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Optimization of *in vitro* model for analysis of tumor cell migration dynamics

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Migration capacity is an important feature of tumor cells. There are several approaches to analyze the dynamics of cancer cell migration *in vitro*. The model of initiation of cell migration from 3D multicellular spheroids onto growth surface is one of the closest to the in vivo conditions. **Aim.** Optimization of the model to study tumor cell mobility for several days. **Methods.** 2D and 3D MCF-7 cell culture, immunofluorescence analysis and image analysis using the Fiji computer software. **Results.** Unification of spheroid size allowed avoiding a significant data deviation. The obtained spheroids spread completely for three days. The highest migration ratio was observed on the second day. The proliferation level was similar during each day of the three-day experiment; it did not exceed 3 %. The validity of the model was tested after migration inhibition by a mTOR signaling inhibitor rapamycin. Additionally, this model was successfully applied to immunofluorescence study of p85S6K1 subcellular localization in moving MCF-7 cells. **Conclusions.** Double filtration of multicellular spheroids allowed unification of their size; this promotes an adequate interpretation of the migration assay. This model allows to study tumor cell migration dynamics and can be further used for development of anticancer drugs.

Keywords: Cancer cell migration assay, 2D and 3D culture, p85S6K1, multicellular spheroids

Introduction

The ability of malignant tumors to form metastases is a critical step of the cancer progression and distinguishes malignant tumor cells from benign or normal ones [1].This feature of tumor cells is determined by their ability to migrate and penetrate surrounding tissues. Often the metastases but not primary tumors lead to the death of organism. That is why the processes of cancer cell migration and invasion are the most important targets of the anti-cancer basic

research and drug development. In normal adult mammalian organism, only immune and placenta cells are able to invade corresponding tissues. However, an ability to migrate is not limited to these cell types. There are also many other cell populations that migrate to their final niche in the course of embryogenesis and post-embryonic development, including the development of cardiovascular system, central nervous system, and many others [2–6].

Nowadays, there are several approaches to evaluate the cell migration and locomotor properties *in vitro*. The most widely employed methods are the transwell migration and invasion assay, the "wound healing" assay and the initiation of cell migration from multicellular spheroids or tissue explants into matrigel or onto growth surface. Each of them has the advantages and disadvantages which leads to the need of their improvement.

The first mentioned method is the transwell migration and invasion assays. The basis of this method is the initiation of directed cell movement through the pores of the transwell membrane toward the chemoattractant. This approach is widely used in cancer research and especially for test of antitumor properties of corresponding drugs or their combination [1]. However, this approach has several disadvantages, in particular time restrictions, which limits the duration of the study to 24–48 hours; relatively high costs, and the need of selecting the optimal conditions for each particular cell type and the type of attractant used. Another widely applied method is the wound healing assay. This method is based on estimation of closure dynamics of the artificially formed free space in the confluent cell monolayer. Alike previous case, the main disadvantage of this method is limitation of experiment duration, usually up to 24 hours to exclude the effect of cell proliferation in freed space. The third experimental strategy of migration estimation is based on the transformation of 3D multicellular spheroid into 2D cellular monolayer colony. This method provides several advantages over above-mentioned approaches; the main of them is that 3D culture of cancer cells more closely reflects *in vivo* conditions [7]. Besides, it also enables easy detection of many of biochemical and morphological properties of moving cells. Depending on the studied cell type, this model provides an opportunity to characterize either collective or single-cell migration. However, there are also several known limitations to this model application. The major difficulty is significant variation of spheroid sizes. It, in turn, complicates the comparison of cell migration kinetics between spheroids with initially different size. Therefore, there is a strong need for further unification of the multicellular spheroid size. There are several approaches to obtain the spheroids of similar sizes including handling drop method or application of special multi-well plates, *etc.*, but they require preliminary preparation and are relatively expensive. From our point of view the double filtration of spheroid suspension through nylon mesh filters could provide strong unification of spheroid size.

Another issue which should be regarded is the input of proliferation in the value of distance or surfaces covered by the cells in migration tests. To compare the dynamics of cell migration at regular terms, it is necessary to ensure that the level of proliferation for the same time period was also similar.

To test the proposed approach, the inhibition of cell locomotion was applied.

The cell migration is strongly affected by a variety of growth factors, hormones, cytokines and other chemical cues, which induce the activation of several signaling pathways within migrating cell. The PI3K/mTOR/S6K cascade has been previously described as an important regulator of mammalian cell migration [8]. In normal tissue this pathway is involved in the control of many intracellular events, including protein synthesis regulation, the G1/S phase cell cycle transition and many others [9, 10]. mTOR/S6K signaling over activation and over expression has been observed in many diseases including cancer, diabetes, and other [11–13]. There are some significant differences in PI3K/mTOR/S6K signaling in 3D vs. a 2D cell culture system [14]. Earlier, we confirmed that rapamycin, an inhibitor of mTOR signaling, decreased the MCF-7 cell locomotion in scratch test. So, it was used in this study for validation of optimized locomotion assay [15].

Additionally, the model of outspreading spheroids can be applied for immunochemical assay. Earlier we revealed the subcellular relocalization of p70S6K1 from the cytoplasm into the nuclei of MCF-7 cells in course of migration from spheroid onto growth surface by immunofluorescent analysis [16]. One of the explanations of the kinase relocalization was its association with the transcription factor TBR2, expressed in actively migrating cells like embryonic, cancer cells and lymphocytes. Kinase of ribosomal protein S6 (S6K1) is one of the key links of the mTOR signaling pathway. S6K1 has several known isoforms: p85(S6K1), the most highly expressed isoform p70(S6K1), and additionally less studied isoforms p60(S6K1) and p31(S6K1). Both p85(S6K1) and p70(S6K1) isoforms have been previously shown to be regulated through phosphorylation by the mTOR/S6K, PI3K/Akt signaling pathway [9]. Initially p85S6K1 was regarded as a nuclear isoform of S6K1, moreover it contained the signal of nuclear localization at N-terminus of molecule. But later it was observed in cytoplasm as well. p70S6K1 and p85S6K1 have both common and different effectors and targets. To compare their subcellular distribution in migrating cells, immunofluorescent revealing of p85S6K1 in outspreading spheroids of MCF-7 cells was performed.

So, the aim of this study was to improve the method of initiation of cell migration from the 3D multicellular spheroids cells onto the growth surface. Namely, the MCF-7 cell spheroid size unification was performed by double filtration through nylon mesh filters with pore diameter 30 and 100 mm; the index of MCF-7 cell proliferation at the 1st, 2nd, and 3rd days in outspreaded spheroids was estimated; the validity of quantitative migration assay was tested using migration inhibition; availability of the model for immunofluorescent analysis was confirmed by corresponding assay of the p85S6K1 relocalization in course of cell migration.The proposed approach could be useful for basic cancer research and anticancer drug development, as well as for other assay of the cell locomotion dynamics.

Materials and Methods

*Cell culture.*MCF-7 cell line derived from metastatic site of malignant breast tumor was used in this study [17]. MCF-7 cells were cultured in Dulbecco's Modified Eagle's me-

dium (DMEM) (Sigma, USA) supplemented with 10 % fetal bovine serum (FBS, CellSera, Australia), 4 mM glutamine (Sigma, USA), 50 U / ml penicillin and 50 g / ml streptomycin (Sigma, USA) at 37 ° C in a 5 % CO2 humidified incubator.

Generation of spheroids. For multicellular spheroids generation, confluent monolayer of MCF-7 cells was detached with 0.25 % trypsin, 0.02 % EDTA in Hank′s Balanced Salt Solution (Sigma, USA) to generate single cell suspension, which was transferred into the 10-centimeter Petri dishes coated with 1 % agarose (Serva, Heidelberg, Germany). Cells were further cultivated for three days. The resulting suspension of spheroids of different size was subjected to the two-step filtration. First, spheroids were passed through a sterile nylon mesh (Spectrum, USA) with a pore diameter of 100 μm to remove large cell aggregates. The second step of the filtration was carried using sterile filter mesh (Spectrum, USA) with a pore diameter of 30μm for single cell elimination.

The transformation of spheroids in a monolayer cell colony. The obtained spheroids of uniform size were transferred into the 6-well plates with a fresh complete medium. The migration and proliferation processes were analyzed at 0, 24, 48 and 72 hours post filtration. The images were acquired using bright field and phase contrast microscopy (CETI Versus inverted microscope, CETI, Belgium, and Leica DM 1000, Leica Microsystems, Germany, Magnification 25x, 100x). Only the colonies of round shape were selected for further analysis. A colony area was determined using Fiji software, and an approximate colony radius was calculated using formula: $r=\sqrt{S}/\pi$. The migration activity was expressed as a difference of colonies radii at corresponding time points. To alter the cell migration dynamics the treatment of MCF-7 multicellular spheroids with 10 nM rapamycin (Calbiochem biochemicals, Los Angeles, USA) was applied and then the cell locomotion was estimated.

Proliferation activity analysis. The number of mitotic cells was calculated at 24, 48 and 72 hours of spheroid outspreading. The images of spheroids were taken at indicated time points, and then the number of proliferating cells of corresponding morphology was calculated. Besides, cultured on cover glasses colonies were fixed with 10 % formaldehyde solution (Thermo Scientific, USA) for 15 minutes and afterwards stained with 2 % Hoechst 33342 (Molecular Probes, USA) in the dark for 25 minutes. Samples were mounted on slides into Mowiol mounting medium (Sigma-Aldrich, St. Louis, USA) containing 2.5 % DABCO (Sigma-Aldrich, St. Louis, USA), and amount of mitotic cells were calculated using fluorescent microscopy by morphological features. Index of proliferation was calculated as the per cent of mitotic cells in the population of spreading spheroid.

Immunofluorescence analysis. Cultured on cover glasses colonies at 0, 24, 48 and 72 hours were fixed with 10 % formaldehyde solution for 15 minutes, as described previously. For membrane permeabilization, the cells were treated with a 0.2 % Triton X-100 in PBS solution and afterwards incubated for 30 min at room temperature in 10 mM cupric sulphate and 50 mM ammonium acetate (pH 5.0) to reduce autofluorescence. Non-specific antibody binding was blocked with 10 % FCS in PBS for

30 min at 37 °C. Anti-p85(S6K1) rabbit polyclonal antibodies were applied in dilution 1:100 overnight at 4 °C [18]. Secondary FITC conjugated anti-rabbit antibodies (Jackson Immuno Research Laboratories, Pennsylvania, USA) were applied in dilution 1:400 for 1h at 37 °C in humidified chamber. Samples were mounted into the Mowiol medium (Sigma-Aldrich, St. Louis, USA) containing 2.5 % DABCO (Sigma-Aldrich, St. Louis, USA). Fluorescent microscopy was performed using Leica DM 1000 fluorescent microscope (Leica Microsystems, Wetzlar, Germany, Canon PowerShots70, Magnification 100x, 400x).

Statistical analysis. All image analysis was performed using the Fiji software [19]. Data analysis was performed using Origin 9. All data are expressed as median +/- SD. Each experiment was repeated at least three times.

Results and Discussion

Unification of spheroid size. One of the main drawbacks of the spheroid to monolayer transition model is the significant size variation of multicellular spheroids generated by the standard liquid overlay method (Fig. 1a). To overcome this obstacle, an additional step of double filtration of generated spheroids suspension was applied. It enabled to unify the size of the colonies used for subsequent analysis, and, so, to perform proliferation and migration measurements more accurately and rapidly (Fig. 1b).

At the first step of method optimization the filtration of spheroid suspension through the nylon mesh with a pore diameter of 100 μm was applied to eliminate large cell clusters in the resulting culture. Subsequent purification from small cell aggregates and single cells was performed using a 30-μm pore diameter nylon mesh. For further analysis of purified colonies, the value of median of spheroid size was determined using image analysis. Average of diameter median of MCF-7 cells spheroids in 5 experiments was 47.65 μm with standard deviation ±21.79μm. It confirmed that filtration

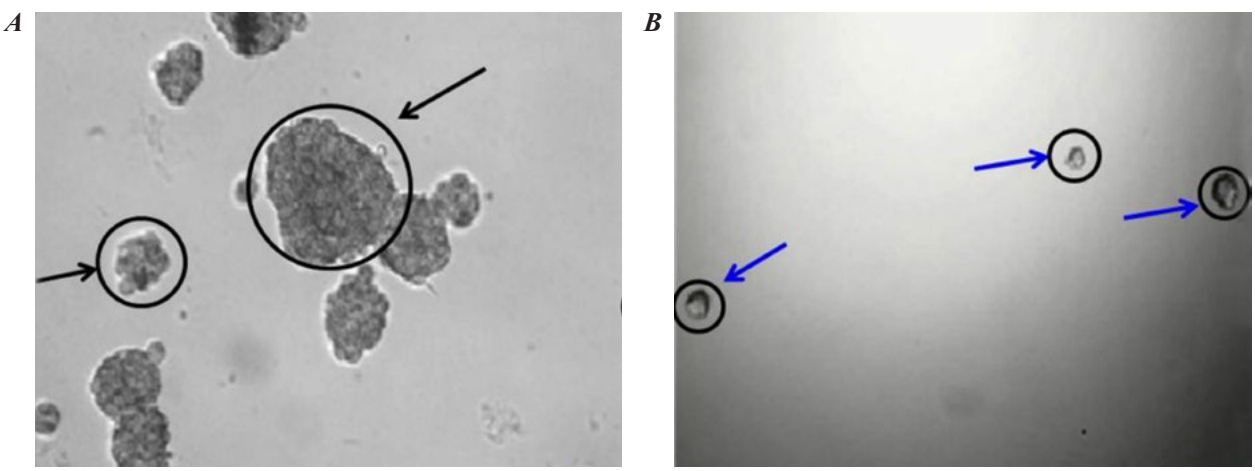

Fig. 1. *A* — Suspension of MCF-7 multicellular spheroids before filtration. Black arrows and circles indicate spheroids of different size. Oc.10x, ob. 10x. *B* — Suspension of MCF-7 multicellular spheroids after double filtration. Blue arrows indicate spheroids of similar size. Oc.10x, ob. 2,5x.

was successful and the population of spheroids of uniform size was generated.

Determination of proliferation activity. Another major technical issue in multi-day migration assays is the proliferation of studied cells. Since proliferation has been shown to affect other migration assays, it was important to compare the proliferation activity of cells in our model on the 1st, 2nd and 3rd days of spheroid spreading. To analyze this parameter in the proposed system, the cell proliferation index after consecutive time periods was estimated .We used two different approaches. In the first case, the number of mitotic cells was calculated directly in course of spheroid spreading using transmitted light microscopy. The mitotic cells in monolayer condition acquire morphology distinct from the interphase cells. Such cells become round, they exhibit condensed chromatin, the morphology of which reflects the corresponding stage of mitosis (Fig 2). The per cent of such cells was determined in each outspreading spheroid.

In the second case, the number of proliferating cells was detected at mentioned time points using Hoechst 33342 staining and fluorescent microscopy (Fig.3).

The proliferation index was expressed as the percentage of dividing cells in each colony analyzed. We detected that the level of cell proliferation after 24, 48, and 72 hours of migration assay did not exceed 3 %. In particular, we obtained the values for 24 hours of 2.54 %, for 48 — 2.6 %, for 72 — 2.94 %. This indicates a similar effect of proliferative activity on the spreading dynamics of the MCF-7 spheroids at every time point, allowing us to neglect a potential proliferation influence at comparison of migration dynamics at neighboring time points.

Migration assay. In order to determine the distance that cells passed during migration process, the radii of outspreading spheroids were measured after 0, 24, 48, and 72 h of cultivation. The difference between the radii values at neighboring time points was further regarded as the length of the cell migration track. From our point of view, the comparison of linear parameters in migration assay is more adequate than of squares since it is more valid characteristic of the directed movement.

For this aim, the area of each spheroid was quantified using Fiji software at all abovementioned time points, and radius was calculated as described in Materials and Methods section. We observed that a migration rate reached the maximum at 48 hours post-filtration and decreased after 72 hours, which morphologically corresponded to the complete spreading

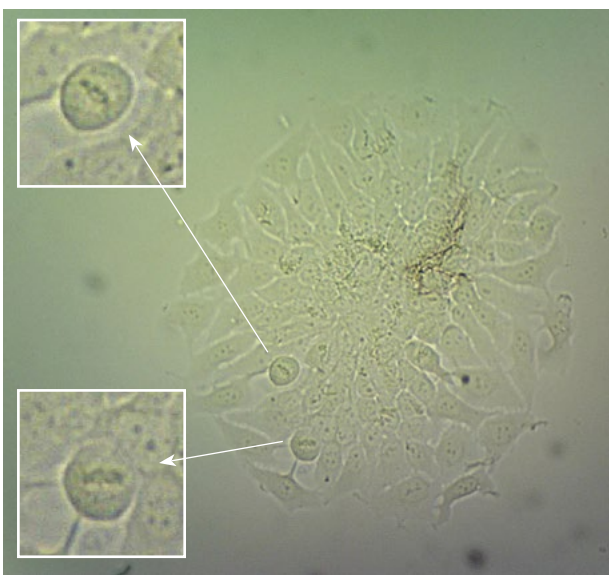

Fig. 2. Determination of mitotic cells in outspreading MCF-7 cell spheroids at the 2nd day of migration initiation. Arrows point out metaphase plates. Transmitted light microscopy. Oc.10., ob.20x.

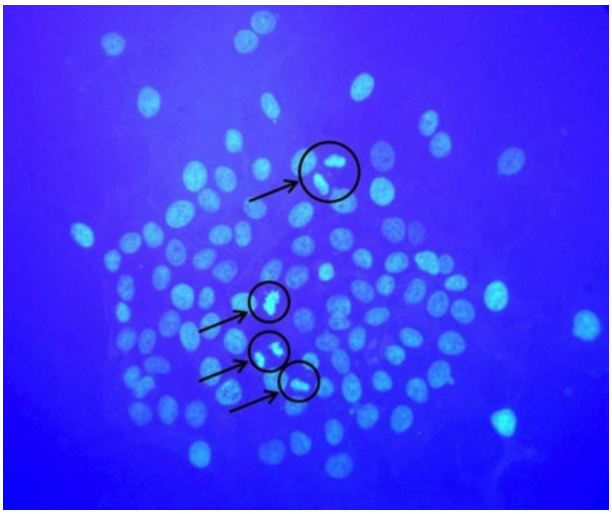

Fig. 3. Detection of mitotic cells in MCF-7 outspreaded spheroid at the 2nd day of experiment. DNA counterstained with Hoechst 33342. Arrows point out proliferating cells. Oc.10x, ob.10x.

of spheroids (Fig. 4, Fig. 5). Thus, the median of migration distance after 24 hours was 7.64 mm, after 48 hours the cells passed another 8.24 mm and after 72 hours another 6.8 mm.

For further validation of the proposed model, the analysis of locomotor properties of MCF-7 cells under effect of Rapamycin (mTOR inhibitor) was applied. Rapamycin and its derivatives are the most well-known inhibitors of mTOR and are currently undergoing clinical trials as novel anticancer agents. These compounds have been shown to inhibit the activity of mTOR complexes and significantly decrease tumor cell motility *in vitro* [20]. Therefore, we analyzed whether rapamycin would affect the MCF-7 cell migration from spheroids onto the growth surface. Noteworthy, the effect of rapamycin on the rate of cell migration during the first day was minimal and cells overcame 8.13 mm, whereas on the second and third days of the experiment, a significant decrease in the cell migration rate was observed, 3.65 mm and 1.94 mm respectively (Fig. 4). It could be potentially attributed to the inhibition of mTORC2 complex involved in the cytoskeleton regulation [19]. So, this result confirmed the suitability of the proposed model for the assessment of cell migration and its inhibition.

Immunofluorescence analysis

The presented model allowed applying an immunofluorescence analysis of intracellular localization of variety antigens in the migrating

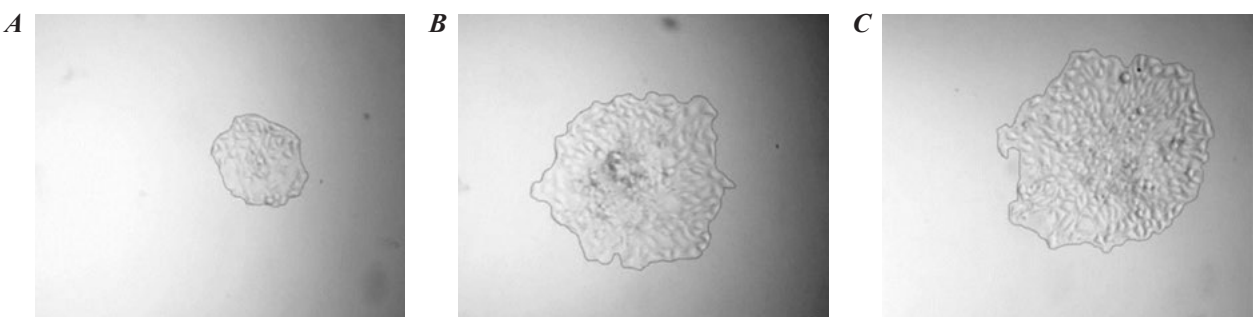

Fig. 4. Cultured multicellular spheroids of MCF-7 cells after 24 (*a*), 48 (*b*) and 72 (*c*) hours of spreading. Transmitted microscopy. Oc.10x, ob.2,5x.

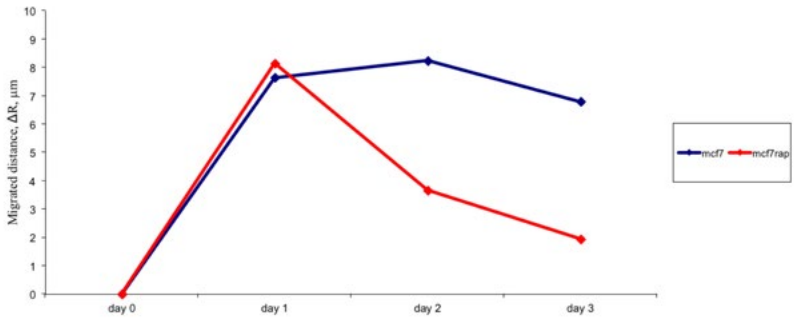

Fig. 5. Dynamics of MCF-7 cell migration from 3D spheroid onto the growth surface at 0, 24, 48, 72 hours post-filtration in standard cell culture conditions (blue line) or under the Rapamycin treatment (10 nM) (red line).

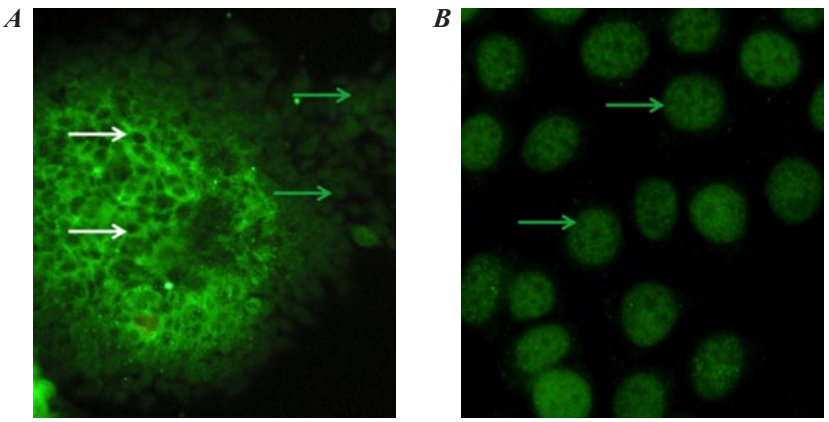

Fig. 6. Immunofluorescence detection of p85S6K1subcellular distribution in MCF-7 cell outspreading spheroid, *a* — Oc.10x, ob. 10x, and *b*–in monolayer culture, oc.10x, ob. 40x.White arrows pointed out negative nuclear reaction, green arrows pointed out positive nuclear reaction.

cells of outspreading spheroids. Earlier we revealed the subcellular relocalization of S6K1 from the cytoplasm into the nuclei of MCF-7 cells after initiation of migration [16]. The presented model was used to determine the distribution of one of S6K1 isoforms namely p85S6K1 in the migrating MCF-7 cells. In 3D conditions MCF-7 cells demonstrated predominantly cytoplasm localization of p85S6K1 (Fig. 6a, white arrows). After initiation of cell migration a bright positive reaction in the nuclei (primarily on the leading edge) appeared for p85S6K1 (Fig. 6a, green arrows). Noteworthy, in 2D monolayer conditions the nuclei of MCF-7 cells were strongly positive for p85S6K1 (Fig. 6b). The obtained results could suggest the important role of p85S6K1 realizing in the nuclei for cell spreading and migration. Besides, the applied model allowed registration of the change of subcellular distribution of the intracellular protein in migrating cell.

Proposed optimisation of cell migration model namely spheroid size unification and estimation of proliferation activity allow to apply this model for detection of locomotor properties of the breast cancer MCF-7 cells during 3 days. Besides, the model is useful for investigation of the subcellular localization of proteins involved in the regulation of cell locomotion. This approach will be helpful for anticancer drug test as well as for study of the basic mechanisms of tumor progression.

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Оптимізація моделі визначення динаміки міграції пухлинних клітин *in vitro*

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Міграційна здатність є важливою ознакою пухлинних клітин. Існує кілька підходів до аналізу динаміки міграції ракових клітин *in vitro*. Однією з найбільш перспективних і близьких до умов *in vivo* є модель ініціювання міграції клітин з тривимірного багатоклітинного сфероїда на ростову поверхню. **Мета.** Оптимізація моделі для адекватної кількісної оцінки міграції пухлинних клітин. **Методи.** 2- та 3-вимірна культура клітин лінії MCF-7, імунофлюоресцентний аналіз, аналіз зображень з використанням комп'ютерної програми Фіджі. **Результати.** Уніфікація розміру сфероїдів дозволила уникнути значного розкиду даних. Отримані сфероїди повністю розпластувались протягом 3 днів. Найвищий показник міграції спостерігався на 2-гу добу розпластування сфероїда. Рівень проліферації клітин за кожну добу 3-денного експерименту був майже однаковим і не перевищував 3 %. Валідність моделі була протестована після пригнічення міграційної активності клітин під впливом рапаміцину (інгібітор сигналізації mTOR). Крім того, запропонована модель була успішно застосована для дослідження субклітинної локалізації p85S6K1 в мігруючих клітинах лінії MCF-7 за допомогою імунофлюоресцентного аналізу. **Висновки.** Подвійне фільтрування багатоклітинних сфероїдів дозволяє уніфікувати їх розміри, що в подальшому сприяє адекватній оцінці міграційного потенціалу клітин. Запропонована модель дозволяє вивчати динаміку міграційних процесів пухлинних клітин і може бути використана для тестування протипухлинних препаратів *in vitro*.

Ключові слова: Міграція ракових клітин, 2- та тривимірна культура клітин, p85S6K1, сфероїди.

Оптимизация модели определения динамики миграции опухолевых клеток *in vitro*

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Миграционная способность является важным признаком опухолевых клеток. Существует несколько подходов к анализу динамики миграции ракових клеток *in vitro*. Одним из наиболее перспективных и близких к условиям *in vivo* является модель инициирования миграции клеток из трехмерного многоклеточного сфероида на ростовую поверхность. **Цель.** Оптимизация модели для адекватной количественной характеристики миграции опухолевых клеток. **Методы.** 2- и 3-мерная культура клеток линии MCF-7, иммунофлюоресцентный анализ, анализ изображений с использованием компьютерной программы Фиджи. **Результаты.** Унификация размеров сфероидов позволила избежать значительного разброса данных. Полученные сфероиды полностью распластывались в течение 3 дней. Самый високий показатель миграции наблюдался на 2-е сутки распластования сфероида. Уровень пролиферации клеток за каждые сутки 3-дневного эксперимента был близьким и не превышал 3 %. Валидность модели была протестирована после подавления миграции под влиянием рапамицина (ингибитор сигнализации mTOR). Кроме того, предложенная модель была успешно применена для исследования субклеточной локализации p85S6K1 в мигрирующих клетках линии MCF-7 с помощью иммунофлюоресцентного анализа. **Выводы.** Двойная фильтрация генерируемых *in vitro* многоклеточных сфероидов позволила унифицировать их размер, что способствует адекватной оценке миграционного потенцала клеток. Предложенная модель позволяет изучать динамику миграционных процессов опухолевых клеток и может бать использована для тестирования противоопухолевых препаратов *in vitro*.

Ключевые слова: Миграция ракових клеток, двух- и трехмерная культура клеток, p85S6K1, сфероиды.

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