.

7. CURRICULUM VITAE

Name	Gabrielis Kundrotas	
Date and place of birth	1986 09 22, Vilnius, Lithuania	
Professional address	Faculty of Natural Sciences Vilnius University M.K.Čiurlionio str. 21/27, Vilnius, LT-03101, Lithuania Phone: +370 5 2 398258	Biobank, National Cancer Institute P. Baublio str. 3b, Vilnius, LT-08406, Lithuania Phone: +370 5 2 190909
E-mail	gabrielis.kundrotas@gf.stud.vu.lt,	gabrielis.kundrotas@nvi.lt.

Educational background

2005 – 2009	Bachelor of Science in Molecular Biology, Vilnius University
2009 – 2011	Master of Science in Genetics, Vilnius University
2011 – 2015	PhD student in Biomedical Sciences, Biology, Vilnius University.

Professional background

2008 – 2010	Part-time Research Student Institute of Biotechnology, Vilnius University
2011 – 2012	Junior Research Associate Institute of Oncology, Vilnius University
2012 – present	Junior Research Associate Biobank, National Cancer Institute.

Internships

2013 03-04	The Cell Factory, Belgium. Aims: MSC techniques & quality control
2013 10 7-11	Comprehensive Pneumology Center, Germany. Aims: cell cultures, 3D live imaging, tissue engineering (de- & recellularisation)
2014 04 5-19	The Cell Factory, Belgium. Aims: MSC GMP bioproduction, EU requirements for bioproduction, validation and clinical use of MSCs.

Memberships Member of Association of Stem Cell Researchers in Lithuania.

8. SUMMARY IN LITHUANIAN

Įvadas

Žmogaus mezenchiminės kamieninės ląstelės (MKL) yra pagrindinės kamieninės ląstelės, naudojamos klinikiniuose tyrimuose. Tyrinėjamas jų potencialas reguliuoti imuninę sistemą ir skatinti audinių regeneraciją. MKL yra daug žadančios gydant degeneracines, imunines ligas, vėžį bei atstatant pažeistus audinius. Nepaisant reikšmingų MKL panaudojimo perspektyvų, vis dar lieka daug neatsakytų klausimų dėl manipuliacijų *in vitro* įtakos MKL savybėms ir paruošto MKL preparato saugumo recipientui.

Kaulų čiulpai yra pagrindinis klinikiniam panaudojimui skirtų MKL šaltinis. Tačiau kaulų čiulpų punkcija yra invazinė ir skausminga procedūra pacientui. Be to, iš kaulų čiulpų išskiriamas ribotas kiekis MKL. Todėl reikalingas efektyvesnis ir mažiau nepatogumų donorams sukeliantis MKL išskyrimo iš kaulų čiulpų metodas.

Siekiant užtikrinti pakankamą MKL terapijos efektyvumą, reikalingi dideli ląstelių kiekiai, o tam ląstelės turi būti ilgą laiką auginamos *in vitro*. Tačiau auginimo sąlygos gali nulemti MKL savybių pokyčius. Visų pirma gausinamos *in vitro* žmogaus MKL pereina į senėjimo fazę, kuri apibrėžiama kaip negrįžtamas ląstelės ciklo sustabdymas. Dėl senėjimo trumpėja ląstelių gyvavimo laikotarpis, sustiprėja uždegimą skatinančių veiksnių sintezė ir sutrinka MKL terapinį poveikį nulemiantis imunomoduliacinis potencialas. MKL senėjimas vertinamas pagal morfologijos, proliferacijos kinetikos pokyčius bei β-galaktozidazės aktyvumą. Tačiau nėra standartinio metodo ląstelių senėjimui įvertinti, todėl reikalingi specifiniai, molekuliniai, žymenys, kurie padėtų nustatyti žmogaus MKL senėjimą.

Galiausiai ilgalaikis auginimas *in vitro* ir su juo susijęs senėjimas didina žmogaus MKL piktybinės transformacijos riziką. Vienų tyrimų rezultatai parodė, kad MKL genomas išlieka stabilus. Atliekant kitus tyrimus nustatyta, kad ilgalaikio auginimo metu išsivysto MKL kariotipo pokyčių. Visuotinai pripažinta, kad genomo nestabilumas sudaro sąlygas ląstelėms įgyti vėžinių ypatybių ir tai yra vienas labiausiai nerimą keliančių aspektų, siekiant žmogaus MKL saugiai panaudoti klinikinėje praktikoje. Nors dalis šokiruojančių publikacijų apie piktybinę žmogaus MKL transformaciją vėliau buvo atšauktos, tačiau diskusijos apie žmogaus MKL genetinį stabilumą ir panaudojimo

klinikinėje praktikoje saugumą išlieka aktualios. Todėl reikia daugiau tyrimų, norint įvertinti žmogaus MKL genomo stabilumą ilgalaikio auginimo metu.

Virkštelės audinys yra alternatyva kaulų čiulpams, kadangi jame gausu MKL ir jų išskyrimas neinvazinis. Norint ateityje panaudoti virkštelės audinį, jį būtina užšaldyti. Tačiau šaldymo–atšildymo procesas gali pažeisti ląsteles ir iki šiol nėra aiški šios technologijos įtaka virkštelės audinio MKL genomo stabilumui.

MKL dažniausiai gausinamos mitybinėse terpėse su fetaliniu veršelio serumu (FVS). Tačiau serumo panaudojimas didina veršelio patogenų perdavimo bei imuninių reakcijų prieš ksenogeninius serumo antigenus riziką. Geriausias šios problemos sprendimas būtų MKL gausinti gyvūninės kilmės komponentų neturinčioje mitybinėje terpėje. Ankstesnių tyrinėjimų metu nustatyta, kad rekombinantiniais baltymais praturtintoje mitybinėje terpėje virkštelės audinio MKL proliferuoja 5–10 kartų greičiau nei įprastoje DME terpėje, papildytoje 10 % FVS. Tikėtina, kad ši pažangiosios terapijos vaistinio preparato gamybai reikšminga savybė susijusi su ląstelių genų raiškos skirtumais.

Tyrimo tikslas buvo ištirti *in vitro* atliekamų manipuliacijų įtaką mezenchiminių kamieninių ląstelių genetinėms savybėms.

Tyrimo uždaviniai

1. Sukurti žmogaus MKL išskyrimo iš likutinės kaulų čiulpų medžiagos ir pagausinimo *in vitro* iki terapijai tinkamo kiekio metodiką;
2. Ištirti ankstyvųjų (3–4) ir vėlyvųjų (6–7) pasažų žmogaus kaulų čiulpų MKL fenotipines, funkcines (morfologiją, imunofenotipą, senėjimą, proliferacijos potencialą) ir genetines (kariotipą ir genų raišką) savybes;
3. Palyginus vėlyvųjų ir ankstyvųjų pasažų ląstelių tyrimų rezultatus, įvertinti ilgalaikio auginimo *in vitro* įtaką žmogaus kaulų čiulpų MKL savybėms;
4. Atrinkti potencialius molekulinius biožymenis žmogaus kaulų čiulpų MKL senėjimui *in vitro* vertinti;
5. Ištirti kontroliuojamo šaldymo įtaką žmogaus virkštelės audinio MKL genomo stabilumui;

6. Ištirti naujos mitybinės terpės, papildytos rekombinantiniais baltymais, įtaką virkštelės audinio MKL genų raiškai ir palyginti su genų raiška virkštelės audinio MKL, augintomis įprastoje terpėje, papildytoje FVS.

Ginamieji teiginiai

1. MKL gali būti sėkmingai išskirtos iš nedidelio žmogaus kaulų čiulpų tūrio ir efektyviai pagausintos iki kiekio, reikalingo terapiniam ląstelių preparatui pagaminti;
2. Ilgalaikis žmogaus kaulų čiulpų MKL auginimas *in vitro* turi įtakos jų morfologijai, proliferacijos greičiui, senėjimui, chromosomų stabilumui ir genų raiškai;
3. Naujus specifinius žmogaus kaulų čiulpų MKL senėjimo *in vitro* biožymenis galima nustatyti tiriant tikslingai atrinktų genų raišką;
4. Kontroliuojamas šaldymas ir atšildymas neturi įtakos žmogaus virkštelės audinio MKL chromosomų stabilumui;
5. Mitybinės terpės, papildytos rekombinantiniais baltymais, sudėtis turi įtakos žmogaus virkštelės audinio MKL genų raiškai.

Tyrimo metodai

MKL iš žmogaus kaulų čiulpų išskyrėme centrifugavimo per fikolio gradientą bei raudonųjų kraujo ląstelių lizės metodais. MKL auginome mitybinėje terpėje, turinčioje 10 % FVS. Žmogaus virkštelės audinio MKL buvo išskirtos ir augintos kompanijoje *The Cell Factory* (Belgija). Kaulų čiulpų MKL identifikuoti ir jų savybėms įvertinti tyrėme ląstelių proliferacijos greitį, morfologiją, imunofenotipą bei β-galaktozidazės aktyvumą. MKL genomo stabilumą vertinome dažydami chromosomas G metodu ir analizuodami jų skaičių bei struktūrą. Genų ir miRNR raišką tyrėme naudojant PGR gardeles. Funkcinę genų ontologijos analizę atlikome naudodamiesi Genų ontologijos konsorciumu.

Rezultatų apibendrinimas

Pirmiausia parodėme, kad iš nedidelio kaulų čiulpų transplantacijos medžiagos tūrio (6 ml) MKL galima išskirti ir pagausinti in vitro iki terapijai tinkamo kiekio

naudojant raudonųjų kraujo ląstelių lizės metodiką taip pat efektyviai kaip ir naudojant dažniausiai taikomą didelio kaulų čiulpų tūrio (60 ml) centrifugavimo per fikolio gradientą metodą. Kad pagausintos ląstelės yra MKL nustatėme pagal tvirtinimąsi prie plastiko paviršiaus, morfologiją ir imunofenotipą.

Tuomet kaulų čiulpų MKL savybes vertinome išsamiau. Tam ląsteles išskyrėme ir gausinome *in vitro*, kol užaugo 3–4 pasažai (P3–P4), siekdami užtikrintai turėti terapijai tinkamą ląstelių kiekį ($>1\cdot10^8$). Vėliau ląsteles auginome papildomus 3 pasažus, kol užaugo vėlyvieji P6–P7, siekdami nustatyti galimos papildomos terapinės dozės MKL savybes. Remdamiesi Tarptautinės ląstelių terapijos draugijos, Europos medicinos agentūros bei JAV maisto ir vaistų administracijos rekomendacijomis, vertinome ląstelių proliferacijos greitį, tvirtinimąsi prie plastiko, morfologiją, imunofenotipą, senėjimo būklę, chromosomų stabilumą ir genų raišką.

Ankstyvųjų pasažų (P3–P4) kaulų čiulpų MKL savybės buvo nepakitusios. Ląstelių populiacijos dalijosi greitai ir padvigubėjo ~8 kartus, išlaikė savybę tvirtintis prie paviršių, būdingą paviršiaus žymenų raišką (CD44, CD73, CD90, CD105, CD34, CD11b, CD19, CD45, HLA-DR), tipišką verpstės morfologiją ir nepakitusį kariotipą.

Vėlyvųjų pasažų kaulų čiulpų MKL proliferacijos greitis ėmė lėtėti, po truputį daugėjo padidėjusių suplokštėjusios formos ląstelių, kol galiausiai P6–P7 beveik pusė populiacijos ląstelių perėjo į senėjimo būklę. Kariotipo pokyčių vėlyvųjų pasažų ląstelėse neaptikome. Palyginę ankstyvųjų ir vėlyvųjų pasažų MKL genų raišką, nustatėme 13 genų ir 33 miRNR, kurių raiška reikšmingai ($p < 0,05$) skyrėsi senstančiose MKL. Funkcinė genų ontologijos analizė patvirtino, kad šių genų produktai dalyvauja su senėjimu susijusiuose ląstelės procesuose.

Išanalizavę iš kontroliuotai šaldyto ir vėliau atšildyto virkštelės audinio (VA) išskirtų ir iki P1–P2 pagausintų MKL kariotipą, jokių chromosomų skaičiaus ar struktūros pokyčių nenustatėme. Naujoje rekombinantiniais baltymais praturtintoje mitybinėje terpėje VA MKL proliferavo 5–10 kartų greičiau ir jose nustatėme 33 genus, kurių raiška reikšmingai ($p < 0,05$) skyrėsi, palyginti su VA MKL, augintose FVS turinčioje mitybinėje terpėje. Funkcinė genų ontologijos analizė parodė, kad dauguma šių genų produktų yra susiję su sekrecija ir užląsteline erdve.

Išvados

1. Kaulų čiulpai – patogus mezenchiminių kamieninių ląstelių (MKL) šaltinis naudoti terapijoje. Eritrocitų lizės metodu MKL gali būti išskirtos iš likutinės kaulų čiulpų transplantacijos medžiagos (6 ml) ir pagausintos *in vitro* iki terapijai tinkamo kiekio ($>1 \cdot 10^8$).

2. Ankstyvųjų pasažų (3–4) kaulų čiulpų MKL fenotipinės, funkcinės bei genetinės savybės (morfologija, proliferacijos greitis, imunofenotipas ir kariotipas) liko nepakitusios. Ankstyvųjų pasažų ląstelėms nebuvo būdingi senėjimo požymiai.

3. Vėlyvųjų pasažų (6–7) kaulų čiulpų MKL buvo genomiškai stabilios. Sulėtėjęs proliferacijos greitis, pakitusi morfologija ir didėjęs β-galaktozidazės aktyvumas rodė, kad 6–7 pasažų MKL perėjo į senėjimo būklę. Nustatyti vėlyvųjų 6–7 pasažų MKL imunofenotipo pokyčiai buvo individualūs kiekvienam donorui.

4. Nustatyti 13 genų, siejamų su ląstelių kamieniškumo palaikymu arba ląstelės dalijimosi stabdymu, ir 33 miRNR raiškos reikšmingi pokyčiai (padidėjimas arba sumažėjimas, $p < 0,05$) senstančiose vėlyvųjų pasažų (6–7) MKL. Tarp jų 8 genų ir 30 miRNR raiškos pokyčiai su MKL senėjimu *in vitro* susieti pirmą kartą.

5. Kontroliuojamas virkštelės audinio šaldymas iš jo išskirtų ir *in vitro* pagausintų MKL kariotipo stabilumui įtakos neturėjo.

6. MKL pagausinimo metodikos pakeitimas pagal Geros gamybos praktikos reikalavimus turėjo reikšmingos įtakos genų, siejamų su ląstelių kamieniškumo palaikymu arba ląstelės dalijimosi stabdymu, raiškai. Nustatyti 33 genų raiškos reikšmingi pokyčiai (padidėjimas arba sumažėjimas, $p < 0,05$) virkštelės audinio MKL, kurios proliferavo greičiau rekombinantiniais baltymais papildytoje mitybinėje terpėje (palyginti su MKL, augintose įprastoje terpėje, papildytoje fetaliniu veršelio serumu).

Darbo mokslinis naujumas ir praktinė reikšmė

Sukūrėme naują metodiką MKL išskirti iš likutinės žmogaus kaulų čiulpų transplantacijos medžiagos ir pagausinti *in vitro* iki terapijai tinkamo kiekio. Taip be papildomos kaulų čiulpų biopsijos galima sukurti nepakitusių savybių ląstelių produktą.

Nustatėme naujus 38 potencialius žmogaus kaulų čiulpų MKL senėjimo molekulinius žymenis, kurie suteikia naujų žinių apie MKL biologiją ir galėtų sudaryti pagrindą ląstelių preparato kokybės kontrolei kurti.

Pirmą kartą parodėme, kad virkštelės audinio kontroliuojamas šaldymas ir atšildymas neturi įtakos MKL genomo stabilumui. Tai patvirtina kriokonservavimo saugumą ilgalaikiam virkštelės audinio saugojimui. Parodėme, kad mitybinės terpės, papildytos rekombinantiniais baltymais, sudėtis turi didelę įtaką virkštelės audinio MKL genų, siejamų su kamieniškumo palaikymu arba ląstelės dalijimosi stabdymu, raiškai.

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