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Scale-out cultivation of human dermal fibroblasts using robotic cell culture system: comparison of manual and automated processing

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Summary

Automation of cell cultivation process allows solving the problems of standardization, reproducibility and cost effectiveness of the large scale manufacturing of cell-based therapy products. In this study we established the protocol for automated (employing CompacT SelecT cell culture platform) *in vitro* expansion of human dermal fibroblasts (here forth, HDFs). We have conducted a series of assays aimed to compare cell morphology, proliferation, viability and secretory activity of human HDFs under manual and automated cell cultivation strategies. Automation of cell cultivation did not have negative impact on HDFs proliferation and provided the higher uniformity of cell yield. Our data indicate that CompacT SelecT is suitable for scale-out HDFs manufacturing and has the potential to be used for various applications, including fibroblast-based regeneration therapies.

Keywords

Cell culture, automated system, dermal fibroblasts, cell proliferation, scale-out manufacturing.

Introduction

Cell therapy is becoming an essential branch of contemporary medicine. Clinical implementation of cell therapy faces the need to solve the challenges of increasing scale of cell manufacturing. The large-scale cultivation of cells may require the use of scale up systems, such as microcarriers-based bioreactor systems, multi-layer bioreactors, hollow-fiber bioreactors. Such approaches are cost effective for the production of a minimum a billion cells *per* lot and hence are the most suitable for the cell cultures applied in allogenic cell therapy [1].

Human dermal fibroblasts (HDFs) of allogenic or autologous origin have a wide range of applications, especially in regenerative medicine. Autologous fibroblasts are clinically used for the correction of age-related skin changes [2, 3], the treatment of acne scars [4] or traumatic scars [5]. The individual dose of fibroblasts usually does not exceed 6×10^7 cells *per* injection session and 12×10^7 cells *per* treatment course. Therefore, the employment of scale up cell cultivation systems for autologous cell therapy is impractical. Considering the requirement for separate manufacturing of autologous fibroblasts for each patient, the scale-out of operations using standard T-flasks is the most preferred method, since this method allows parallel manufacturing of several small batches of adherent cells.

However, the scale-out production of autologous cells using culture flasks is labor intensive, costly, time-consuming, increases the risk of microbial contamination, and, moreover, often fails to reproduce cell cultivation processes. These drawbacks could be minimized or even completely eliminated by using automated cell culture platforms operating with T-flasks.

CompacT SelecT CellBase automated cell culture system (here forth referred to as CompacT SelecT, TAP Biosystems/

Sartorius, Royston, UK) is used to automatically cultivate and maintain adherent cell lines using polystyrene cell culture flasks. It consists of two main parts: CO_2-incubator chamber containing a carousel to handle cell culture flasks and the laminar flow chamber with robotic manipulator equipped with a gripper, performing the work steps of a human operator (Fig. 1). Liquids, such as growth media, can be added *via* peristaltic pumps, while in order to transfer liquids from one flask to another one, CompacT SelecT uses 10 mL disposable serological pipettes. The counting of viable cell number is performed by the integrated Beckman Coulter Vi-CELL XR Cell Viability Analyzer in the semi-autonomous mode.

CompacT SelecT has been successfully employed to cultivate mesenchymal stem cells (MSCs) [6,7], induced pluripotent stem cells (iPSCs) and their derivatives [8,9], embryonic stem cells (ESCs) [10], human Caucasian osteosarcoma cells (HOS) [11], as well as transfected cell lines [12]. To our knowledge, however, cultivation of HDFs on CompacT SelecT platform has not been reported previously.

Our study aimed (i) to test the automated process of long-term cultivation of autologous HDFs using the CompacT SelecT platform, and (ii) to compare morphology, proliferative activity, viability and secretory activity of HDFs cultured under manual and automatic modes.

Materials and methods

HDFs culture

The HDFs lines were isolated from skin samples (area ≈0,5 cm^2), which were surgically excised from 6 donors (age 25-35) during surgery. Prior to the surgery patients provided a written informed consent. The study was approved by the Ethics Committee of the Institute of Medical Cell Technologies (Ekaterinburg, Russian Federation) (approval No.5-16). Primary HDFs cultures were obtained *via* tissue enzymatic dissociation method. Briefly, dermal biopsy sample was cut into small pieces (about 1 mm in all three dimensions) and incubated in 3 mL of collagenase I solution (1000 U/mL, Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 2.5 hours. Following enzyme inhibition by mixing with 4-fold volume of growth medium, cells were dissociated by vigorous shaking. Cell suspension was centrifuged at 270 g for 10 minutes, the pellet was resuspended in complete growth medium and transferred to T25 culture flask (Nunc, Roskilde, Denmark). The growth medium consisted of the mixture of Advanced DMEM and F-12 (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) in the ratio 1:1, supplemented with 12% fetal calf serum (Biosera, Nuaille, France), 0.03% Glutamine (Gibco, USA) and gentamycin (50 μg/mL). Fibroblasts were subcultured when the monolayer reached 70-80% confluency.

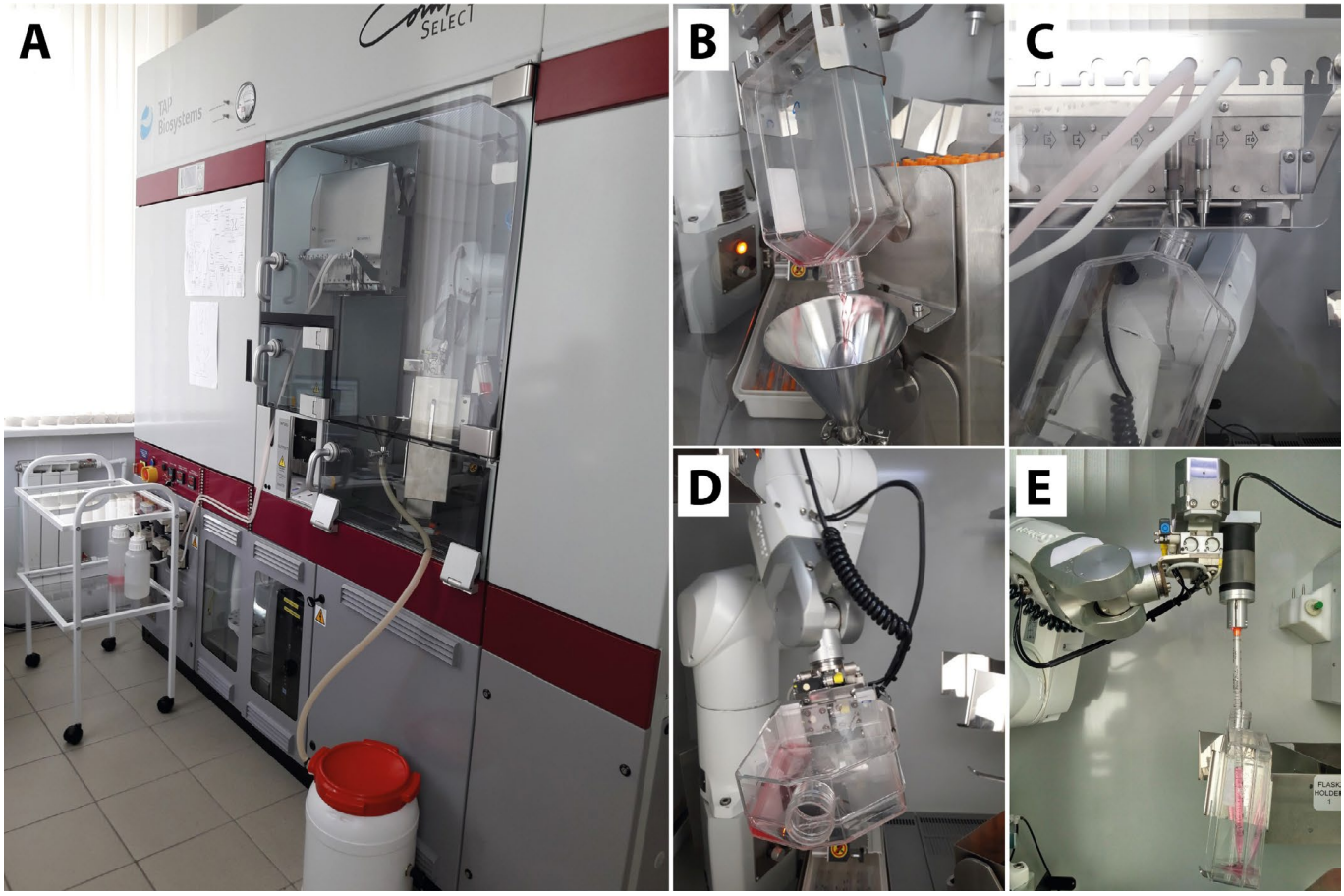

Figure 1. CompacT SelecT automated cell culture system (A) and its key functions: (B) spent medium is poured to waste funnel; (C) media, trypsin or other process liquids can be precisely dispensed by peristaltic pumps from nozzles; (D) flasks are swirled to distribute enzyme or rinse the cell monolayer; (E) the pipette is used to transfer cells from flask

For long-term preservation, cells were stored in liquid nitrogen. Thawed cells were passaged at least twice prior to their usage. In this study, the cells from passages 4-7 were used.

Manual HDFs subculture protocol

The protocol for manual subculturing of HDFs in T175 culture flask consisted of the following steps: (i) growth medium from flask was aspirated, cells were washed with 15 mL of DPBS without Ca^{2+}, Mg^{2+} (Biolot, Russia); (ii) cells were incubated with 5 mL of 0.25% trypsin/EDTA (Gibco, USA) at 37°C, 5% CO_2 for 8 min., cell detachment was controlled visually; (iii) trypsin was neutralized with 10 mL of growth medium; (iv) cell number was counted using the ViCell XR cell counter, integrated into CompacT SelecT; (v) cell suspension containing 525,000 cells was transferred into a new culture flask (seeding density 3000 cells/cm^2); (vi) growth medium was added to the new flask to reach the final volume of 30 mL. Cells were incubated in CO_2-incubator (Sanyo/Panasonic, Moriguchi, Osaka, Japan) at 37°C, 5% CO_2.

Automated HDFs subculture protocol

The sequence of CompacT SelecT automated protocol operations is presented in Fig. 2.

Secretory products assay

Spent medium samples were collected from flasks before subculturing, centrifuged at 6800g for 10 min and stored at -20°C until further analysis. Quantitative ELISA assay was used to evaluate the levels of IL-6 (Interleukin-6-ELISA-BEST №A8768, Vector-Best, Novosibirsk, Russia), IL-8/CXCL8 (Interleukin8-ELISA-BEST №A8762, Vector-Best, Russia) and α1 chain of procollagen I (Human Pro-Collagen I alpha 1 DuoSet ELISA №DY6220-05, R&D Systems, Minneapolis, MN, USA). The assays were performed according to the manufacturer's protocols.

HDFs adhesion and growth in T175 flasks from different manufacturers

The operations were performed in CompacT SelecT using a modified protocol for automated subculturing. For cell adhesion assay, HDFs were seeded at a density of 8000 cells/cm^2 into the flasks and incubated in CompacT SelecT CO_2-incubator (37°C, 5% CO_2) for 18 hours; adherent cells were detached with trypsin and counted automatically by using the shortened version of protocol (Fig. 2) without steps of seeding cells to new flask. To perform proliferation assay, the seeding density was 1500 cells/cm^2, incubation in CompacT SelecT lasted 6 days with the single medium change on day 3. After 6 days fibroblasts were automatically detached and counted in the same way.

Comparative analysis between manual and automated HDFs cultivation

HDFs after thawing were seeded into T175 Nunc culture flasks. After reaching 80% confluency of monolayer, cells from all flasks were harvested and seeded into new T175 Nunc flasks, according to scheme in Fig. 3, with the seeding density 3000 cells/cm^2. Six flasks were loaded into CompacT SelecT for automated cultivation; the other 6 flasks were placed in CO_2-incubator for manual cultivation. Cells were subcultured after 7 days of growth with the single growth medium change on day 4. During subculturing HDFs were seeded from mother flask to daughter flask without mixing cells from different flasks for both manual and automated protocol. HDFs were grown for 4 consecutive passages. Seeding density was always 3000 cells/cm^2. All operations with manually and automatically processed flasks were synchronized (Fig. 3).

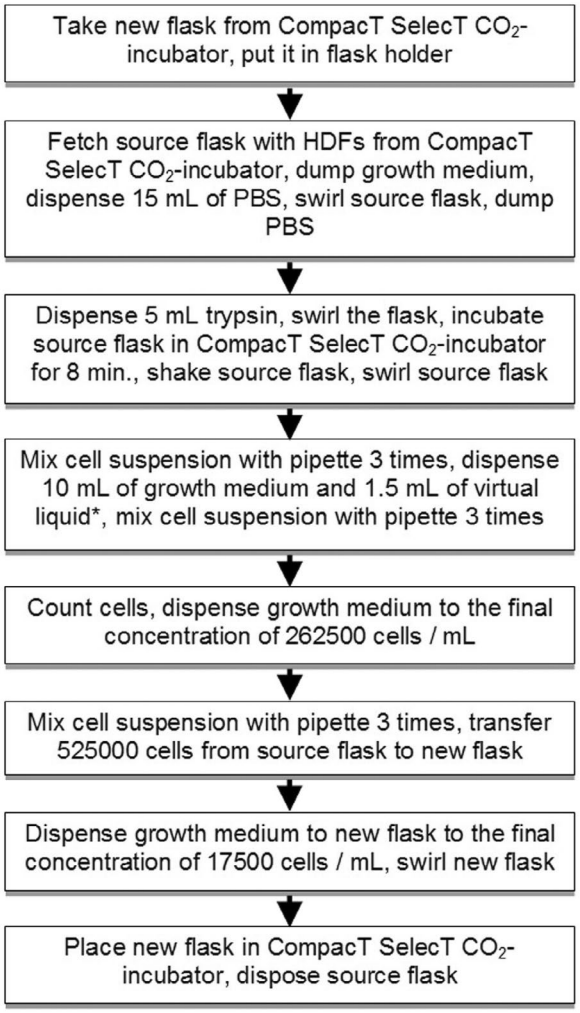

Figure 2. The process flow diagram of automated cell subculturing protocol for CompacT SelecT

*After dumping the flask contains residual 1.5 mL of liquid, which is not taken into account by machine software. Dispensing 1.5 mL of virtual (non-existing) liquid by empty pump corrects the liquid volume calculations by software.

Statistics

All quantitative values are presented as X±m, where m is the standard error of the mean X. The relative standard deviation (RSD) was calculated as a ratio of standard deviation of the value to its mean X. The statistical significance of differences between 2 groups was analyzed by non-parametric Mann-Whitney U-test, for pairwise comparison of 3 groups the non-parametric variant of Newman-Keuls test was used. Statistical analysis was performed using MS Excel 2010 and STATISTICA 6.0. The level of statistical significance was set at $p<0.05$. All experiments were performed in six replicates.

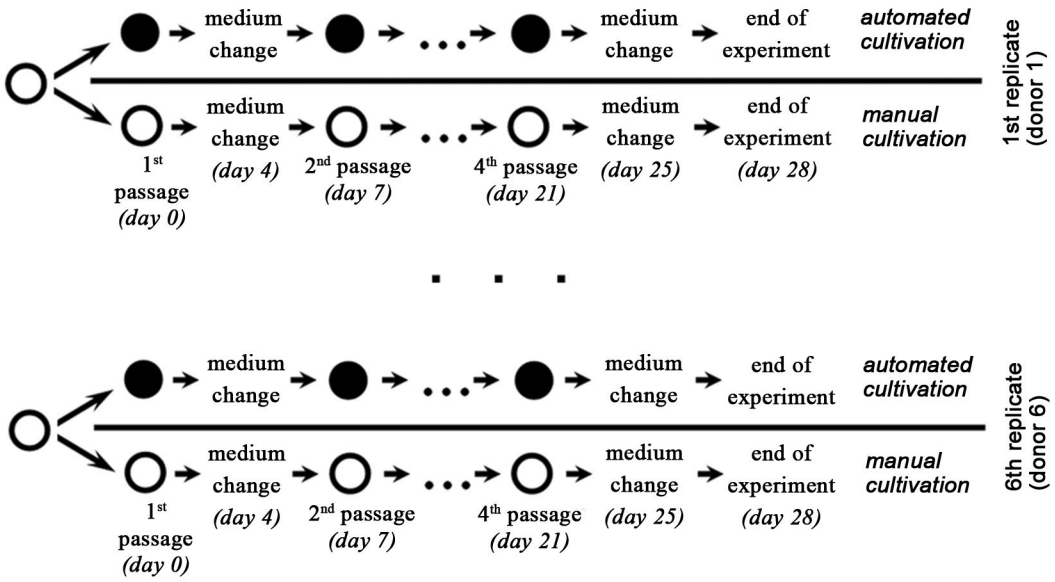

Figure 3. Schematic representation of automated (filled circles) and manual (empty circles) cultivation of human dermal fibroblasts

Results

Selection of T175 cell culture flasks for HDFs automated cultivation

In the first series of experiments the culture flasks which are the most suitable for the automated HDFs cultivation were selected. CompacT SelecT software allows to use T175 flasks from three manufacturers: (i) Nunc (Denmark), № 178983; (ii) Corning (USA), № 431306; and (iii) Becton Dickinson (BD) (USA), № 353118. The percentage of adhered HDFs and their proliferation on these types of flasks were measured using CompacT SelecT platform.

The variations in the percentage of adhered cells between all types of flasks were not statistically significant ($p>0.05$) (Table 1). At the same time, HDFs, grown in Corning flasks, showed the lowest ratio of monolayer density / seeding density, while the differences between BD and Nunc flasks were not statistically significant ($p>0.05$). Hence, BD and Nunc T175 flasks proved to be the most suitable for HDFs manufacturing in CompacT SelecT. To conduct a comparative analysis of manual and automated HDFs cultivation, we have used Nunc flasks.

Automated and manual cultivation: cell morphology and proliferation

In the next series of experiments, we compared morphology, proliferation and secretory activity of HDFs, cultivated in T175 Nunc culture flasks manually and automatically in CompacT SelecT. The manual and automated protocols contained similar sets of operations. CompacT SelecT is not designed to use the centrifuge. For this reason, in both protocols following cell detachment, trypsin was neutralized by the dilution with growth medium.

The cells grown manually and automatically had the typical fibroblast-like shape without any differences in their shape (Fig. 4). Besides, in both cases cells were distributed on culture plastic surface rather evenly, without the formation of large aggregates. This indicates that the protocol for automated cell cultivation provides the even mixing of cells in suspension after their detachment from plastic surface by trypsin.

The comparison of HDF sizes (cell diameters in suspension) also did not reveal significant differences between manual and automated cell cultivation at all four passages (Fig. 5).

Table 1. HDFs adhesion and proliferation on three types of T175 culture flasks (Nunc, BD, Corning), appropriate for CompacT SelecT

Flask type	Cell adhesion		Cell proliferation	
	Percentage of adherent cells, %	Statistical significance	Ratio monolayer density/ seeding density	Statistical significance
Nunc	64.2 ± 2.0	-Corning $p>0.05$	15.9 ± 0.7	-Corning $p<0.05$
BD	66.5 ± 4.9	-Nunc $p>0.05$	18.2 ± 0.7	-Nunc $p>0.05$
Corning	71.5 ± 5.8	-BD $p>0.05$	12.0 ± 0.8	-BD $p<0.05$

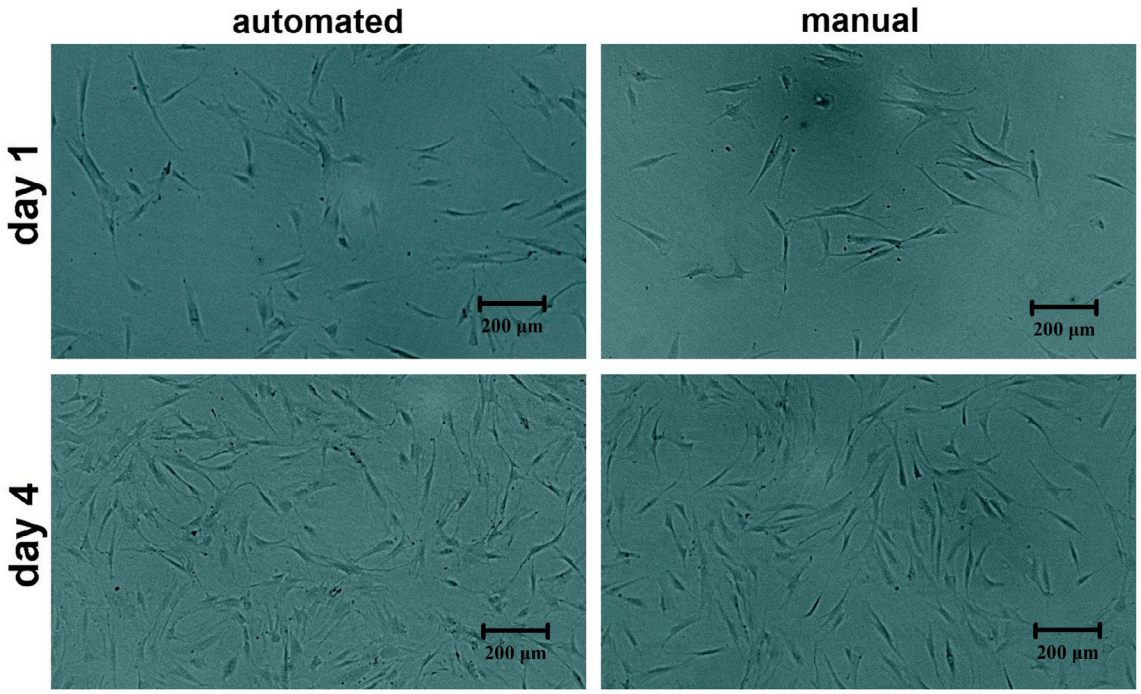

Figure 4. HDFs shape at automatic and manual cultivation modes, passage 2

The cell viability was 97% or higher at all the passages. At the 2nd and 3rd passages the viability of automatically cultivated cells was statistically higher than that of manually grown cells ($p<0.05$), however, that difference was less than 1% and hence can be considered negligible (Fig. 5).

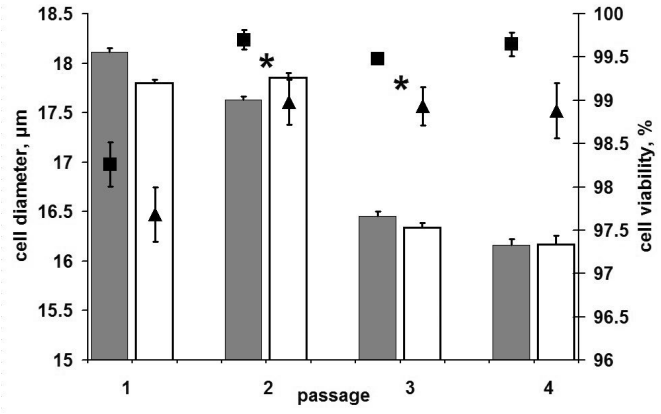

Figure 5. Average diameter and viability of HDFs cultured automatically and manually

Cell diameter is represented by grey bars (automated cultivation) and white bars (manual cultivation). Cell viability is shown by squares (automated cultivation) and triangles (manual cultivation). Statistically significant differences of cell viability between manual and automated cultivation modes are indicated by asterisks ($0.01<p<0.05$). Differences in cell diameter were not statistically significant ($p>0.05$).

The HDF monolayer densities are presented in Fig. 6. The cell yields demonstrated the tendency of decreasing from the 1st to the last passage, which is obviously due to the aging of culture. At all of the four passages the average quantity of automatically grown cells was 1.25-1.5 times higher than of that of cells grown manually ($p<0.01$).

The technical flask-to-flask variations of cell diameter and cell number for both cell processing techniques are presented in Fig. 7. The RSD in cell density fell in the range from 0.047 to 0.130 for automated culturing and from 0.097 to 0.185 for manual culturing; the RSD in cell diameter ranged from 0.0019 to 0.0038 and from 0.0020 to 0.0053 for automated and manual culturing, respectively. At all the passages, the RSDs in cell diameter and cell number for automated protocol were lower in comparison with the data for manual protocol.

Automated and manual cultivation: cell secretion assay

The paracrine function of HDFs was estimated by the quantity of secreted interleukin-6 (IL-6) and interleukin-8 (IL-8/CXCL8) *per* 10^6 cells. The concentration of IL-8 was 1.5-3 times higher for manual cultivation in comparison to the automated mode at all passages. The quantity of IL-6 was also higher for the manual cell cultivation albeit the differences observed were less significant (maximum 1.8 times) (Table 2).

The pro-collagen Iα assay was used to estimate the synthesis of extracellular matrix (ECM) components by HDFs. As with cytokines, the quantity of pro-collagen secreted by manually grown cells was higher than that recorded in the case with automatically cultivated cells. The differences between the two modes were statistically significant at 1, 3 and 4 passages (Table 2).

Discussion

The procedure of obtaining fibroblasts for autologous therapy consists of 3 main steps: (i) isolation of primary fibroblasts culture, (ii) expansion of cells and (iii) preparation of cells for clinical usage and / or cryopreservation of cell yield.

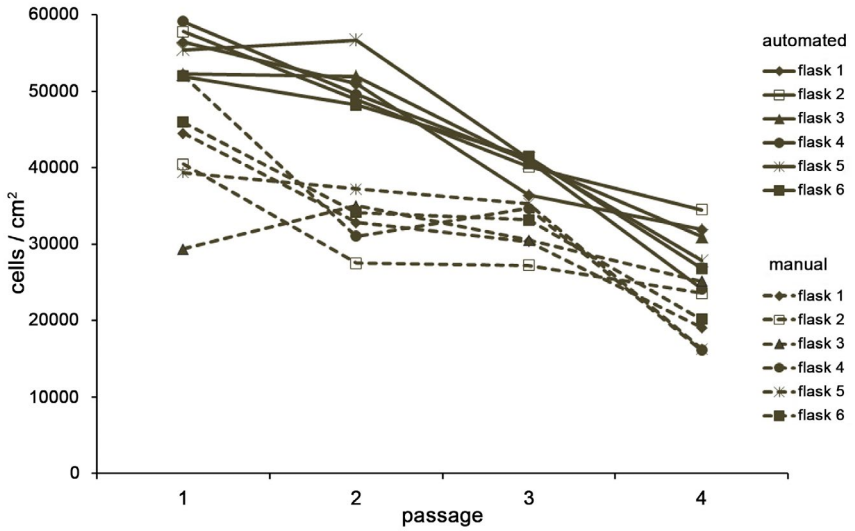

Figure 6. HDF densities in flasks during four passages: automated and manual procedures are shown by solid and dotted lines, respectively. Differences between automated and manual cultivation were statistically significant at all four passages (p<0.01)

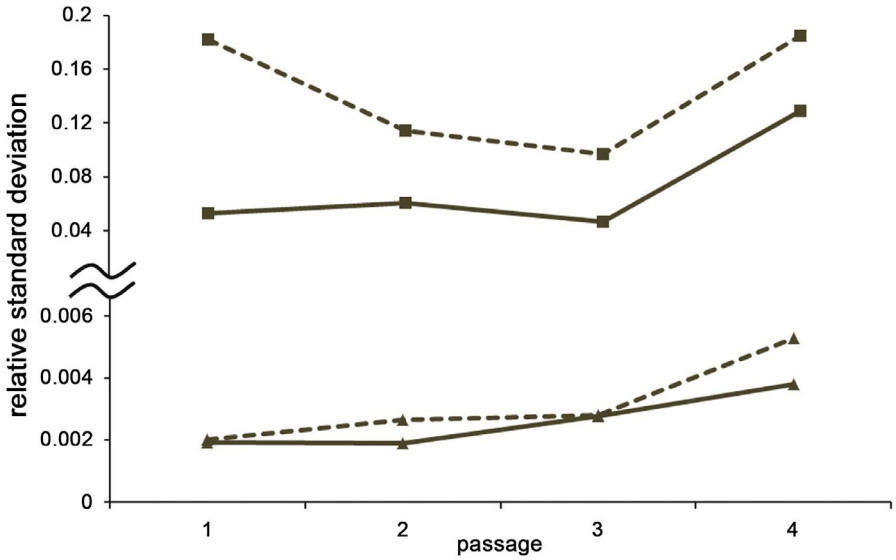

Figure 7. The RSD in cell diameter (lower lines with triangle markers) and in cell monolayer density (upper lines with square markers) of HDFs grown automatically (solid lines) and manually (dotted lines)

Table 2. Secretion of cytokines (IL-6, IL-8) and pro-collagen I α-chain by HDFs. Statistically significant (p<0.05) differences between manual and automatic cultivation are indicated by bold underlined font

passage	IL-6, pg/10^6 cells		IL-8 / CXCL8, pg/10^6 cells		pro-collagen-Iα, ng/10^6 cells	
	manual	automated	manual	automated	manual	automated
1	201.9 ±22.3	192.6 ±34.5	**578.2 ±62.3**	**389.4 ±13.8**	**1010.5 ±58.7**	**678.3 ±52.1**
2	1486.1 ±136.7	1194.7 ±41.3	**787.3 ±63.4**	**475.9 ±23.9**	998.5 ±62.6	992.4 ±61.7
3	2849.6 ±160.7	2789.1 ±223.4	**946.0 ±110.5**	**570.7 ±43.6**	**1282.8 ±73.1**	**949.3 ±73.5**
4	**4504.3 ±458.0**	**2516.0 ±334.6**	**927.0 ±69.9**	**311.1 ±27.3**	**986.4 ±89.9**	**482.4 ±56.3**

The technical characteristics of CompacT SelecT allow its use for the automation of step 2. We developed the protocol to cultivate human skin fibroblasts using CompacT SelecT robotic station and imitated its application for the scale-out cultivation of autologous cells which can be further applied in clinical practice.

Automation of cell cultivation processes allows to standardize and multiply cell cultivation and to overcome the problems of manual operations with cells related to individual features of operator skills and possible missteps occurring during work [13, 14, 15]. Thus, it is expected that automated cultivation would reduce the flask-to-flask variations resulting in more homogenous and standardized cell yield.

In this study, we demonstrated the possibility of long-term automated scale-out manufacturing of HDFs applied in autologous cell therapy using CompacT SelecT. Cells were successfuly subcultured for 4 passages, in the first 3 passages (7-10)×10^6 cells *per* flask were obtained. Thus, manufacturing the batch of 10^8 HDFs for the patient requires harvesting cells from about 15 flasks. Using the seeding density of 3000 cells/cm^2 (applied in this study) allows the production of such HDFs batch after 1-2 passages from a single T175 flask. CompacT SelecT platform is capable of handling up to 90 flasks simultaneously, which is sufficient to process up to 8 batches in parallel (starting batches processing at different time and using more sophisticated types of flasks can increase this number).

We have also compared the manual and automated methods of cell cultivation. The automated protocol repeated the basic steps of manual protocol. With the both modes having been applied, trypsin was neutralized by dilution with serum containing growth media, hence, the differences in the data obtained after studying of two cultivation modes cannot be explained by different ways of trypsin removal. The comparison demonstrated similarity in cell shape, cell size and viability of manually and automatically processed HDFs cultures. However, the cell yield on robotic platform was significantly higher at all passages.

The quantities of cells grown manually and using CompacT SelecT platform were analyzed in other studies. The results of such studies varied: the yield of human MSCs [7] and HOS cells [11] was greater in manual culture, while the human iPSC-derived neuroepithelial-like stem cells [8] had more population doublings when grown automatically. On the other hand, the growth profiles of manual and automated neural stem cells (CTX0E03) cultures were almost identical [16]. These findings are not surprizing. They may be due to significant discrepancies between manual and automated protocols. Firstly, in manual protocols of all these studies, trypsin was discarded by centrifugation, while in automated protocols it was either diluted by growth medium or neutralized and dumped after cells adhered to plastic. The different ways of trypsin neutralization could significantly affect cell proliferation. Secondly, CompacT SelecT robotic arm imitates the work of human operator in laminar flow hood, but the development of protocol for automated cell cultivation is limited by manipulations performed by the machine and by the inability to monitor cells during protocol execution. For example, one of the most significant problems was the high rate of cell aggregation after detachment with trypsin. The aggregates were ineffectively broken by CompacT SelecT pipette, which inevitably affected the cell yield. The same problem was observed in other researches [17]. High cell aggregation rate is most probably due to the prolonged incubation in trypsin during the protocol run, since the inability to control cell detachment requires the increase in trypsinization time. In our protocol, this problem was solved by using the complicated scheme of mixing of trypsinized fibroblasts with growth medium. The quantity of cells obtained in automated platform greatly depends on the design of the protocol and its fitness for cell culture [18, 19]. Hence, the evaluation of advantages/disadvantages of the automated/manual cell cultivation using the data on the cell yield from various studies must be done with caution.

The clinical effect of transplanted fibroblasts is mostly due to the synthesis of ECM components [20, 21] and to the paracrine secretion of growth factors and cytokines affecting the migration and proliferation of patient skin cells [22, 23]. IL-6 is involved in both pro- and antiinflammatory activities, but it also stimulates the proliferation of keratinocytes [24]. Apart from inducing chemotaxis in neutrophils, IL-8/CXCL8 is a pro-angiogenic factor [25]. The concentration of these cytokines tended to grow from the first to the last passage at the both types of HDFs culture techniques used, which was most likely caused by the progressive approaching of cells to senescent phenotype [26, 27]. Though it should be noted that automatically cultured HDFs produced lower amounts of both cytokines and pro-collagen I (*per* 10^6 cells) than cells cultured manually. This may be due to the difference in cell monolayer density in flasks processed manually and automatically: in some researches fibroblasts with higher monolayer density secreted lower levels of cytokines and collagen *per* cell [28, 29, 30].

A higher reproducibility of automated operations in scale-out manufacturing of cells is expected to provide a higher uniformity of cells phenotype and cell yields for different flasks from the batch. We compared the flask-to-flask variation of HDFs cell diameter and cell yield. In all four passages, the RSD in cell monolayer density and RSD in cell diameter were lower in the case of automated process. The RSD data showed that flask-to-flask variations of cell size and cell yield for automated culturing were less apparent, which supports a higher consistency of automated HDFs manufacturing.

Conclusion

The automation of scale-out cultivation of cells for clinical applications is one of significant challenges in regenerative medicine. In this study, we demonstrated a successful long-term cultivation of HDFs in the CompacT SelecT robotic platform. Cells, manufactured automatically, preserved the typical fibroblast-like shape, high proliferation activity and the capability to produce cytokines and pro-collagen. Comparison of manual and automated cultivation confirmed a higher uniformity of cell yield after automated manufacturing.

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Conflicts of interest

The authors declare no conflict of interest.

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Выращивание фибробластов кожи человека с использованием роботизированной системы: сравнение технологий ручного и автоматизированного культивирования

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Резюме

Автоматизация культивирования клеток позволяет решить вопросы стандартизации, воспроизводимости и снижения себестоимости данного технологического процесса при получении клеточной культуры для терапевтического применения. В данной работе был разработан протокол автоматизированного культивирования фибробластов кожи человека с использованием роботизированной станции CompacT SelecT и проведено сравнение морфологии, скорости пролиферации, жизнеспособности и секреторной активности фибробластов, выращиваемых вручную и в автоматическом режиме. Автоматизация процесса культивирования не оказала негативного влияния на скорость пролиферации фибробластов, но при этом обеспечивала большую стабильность, как объемов клеточного урожая, так и морфологических характеристик клеток. Таким образом, применение роботизированной станции позволяет масштабировать производство фибробластов. Технология автоматизированного культивирования клеток может находить применение в различных областях, в том числе, для получения фибробластов, используемых в регенеративной медицине.

Ключевые слова

Культивирование клеток, автоматизированная система, фибробласты кожи, пролиферация клеток, масштабирование производства.