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Isolation, characterization and analysis of *TGFβ* gene expression in adipose-derived precursor cells cultured in different growth media

Inese Čakstiņa[1,3]*, Una Riekstiņa[2,3], Ruta Muceniece[2,3], Jānis Ancāns[1,3]

[1]Faculty of Biology, University of Latvia, Kronvalda Bulv. 4, Rīga LV-1586, Latvia
[2]Faculty of Medicine, University of Latvia, Šarlotes 1a, Rīga LV-1001, Latvia
[3]Laboratory of Bioanalytical Methods, University of Latvia, Rātsupītes 1, Rīga LV-1001, Latvia

*Corresponding author, E-mail: inese.cakstina@lu.lv

Abstract

Human adult (autologous) stem cells which are capable of self-renewal and differentiation into other cell types, can be isolated from various tissue and are a promising source for cell therapy, since there are no ethical and rejection problems as in the case of embryonic stem cells. The human body contains a great amount of adipose tissue which consists also of several mezenchymal stem cell populations. According to the existing data Transforming Growth Factor β (TGFβ) is one of the molecules involved in MSC differentiation. The aim of this work was to study the capablility of human adipose-derived precursor cells to differentiate into other cell types and to study the effect of various cell media on the expression of *TGFβ* in adipoze-derived precursor cells. Here we show that in serum-free medium the morphology of cells differs and the expression level of *TGFβ* is significantly higher than in serum-supplemented media. Our work also shows that cell populations isolated from human adipose tissue can differentiate not only into mezenchymal lineage cells but also into neuronal lineage cells, prooving the plasticity of adipose derived cells. We conclude that cell populations isolated from human adipose tissue contain a number of precursor cells with characteristic features of stem cells – ability to differentiate into more than one cell type. Cell cultivation medium affects population growth and morphology. Cells in serum-containing medium grow faster and develop a monolayer, while cells in serum-free medium grow slower and tend to form spheres. *TGFβ* expression is significantly higher in cells grown in serum-free medium which is more suitable for maintenance of cells than for promoting rapid growth.

Key words: adipose derived precursors, stem cells, transforming growth factor

Introduction

Stem cells can continue cell division and renewal for long periods by stable maintenance of unspecialized status and by differentiaion into specialized cell types (Fuch et al. 2004; Toma et al. 2005). Future stem cell therapy is focused on using the patient's own adult stem cells to regenerate tissue and to treat a variety of disorders. The human body is a good source of adult (or autologous) stem cells, since almost every organ contains smaller or larger populations of immature cells capable of differentiation into some cell types

(Aejaz et al. 2007). Adipose tissue, like bone marrow, is derived from the mezenchyme and contains a supportive stroma that is easily isolated. Considering the above adipose tissue may represent a source of stem cells that could have far-reaching effects in several fields of research and medicine (Zuk et al. 2002). The many investigations on adipose tissue-derived mezenchymal stem cell populations have shown their potential to differentiate into diverse cell types, such as adipogenic, osteogenic, chondrogenic and myogenic lineages. The transforming growth factor beta (TGFβ) signaling pathway induces adipoze tissue cells to differentiate into smooth muscle-like cells (Jeon et al. 2006).

The multifunctional cytokine superfamily of the TGFβ is a large group of structurally related proteins that are major regulators of normal growth and development in multicellular eukaryotic organisms (Piek et al. 1999; Chang et al. 2002). Almost all cells produce TGFβ and its cell surface receptors, and convey their signals to the intracellular mediators, the Smads (Shi, Massague 2003). Several growth factors belonging to the TGFβ superfamily have the potential to regulate cell proliferation, differentiation and migration and their interaction with extracellular matrix molecules (Roberts et al. 1992; Shi, Massague 2003). The TGFβ isoforms 1, 2 and 3 each have been proposed to have specific, non-overlapping roles in development of eykariotic organisms (Dickson et al. 1993; Kaartinen et al. 1995; Proetzel et al. 1995).

TGFβ1 performs many cellular functions, including control of cell growth, cell proliferation, cell differentiation and apoptosis. There is evidence that TGFβ1 is involved in wound healing processes (Assoian et al. 1983). TGFβ1 is produced by every leukocyte lineage, including lymphocytes, macrophages, and dendritic cells, and its expression serves in both autocrine and paracrine modes to control the differentiation, proliferation, and state of activation of these immune cells (Letterion, Roberts 1998).

In animal models TGFβ3 regulates molecules involved in cellular adhesion and extracellular matrix (ECM) formation during the process of palate development as well as controls wound healing by regulating the movements of epidermal and dermal cells in injured skin (Taya et al. 1999; Bandyopadhyay et al. 2006).

TGFβ2 is the isoform most strongly linked to heart morphogenesis (Yamagishi et al. 1999), supported by the involvement of TGFβ2 signaling in epithelial-mezenchymal cell transformation during avian and mouse heart development (Mercado-Pimentel, Runyan 2007). It has been reported that TGFβ2 also plays a role in normal hematopoiesis (Majka et al. 2001) and is expressed in multipotent neural progenitor cells (Klassen et al. 2003). Recent work (Im et al. 2003; Wang et al. 2003; Lou et al. 2007) has shown the role of TGFβ2 in neocartilage formation from predifferentiated human adipose-derived stem cells in vivo, as well as in human bone marrow-derived mezenchymal progenitor cells. Therefore, TGFβ2 is one of the key molecules in the cell differentiation, which is an important process in stem cell research field.

Since TGFβ is involved in so many cellular processes, it is important to understand its role and molecular mechanisms within the different cell populations and various growth conditions. In this paper we investigate the effect of different growth conditions on the cell morphology and expression of cellular *TGFβ* within adipose-derived precursor cell (hADPC).

Materials and methods

hADPC isolation and cultivation

Subcutaneous adipose tissue was obtained from elective surgery with patient consent as approved by the Central Ethical committe of Latvia.

Human adipose-derived precursor cells (hADPC) were isolated as previously described (Lee et al. 2004) with minor modification (Hoogdjuin M., personal communication). Briefly, adipose tissue was mechanically disrupted with a scalpel knife and, after two washes with phosphate - buffered saline (PBS), digested with sterile filtered 0.5 mg ml^{-1} collagenase type XI in DMEM/F12 (3:1, v/v) for 30 min at 37 °C with intermittent shaking. Medium with 10 % fetal bovine serum (FBS) and 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin was then added and the floating adipocytes were separated from the stromal - vascular fraction by centrifugation at 500 × g for 5 min. The cell pellet was resuspended in DMEM:F12 medium with serum as described above, and cells were plated in 25-cm^2 tissue culture flasks (T-25; Sarstedt) and grown 2 - 3 weeks in 37 °C, 5 % CO_2 until 80 % confluence. The passage number of human adipose-derived precursor cells used in these experiments was 5 - 7. Different growth medium were used starting with passage number 3. Cells were counted using a hemacytometer.

Media

Serum medium was based on DMEM/F12 (3:1, v/v) supplemented with 1 % antibiotic mix (penicillin/streptamycin, Invitrogen) and fetal bovine serum (10 % and 30 %) alone, or in some samples, with 5 ng ml^{-1} basic fibroblast growth factor (bFGF; R&D Systems). Cells were grown also in serum medium diluted with serum-free medium (described below) supplemented with leukemia inhibitory factor (LIF) as 1:1 and 3:1, respectively.

Serum-free medium consisted of DMEM/F12 (3:1, v/v) 1 % antibiotic mix supplemented with 40 ng ml^{-1} bFGF, 20 ng ml^{-1} epidermal growth factor (EGF; R&D Systems), 2 % B27 (Invitrogen) and 10 ng ml^{-1} LIF (Millipore/Chemicon).

In vitro *differentiation of hADPC*

For induction of neuronal differentiation, 5×10^4 cells per well were seeded onto four-well chamberslides (Nunc). Cells were cultured in basal neuronal differentiation medium containing 40 ng ml^{-1} bFGF, 20 ng ml^{-1} EGF and 2 % B27. To induce oligodendritic and astrocyte differentiation, 48 h after initial plating, cell culture medium was replaced with basal medium supplemented with neural differentiation supplement (Human Neural Stem cell Functional Identification Kit, R&D Systems). Cells were fixed for immunohistochemical analysis of nestin expression 48 h after initial plating; immunohistochemical analysis of tubulin βIII, O4 and GFAP expression was performed after seven-day incubation in the neural differentiation medium.

Immunocytochemical analysis

Neural lineage markers were determined using monoclonal antibodies against human neural lineage markers nestin, GFAP, O4, tubulin βIII (all included in Human Neural Stem Cell Functional Identification Kit, R&D Systems) following manufacturer instructions. Cells, when confluence of 70 % was reached, were fixed in 4 % paraformaldehyde in DMEM for 20 min at room temperature. Monoclonal antibodies were applied to specimens and

incubated overnight at 4 °C.

Following incubation with primary antibodies, horse-readish peroxidase-conjugated secondary reagent and diaminobenzidine substrate was applied to all specimens according to manufacturer instructions (DakoCytomation LSAB+ System-HRP). Cells were counterstained with Mayer's hematoxylin (Lilie's modification, DakoCytomation), mounted (DakoCytomation) and examined under microscope (Leica DMI4000 B). Images were made using Image-Pro® Express software.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted by the TRIzol method (Sigma) and the concentrations were obtained using a Nanodrop spectrophotometer. cDNA synthesis was performed as described previously (Lemos et al. 2003). Total RNA was denatured for 5 min at 80 °C and then chilled on ice. The reaction took place at 42 °C for 90 min in a volume of 50 ml. The reaction mixture contained 10 ml of 5 × first-strand buffer (Life Technologies-BRL), 25 pmol of each dNTP (Amersham Pharmacia Biotech Benelux), 0.25 mg of random primers (Promega), 500 U of M-MLV reverse transcriptase (Life Technologies-BRL), 20 U of RNAse inhibitor (Promega), and 0.5 nM of dithiothreitol (Life Technologies-BRL). After the cDNA reaction, the M-MLV reverse transcriptase was inactivated by a 5 min incubation at 95 °C.

Real-time quantitative PCR

Real-time PCR analysis was conducted as previously described (Lemos et al. 2003). The mRNA levels of TGFβ and the housekeeping gene GAPDH were quantified in the hADPC using real-time PCR in the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Predeveloped TaqMan assays (Applied Biosystems) were used for *TGFβ* and *GAPDH*. Amounts of primers and probes of the predeveloped kits were added according to manufacturer instructions. PCR conditions were incubation for 2 min at 50 °C, incubation for 10 min at 95 °C, followed by 40 cycles of 15 sec denaturation at 95 °C, 1 min annealing and extension at optimal temperature 60 °C.

Relative quantification was made against the expression levels of *GAPDH* used as a housekeeping gene. Copy numbers were calculated for 20 ng of total RNA for each sample.

Results

hADPC isolation and viability

Isolated cells from subcutaneous adipose tissue were transferred to culture flasks. After three to 10 days, cells with fibroblast morphology appeared. Within a week it was observed that the number of cells rapidly increased in serum containing medium. Isolated adipose-derived precursor cells were cultured in defined FBS-containing medium. The medium was first changed after one week, when together with the medium dead cells were washed away from the cultures. Over the first two weeks most cells had adhered to the tissue flasks and a significant amount of dead cells was no longer noticed. Approximately 35 0000 - 80 0000 viable cells per T-25 flask were observed when 80 % confluence was reached. The estimated cell doubling time was seven to 10 days according to cell counts under the microscope. Cells were grown continuously in culture for six months and passaged more

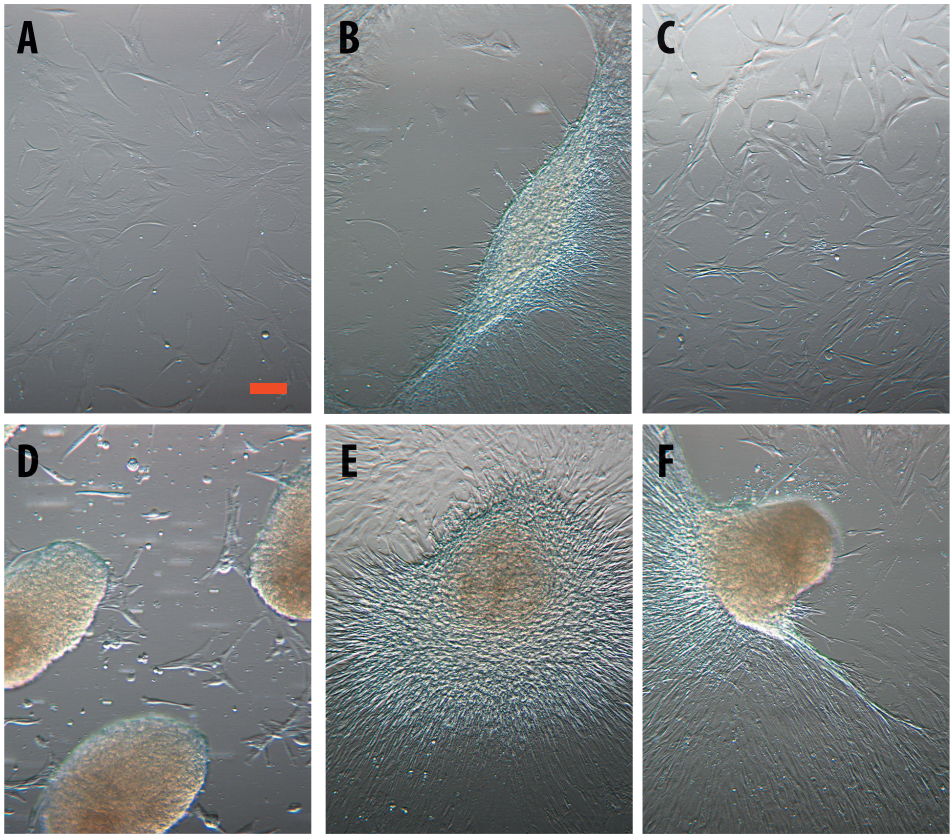

Fig. 1. Morphology of adipose derived precursor cells used for TGFß expression analysis. A, 10 % FBS; B, 30 % FBS; C, 10 % FBS + FGF; D, LIF spheres; E, 10 % FBS + LIF (1:1); F, 10 % FBS + LIF (3:1). The bar represents 100 μM.

than seven times. Comparison of subcutaneous adipose tissue cells to those from heart, spleen, and cultured bone marrow-derived MSCs obtained from Dr. Martin Hoogdjuin (Erasmus Medical Center, Rotterdam, Netherlands) showed similar morphology.

Visual examination of adipose-derived cell cultures revealed significant differences in dependence of medium used for cultivation (Fig. 1). In serum medium with different concentrations of FBS cells had fibroblast-like morphology (Fig. 1 A - C). In serum-free medium supplemented with LIF cells resembled floating spheres (Fig. 1 D), and in serum media diluted with serum-free media supplemented with LIF in different dilutions (1:1 and 3:1, Fig. 1 E - F) – rounded cell bodies and dendritic-like sprouts with some floating spheres, respectively. In some cases cells tended to overgrow themselves forming clumps as in the case of 30 % FBS. Cells forming spheres observed in serum-free medium culture supplemented with LIF grew much slower than cells in serum medium. Nevertheless, when cell spheres from serum-free media supplemented with LIF were seeded back in serum medium (even when diluted with serum-free media supplemented with LIF), they tended to attach to the surface and cells from the spheres grew forming a monolayer (Fig. 1 E - F), suggesting that serum-free media supplemented with LIF could serve as stem

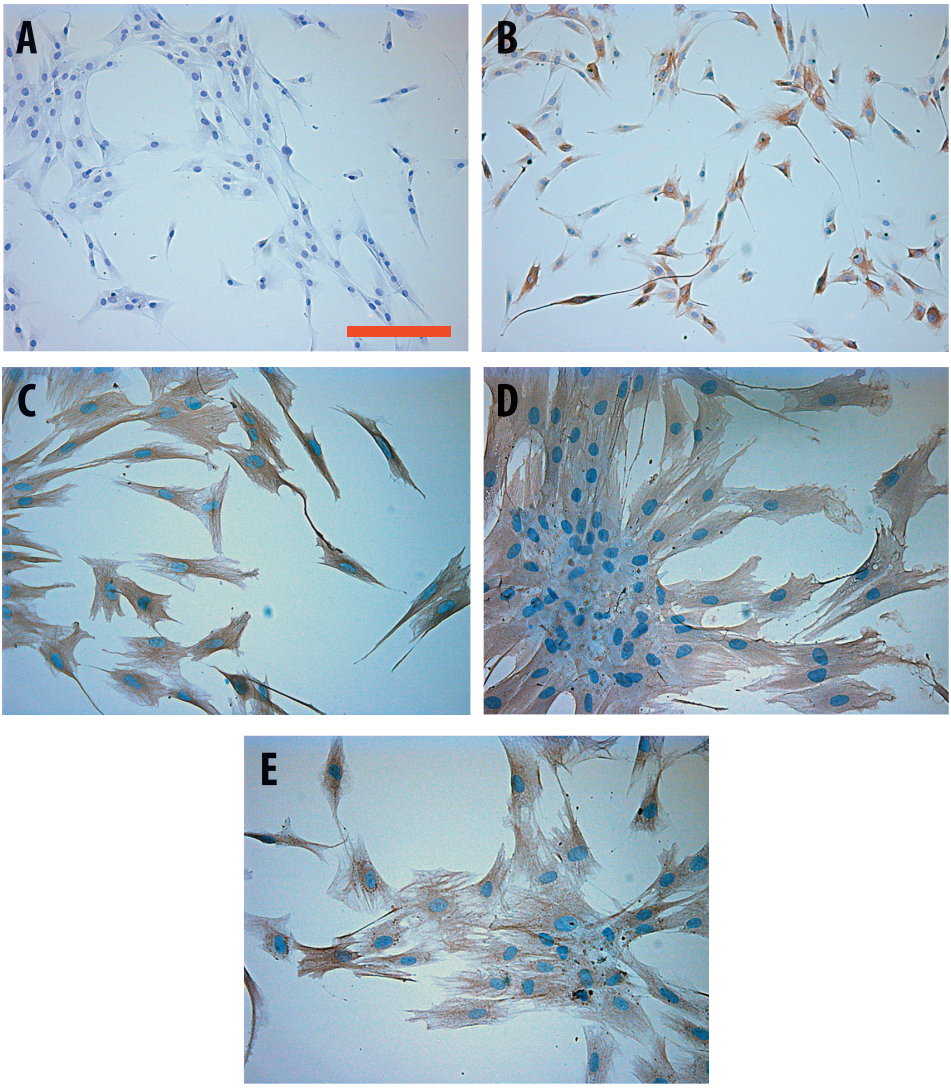

Fig. 2. *In vitro* differentiation of adipose-derived precursor cells. The following markers were used for immunocytochemical staining: control (A), nestin (B), βII tubulin (C), GFAP (D), O4 (E). The bar represents 100 μM.

cell culture-maintenance medium. To investigate whether the isolated cells had stem cell characteristics, their ability to differentitate into mezenchymal and other cell types were examined by *in vitro* diferentiation to neuronal lineage cells. The expression levels of *TGFβ* were examined by real-time PCR analysis.

In vitro *differentiation potential hADPC*

Human adipose-derived precursor cells tested for nestin, a neuronal precursor marker, expressed strongly positive phenotype after 48 h growth (Fig. 2).

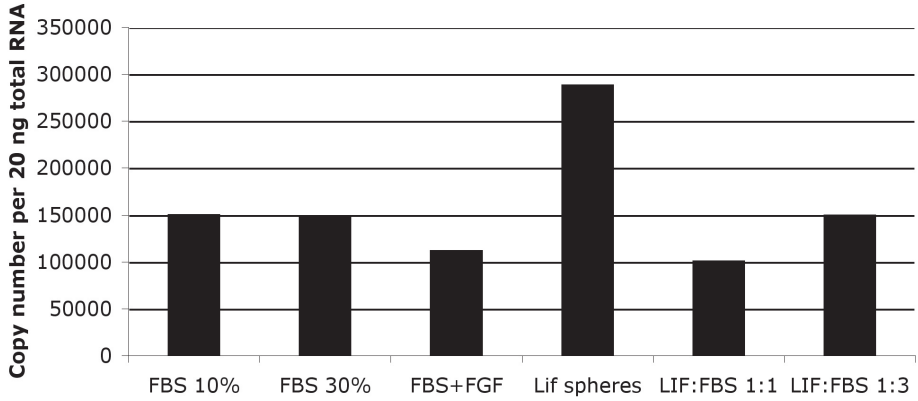

Fig. 3. Expression level of intracellular *TGFβ* in adipose-derived precursor cell line cultivated under different medium.

Nestin staining was observed in approximately 70 % of the cells in an observed area. Following seven-day differentiation into neural differentiation medium, all adipose-derived precursor cell samples tested were positive for neuronal marker tubulin βIII astrocyte marker GFAP and oligodendrocyte marker O4 (Fig. 2). We suspect that under these culture conditions adipose-derived precursor cells probably differentiate to a higher extent towards a neuronal precursor phenotype. Simultaneous expression of nestin, tubulin βIII, GFAP and O4 would suggest an immature neuronal precursor phenotype of these cells.

Real-time quantitative PCR analysis of the expression of TGFβ

Real-time PCR analysis was conducted to determine the level of expression of intracellular *TGFβ*. The expression levels were synchronized to the houskeeping gene *GAPDH* expression level for each sample. Since the cell count was not made before harvesting cells for the RNA isolation, real-time-PCR results were calculated as copy number per 20 ng of total RNA (Fig. 3).

Interestingly, the expression level of the *TGFβ* was highest in serum-free media supplemented with LIF cultivated cells, but did not change in serum medium with different FBS concentrations and in serum medium diluted with serum-free media supplemented with LIF 3:1. In cells cultured in serum-free media supplemented with LIF 1:1, the level of *TGFβ* was significantly lower than in cells cultured in FBS medium containing FGF, suggesting that perhaps FGF could have a negative regulatory effect on the gene expression of *TGFβ*.

Discussion

In this work we showed that some populations of cells isolated from subcutaneous fat can grow *in vitro* for many passages. Under different media conditions they develop specific morphology as in the case of serum and serum-free media. They can not only grow in culture for longer periods but they also can differentiate into cell types other than mezenchymal cells, such as into neuronal cells, which is consistent with the literature (Kokai

et al. 2005; Jeon et al. 2006), indicating that isolated hADPC have features characteristic to stem cells.

In this work we showed that some of the hADPC cultivated in various medium not only changed their phenotypical appearance, but also differed in gene expression level, as in the case of *TGFβ* and other genes (*GAPDH*, *CXCL12* and *CXCR4*; Cakstina et al., unpublished data) in this cell line. Real-time PCR analysis of *TGFβ* also showed that there was no difference between 10 % FBS of 30 % FBS cell cultivation in terms of *TGFβ* (and also *GAPDH*) expression levels. However, *TGFβ* expression in cells cultivated in serum-free medium supplemented with LIF, in which the cells had a tendency to form neurospheres, was significantly higher than in cells grown in serum containing media (Fig. 3). We suggest that FBS may contain some factors that negatively regulate the expression of *TGFβ*, particularly in the case when endogenous FGF is added to the serum medium. There is some evidence in the literature of interaction between *TGFβ* and FGF, suggesting a negative effect of FGF on *TGFβ* expression in quail heart tubulogenesis (Holifield et al. 2004).

Nevertheless, more research is needed in order to characterize isolated cells and to study the role of exogenous TGFβ in hADPC and other putative stem cell lines isolated from different tissue. Therefore, we first needed to examine the level of endogenous TGFβ and the influence of various media on the expression of *TGFβ*. The global aim of the project is to investigate the role and possible use of TGFβ and other factors in controled differentiation towards other important cell types for which the adipoze-derived precursor cells hold a great potential (Roche et al. 2007). Further research is aimed towards characterization of other cell lines isolated from various tissue samples by using not only FACS but also looking at different important signaling pathway and cell cycle regulatory gene expression patterns, such as *TGFβ*.

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Dažādās vidēs kultivētu no taukaudiem iegūto priekšteču šūnu izolēšana, raksturošana un *TGFβ* gēna ekspresijas analīze

Inese Čakstiņa[1,3]*, Una Riekstiņa[2,3], Ruta Muceniece[2,3], Jānis Ancāns[1,3]

[1]Bioloģijas fakultāte, Latvijas Universitāte, Kronvalda bulv. 4, Rīga LV-1586, Latvija
[2]Medicīnas fakultāte, Latvijas Universitāte, Šarlotes 1a, Rīga LV-1001, Latvija
[3]Bioanalītisko metožu laboratorija, Latvijas Universitāte, Rātsupītes 1, Rīga LV-1001, Latvija
*Korespondējošais autors, E-pasts: inese.cakstina@lu.lv

Kopsavilkums

Pieauguša cilvēka, jeb autologās, cilmes šūnas, kas ir spējīgas pašatjaunoties un diferenciēties dažādos šūnu tipos, ir perspektīvs izejmateriāls šūnu terapijā, jo tās var izolēt no visdažādākajiem pieauguša cilvēka audiem, kā arī to pētīšana un turpmāka izmantošana terapijā (piem., mazāks atgrūšanas risks) saskaras ar ievērojami mazāk ētikas dabas jautājumiem nekā darbs ar embrionālajām cilmes šūnām. Mūsu ķermenī ir daudz taukaudi, kas satur vairākas mezenhimālo cilmes šūnu populācijas. Pēc literatūras datiem, transformējošais augšanas faktors beta (TGFβ) ir viena no daudzajā molekulām, kas saistīta ar mezenhimālo cilmes šūnu diferenciāciju. Šī darba mērķis ir izpētīt no cilvēka taukaudiem izdalīto mazdiferenciēto aizmetņa šūnu spēju diferenciēties citos šūnu tipos, kā arī pētīt šūnu kultivēšanas vides ietekmi uz šūnu morfoloģijas izmaiņām un *TGFβ* gēna ekspresiju. Darbā iegūtie dati parāda, ka, kultivējot no taukaudiem izdalītās šūnas serumu saturošā un bezseruma vidē, šūnu morfoloģija, kā arī *TGFβ* ekspresijas līmenis ievērojami izmainās. Pētījuma gaitā veikti eksperimenti attaino iegūto šūnu populāciju spēju diferenciēties ne tikai mezenhimālās līnijas šūnās, bet arī neironālās līnijas šūnās, kas norāda uz šo populāciju diferenciācijas plastiskumu. Mēs varam secināt, ka no cilvēka taukaudiem izdalīto šūnu populācijas satur noteiktu daudzumu šūnu ar cilmes šūnām raksturīgām īpašībām – spēju diferenciēties dažādos šūnu tipos. Šūnu kultivēšanas videi ir liela nozīme populācijas augšanā un morfoloģijas izmaiņās – serumu saturošā vidē šūnas aug ātrāk un veido monoslāni, savukārt bez seruma vidē šūnām ir tendence veidot sfēras un vairoties daudz lēnāk. *TGFβ* ekspresija ir augstāka tieši šūnās, kas vairojas lenām un ir kultivētas bez seruma vidē.