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## **Sensitivity of MCF-7 cells with differential expression of S6K1 isoforms to the regulatory impact of fibroblasts**

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**Aim.** To investigate the involvement of mTOR/S6K1 cell signaling network, with focus on S6K, in response of tumor cells to regulatory impact of fibroblasts. **Methods.** Cell culture, including co-cultivation of fibroblasts and tumor cells, immunofluorescence analysis, Western blot analysis, assessment of cell migration by scratch test, and transformation of multicellular spheroid in monolayer cell colony. **Results.** The present work showed the positive effect of stromal cells on the phosphorylation level of the components of mTOR/S6K1 signaling cascade: p85S6K1, p70S6K1 and mTOR in human breast adenocarcinoma MCF-7 cells. To determine, which of the S6K1 isoforms, p85S6K1, p70S6K1 or p60S6K1, is the most sensitive to the extracellular environment, the stable MCF-7 cell lines with edited expression of S6K1 isoforms were used. It was found that selective expression of p60S6K1 led to the changes in morphological features of tumor cells under both two- and three-dimensional culture conditions. These cells also exhibited high levels of focal adhesion kinase (FAK) phosphorylation and large protein content of Zo-1, CD29, CD44 compatible with their high migration potential in the scratch test. Besides, the cells, selectively expressing p60S6K1, were resistant to fibroblast-producing factors and rapamycin. It was also demonstrated that fibroblasts increased tumor cell motility in scratch test and spheroid outspreading assay under co-cultivation conditions in paracrine manner, whereas the direct contact of tumor spheroids with the fibroblast monolayer significantly reduced the velocity of spheroid outspreading. **Conclusions.** The data obtained indicate that not only the differential expression of S6K1 isoforms in MCF-7 cells but also their ratio are important signaling parameters determining cell survival and response to microenvironment factors.

**Keywords:** S6K1, migration, tumor microenvironment.

## Introduction

Numerous studies have shed light on the role of tumor microenvironment in carcinogenesis [1]. It has been found that, depending on the stage of tumor progression and other physiological conditions, tumor stroma may act as a suppressor or activator of the tumor development [2]. Tumor stroma includes fibroblasts, macrophages, histiocytes, pericytes, vascular endothelium (mainly microvessels), cells of the immune system, nerve endings, extracellular matrix. These cells exert a direct as well as indirect predominantly humoral effects on the tumor cells. Heterotypic interactions between fibroblasts and malignant cells are considered as the most studied tumor-stroma interplay [3]. Thus, it has been shown that the co-culture of fibroblasts and myofibroblasts derived from benign prostatic hyperplasia tissue has a significant antitumor effect on the cells originating from prostate cancer. This effect is realized through the paracrine effects, first of all, the production of IL-6 [4]. On the other hand, cancer-associated fibroblasts (CAFs) promote breast carcinogenesis through the enhanced GREM1 (gremlin1, bone morphogenic protein antagonist) production, which resulted in the intensification of tumor invasion and extravasation processes [5]. Besides, it has been found that CAFs induce epithelial-mesenchymal plasticity of tumor cell clusters in breast cancer [6]. Noteworthy also significant interest paid to the study of the influence of macrophages / histiocytes on the development of tissue malignancy [7].

The signal transduction from extracellular factors into the cells is mediated through intracellular signaling networks. One of the net-

works undergoing significant changes in malignant transformation is the mTOR/S6K signaling pathway [8,9]. The ribosomal protein S6 kinases - S6K is among the key components in this signaling cascade. S6K belongs to the family of serine/threonine protein kinases, including protein kinase C, protein kinase B, SGK, and p90S6K. It is involved in the regulation of protein synthesis and G1/S cell cycle transition. There are two forms of S6K, S6K1 and S6K2 (S6K $\alpha$ , S6K $\beta$ ) that share high level of homology. S6K activation has been shown to be regulated by phosphorylation/dephosphorylation in response to various extracellular stimuli, including growth factors, cytokines and hormones [10]. There are several S6K1 and S6K2 isoforms formed through alternative splicing and alternative translation start processes. To date, the most studied isoforms are alternatively translated p70S6K1 and p85S6K1, which have N-terminal extension (23 a.a.) where NLS (nuclear localization signal) is located. Additionally, a new 31 kDa S6K1 isoform (p31S6K1) was also discovered, which is an mRNA splicing variant. Although p31S6K1 protein has been shown to lose the kinase domain, it has oncogenic properties [11]. Recently, a novel p60 isoform of S6K1 was found that has the potential of oncogenic properties [12-14].

The content of phosphorylated S6K1 (Thr389) has been shown to be inversely proportional to the response to irradiation therapy in patients with breast cancer. So, the phospho S6K1(Thr389) level was regarded as a possible factor for prediction of the ovarian irradiation therapy efficacy [15, 16]. Phosphorylated

S6K1 is shown to bind directly to F-actin in ovarian cancer cells, suggesting a role for S6K1 in actin reorganization [17]. However, the effects of S6K1 may be cell type dependent. For example, in fibroblasts Rac1 (Ras-related C3 botulinum toxin substrate 1) and Cdc42 (CDC42 small effector protein 1), two GTPases of Rho family that regulate cytoskeletal reorganization, acted as the activators of S6K1. In ovarian cancer cells, this effect was not observed [17].

The important role of S6K1 in malignancies was confirmed by a study showing that inhibition of S6K1 impeded the migration of breast cancer cells. This fact indicates the role of S6K1 in the processes of breast cancer metastasis [18]. There is another evidence that S6K1 may be involved in the regulation of normal and malignant cell motility, as knockdown of p70S6K1 or inhibition of S6K1 kinase activity caused a significant decrease in the rate of migration of prostate, breast, and ovarian cancer cells *in vitro* [17, 19]. Moreover, the activation of p70S6K1 in human ovarian cancer cells in response to stimulation with hepatocyte growth factor (HGF) led to an increased expression of matrix metalloproteinase 9 (MMP9) and elevated migration activity of these cells [20].

So, the question is: whether there is a direct relationship between the influence of tumor microenvironment and the functioning of the mTOR/S6K signaling network within the tumor cells. A number of studies indicate that such a link exists. For example, it has been shown that, unlike normal fibroblasts, CAFs produce an increased amount of interleukin 22, which leads to the activation of the mTOR/S6K signaling cascade in lung cancer cells,

and thus to a significant increase in the level of proliferation, migration and invasion of these cells [21]. Besides, it has been found that pre-operative irradiation in rectal cancer leads to the activation of CAFs, which in paracrine manner through IGF1/IGF1R, trigger mTOR/S6K signaling and increase tumor progression [22]. It has also been found that CAFs in pancreatic cancer produce IL-6 that activates mTOR/4E-BP1 signaling followed by the development of chemoresistance. Moreover, the paracrine factors, including IL-6, produced by CAFs induce the expression of survivin by tumor cells, which ultimately leads to the inhibition of apoptosis [23].

So, in the present work, we evaluated the effect of fibroblasts on the phosphorylation status of the components of mTOR/S6K signaling pathway in MCF-7 cells originated from breast cancer. The stable cell lines derived from MCF-7 cells with edited expression of S6K1 isoforms by CRISPR/Cas9 technology were used to evaluate their role in the manifestation of the malignant cell phenotype. Initially we found that down-regulation of the p70 and p85 isoforms expression with sustained expression of p60 S6K1 dramatically enhances the locomotor activity of MCF-7 cells [14]. The migration potential of these cells with direct and indirect influence of fibroblasts was also analyzed.

## Materials and Methods

**Cell culture.** MCF-7 breast cancer cell line and its mutant derivatives MCF-7 *p85-/p70+/p60+* (clone F1), MCF-7 *p85-/p70-/p60+* (clone F2), MCF-7 *p85-/p70-/p60-* (clone F3) [12-14] were cultured at 37 °C (5 % CO<sub>2</sub>) in Dulbecco's Modified Eagle's Medium (DMEM) supple-

mented with 10 % fetal bovine serum (FBS, GIBCO), 50 units/ml penicillin and 50 µg/ml streptomycin, 4 mM glutamine.

Human dermal fibroblast cultures were obtained as it was described in [24]. Briefly, specimen of skin was cut in sterile conditions to obtain pieces about 1x1x1 mm. Five pieces were put in 60 mm Petri dish precoated with 1 % gelatin and cultivated for 10 days in F-12 medium with 10 % fetal bovine serum, 50 units/ml penicillin, 50 µg/ml streptomycin, 4 mM glutamine. After active fibroblast migration they were subcultivated. To obtain the conditioned medium, human dermal fibroblasts were cultured under the same conditions as MCF-7 cells. The medium conditioned by fibroblasts was obtained after 24 h of cultivation of subconfluent monolayer of human dermal fibroblasts, then it was centrifuged at 2000 rpm for 10 min and filtered through 22 µm pore sterile filter.

*May-Grunvald Giemsa staining.* Monolayer cultures were fixed with methanol for 5 min and stained with mixture of May-Grunvald and Giemsa dyes 1:3 in PBS pH 7.8 (2 parts of dye mixture and 5 parts of PBS) for 20 min.

*Scratch assay.* MCF-7 cells and obtained clones were cultured 72 h before scraping. Sub-confluent cell monolayer was scrapped by blue tip in order to create an experimental wound. Detached cells were removed by aspiration. Medium supplemented with 10 nM rapamycin, or 20 % conditioned medium, or their combination was added into corresponding dishes. The images of 10 randomized fields of observation of each scratch were captured using a microscope immediately after scraping and after 24 hours of cultivation. The size of widths was determined (as ratio experimental

wound area/width of field of observation) from these images using Icy 1.3.2.0 software [25]. Migrated distance was calculated as width difference at start point and after 24 h of migration.

To detect the influence of direct co-culture of fibroblasts and tumor cells the described model was applied [26]. Human dermal fibroblasts ( $2 \times 10^6$  cells in 1 ml of DMEM/FBS) were seeded and distributed at the periphery of 10 cm Petri dish. Cells were incubated during 1.5 h at 37 °C. Non adhered cells were removed by repeated washing with culture medium. After that MCF-7 cells or clones were added to the Petri dish (1/5 of confluent monolayer per dish) in the complete growth medium. Fibroblasts and cancer cells were co-cultured for 48–72 h before scraping. The subconfluent cell monolayer was scrapped by tip in order to create an experimental wound. After removing the damaged cells, the cultures were washed with DMEM complete medium. 10 ml of DMEM complete medium with or without 10 nM rapamycin were added to corresponding dishes. The scratch images of 10 randomized fields of observation in the center of the dish were captured with a microscope just after scraping and after 24 h of cell migration.

*Generation of spheroids.* For multicellular spheroids generation, a confluent monolayer of MCF-7 cells or corresponding clones was detached with 0.25 % trypsin, 0.02 % EDTA in Hank's Balanced Salt Solution (Sigma, USA), a single cell suspension 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 filtra-

tion. First, spheroids were passed through a sterile nylon mesh (Spectrum, USA) with a pore diameter of 100  $\mu\text{m}$  to remove large cell aggregates. The second step of the filtration was carried out using sterile filter mesh (Spectrum, USA) with a pore diameter of 30  $\mu\text{m}$  for the single cell elimination [27].

*The transformation of spheroids in a monolayer cell colony.* The obtained spheroids of uniform size were transferred either onto sterile cover slip in 24-well plates or onto human dermal fibroblast monolayer on cover slip in 24-well plate. Tumor cell dissemination was analyzed after 24 hours of spheroid outspreading. The images were acquired using the bright field and phase contrast microscopy (CETI Versus inverted microscope, CETI, Belgium, and Leica DM 1000, Leica Microsystems, Germany, Magnification 25x, 100x and Zeiss 510 META confocal microscope, Germany). A colony area was determined using Fiji software [28]. The dissemination capability was expressed as a difference of colonies areas on Day1 and Day 0.

*Immunofluorescence analysis.* The colonies cultured on cover glasses at 0 and 24 hours or the monolayer cultures were fixed with 10 % formaldehyde solution for 15 minutes. For membrane permeabilization, the cells were treated with 0.2 % Triton X-100 in PBS and afterwards incubated for 30 min at room temperature in 10 mM cupric sulphate and 50 mM ammonium acetate (pH 5.0) to reduce auto-fluorescence. Non-specific antibody binding was blocked with 10 % FCS in PBS for 30 min at 37 °C. Anti-pan cytokeratin (Clone C11, Sigma) mouse monoclonal antibodies were applied in dilution 1:100 overnight at 4 °C. Secondary FITC conjugated anti-rabbit

antibody (Jackson Immuno Research Laboratories, Pennsylvania, USA) was applied in dilution 1:400 for 1h at 37 °C in humidified chamber.

For Zo-1 detection the fixed cells were blocked and permeabilized in PBS containing 3 % bovine serum albumin, 5 % FBS, 0.3 % Triton X-100 for 40 min at room temperature. The primary and secondary antibodies were diluted in PBS containing 3 % BSA, 1 % FBS and 0.1 % Triton X-100. Zo-1 antibody (Invitrogen) 1:100 was incubated overnight at 4 °C followed by incubation with secondary FITC conjugated anti rabbit antibody (Jackson Immuno Research Laboratories, Pennsylvania, USA) for 1 h at 37 °C in humidified chamber.

Cell nuclei were counterstained with Hoechst 33342. Samples were mounted into the Mowiol medium (Sigma-Aldrich, St. Louis, USA) containing 2.5 % DABCO (SigmaAldrich, St. Louis, USA). Fluorescent microscopy was performed using Zeiss 510 META microscope (Germany).

*Western blot analysis.* Cells were washed with ice-cold PBS and lysed on ice for 30 min in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5 % Triton X-100, supplemented with a Complete EDTA free protease inhibitor cocktail tablet (Roche) and phosphatase inhibitors (Sigma-Aldrich). Whole-cell lysates were centrifuged at 12,000 rpm for 15 min at +4 °C, and the supernatant was collected. The protein concentrations were determined using Bradford assay. Equal amounts of protein (10  $\mu\text{g}$ ) were resolved on 10 % SDS-PAGE and electro transferred on polyvinylidene difluoride (PVDF) membranes for immunoblotting. The antibodies were diluted for use as follows: anti-S6K1-

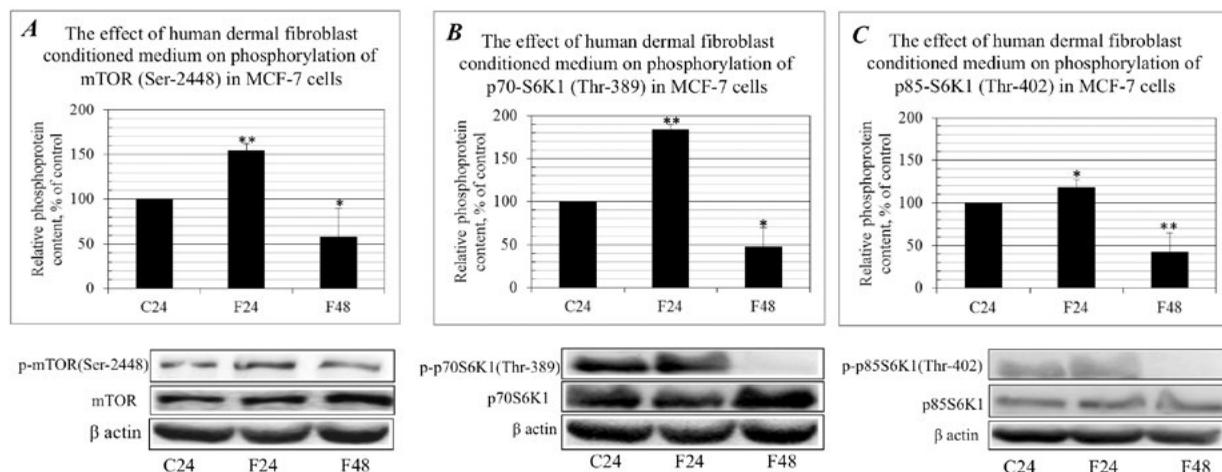
C-terminus (generated as described in [29]) 1:2500, anti- $\beta$ -actin (clone 13E5, Sigma) 1:20000, anti-phospho-p70 S6 Kinase (Thr389) Antibody 1:1000 (Cell Signaling Technology), anti-ZO-1 antibody 1:1000 (Invitrogen), anti- $\beta$ -Tubulin Antibody 1:1000 (Cell Signaling Technology), anti-phospho-Focal Adhesion Kinase (Tyr925) antibody 1:1000 (Milipore), anti-Integrin beta-1/CD29 antibody 1:500 (CiteAb), anti-human CD44 antibody 1:500 (BD Pharmingen), anti-phospho rpS6 (Ser 235/236) antibody 1:1000 (Cell Signaling Technology). The process of detecting the specific binding of secondary antibodies was performed using the enhanced chemoluminescence (ECL) reaction. Densitometric analysis was performed using ImageLab 6.0.1 software.

**Statistical analysis.** The data were analyzed by Student's *t* test using GraphPadPrism 6.01.

The results were expressed as the mean ( $\pm$ SD) or median. The differences were considered significant at  $p < 0.05$ . Each experiment was repeated 3 times.

## Results and Discussion

**Analysis of human fibroblasts effect on the mTOR/S6K signaling network of MCF-7 tumor cells.** To evaluate the possible modulation of mTOR/S6K signaling in MCF-7 cells by fibroblasts, the phosphorylation levels of mTOR and S6K1 kinases under the influence of fibroblast conditioned medium were detected using Western blot analysis. Thus, it was found that the effect of fibroblast-produced factors caused a statistically significant increase in the level of mTOR (Ser2448) phosphorylation, which is an evidence of the activation of this kinase (**Fig. 1 A**). Additionally, the paracrine effect



**Fig. 1.** Phosphorylation pattern of the components of mTOR/S6K signaling pathway of MCF-7 cells under the effect of fibroblast conditioned medium. Densitometric assay of Western Blot analysis data of mTOR/Ser2448 (**A**), p70-S6K1/Thr389 (**B**) and p85-S6K1/Thr402 (**C**) in MCF-7 cells after 24 and 48 h treatment with human dermal fibroblast conditioned medium. C24 – control MCF-7 cells cultured in standard conditions for 24h, F24 – treatment with 20 % conditioned medium of human dermal fibroblasts for 24h, F48 – treatment with 20 % conditioned medium of human dermal fibroblasts for 48h. Results were regarded as statistically significant (\* –  $p < 0.05$ ; \*\* –  $p < 0.01$ ) using unpaired Student's *t* test.

of fibroblasts led to a considerable statistically significant elevation in the level of phosphorylation of p70S6K1 and p85S6K1 kinases on threonine 379 and 402, respectively (**Fig. 1 B, C**). Usually, the estimation of the activation of these kinases is performed 30-60 minutes after their activation. However, whereas the cell response in migration test was examined after 24 hours, the phosphorylation level of these kinases was assessed after 24 and 48 hours. Obtained results convincingly indicate the activating paracrine effect of human fibroblasts on the mTOR / S6K signaling network of MCF-7 cells.

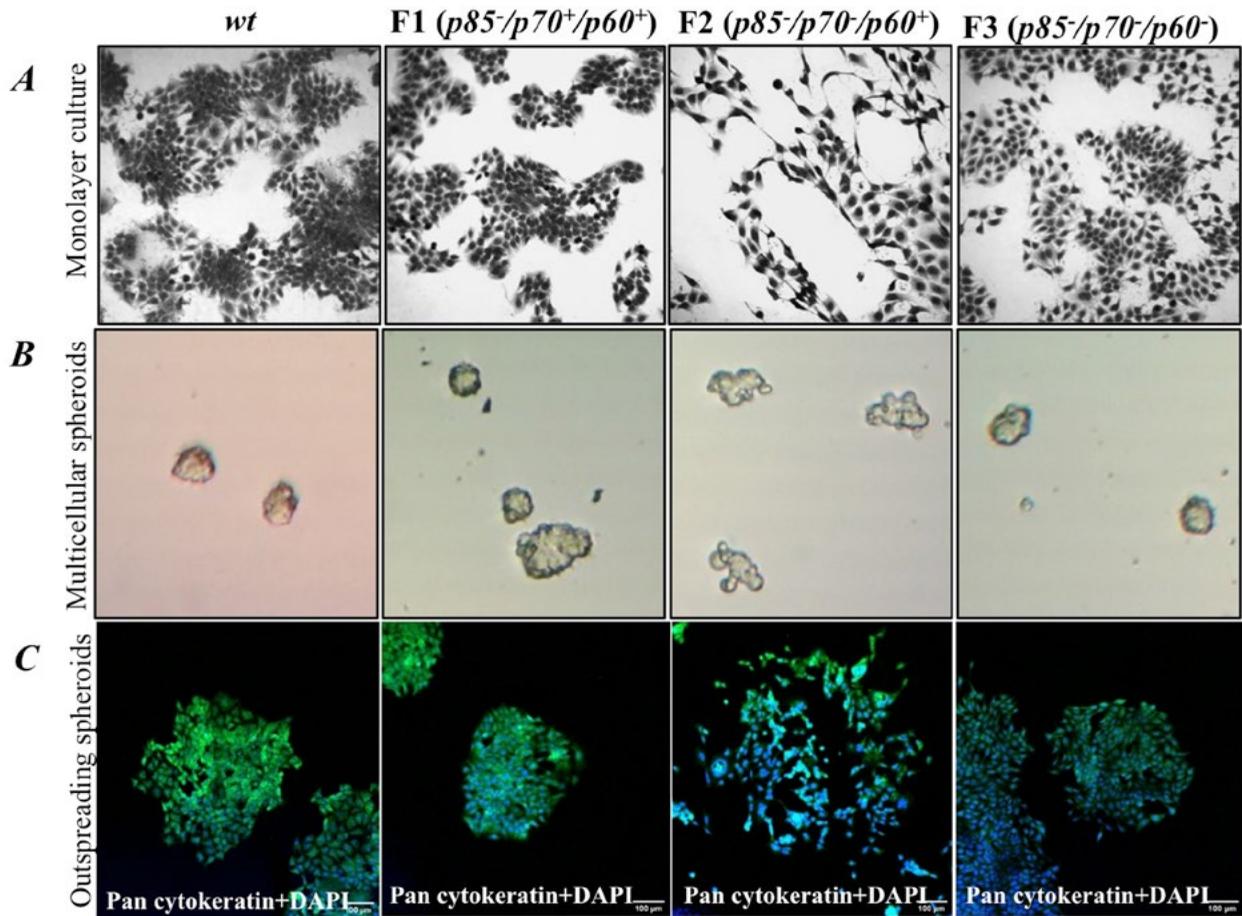
*Morphological analysis of tumor cells with altered expression of S6K1 isoforms under 2D and 3D culture conditions.* Recently we claimed generation of MCF-7 cells with edited by CRISPR/Cas9 technology expression of p60, p70 and p85 S6K1 isoforms: F1 (*p85<sup>-</sup>/p70<sup>+</sup>/p60<sup>+</sup>*), F2 (*p85<sup>-</sup>/p70<sup>-</sup>/p60<sup>+</sup>*), and F3 (*p85<sup>-</sup>/p70<sup>-</sup>/p60<sup>-</sup>*) [13,14]. It was demonstrated that alterations in the S6K1 isoforms expression differently affects cell migration [14]. Down regulation of the main S6K1 isoforms p70 and p85 with sustained expression of p60 S6K1 dramatically enhances the locomotor activity of MCF-7 cells. Interesting, down regulation of all S6K1 isoforms (clone F1) does not have such effect indicating the prominent role of p60 isoform. The further morphological analysis revealed differences in the shape and type of cell growth of different clones. The most significant differences were observed in clone F2 (**Fig. 2.A**). Immediately after passage in monolayer culture, these cells demonstrated elongated fibroblastic form, unlike other clones, which had morphological features of epithelial cells. However, over time, when the

cell density and the number of cell contacts got increased the cell morphology switched again to rather epithelia like, but remained elongated and polygonal rather than rounded (**Fig. 2 A**), which may indicate the epithelial-mesenchymal plasticity of these cells.

The differences were also detected in the growth type of different clones under three-dimensional culture. In course of multicellular spheroid generation from MCF-7 cells and derivatives on the agarose surface, it was found that MCF-7, F1 and F3 clones formed round shaped spheroids, in contrast, the cells of F2 clone formed irregular-shaped aggregates with signs of multifocal growth (**Fig. 2.B**). The sphericity index of their shape was reduced by about 25 % (data not shown) in comparison to wild type cells, F1 and F3 clones.

Morphological analysis of migrating cells also revealed significant changes in the pattern of cell migration of different clones (**Fig. 2.C**). For evaluation it was applied a method of initiation of cell migration from 3-dimensional multicellular spheroid onto growth surface. The obtained results showed a different migration mode for the multicellular spheroids of F2 clone compared to the wild-type MCF-7 cells. Visually the cells of clone F2 migrated more actively to the growth surface, they significantly lost intercellular connections, retaining strong contacts only between the cells in small groups. The cell migration pattern of clones F1 and F3 was almost indistinguishable from that of the wild-type MCF-7 cells.

The obtained data prompted an assessment of the expression levels and activation status of some molecules responsible for the adhesion and migration. Therefore, for further analysis of the cell migration activity, the phosphoryla-



**Fig. 2.** Morphological analysis of monolayer culture of MCF-7 wild-type (MCF7) cells and stable cell lines F1 ( $p85^+/p70^+/p60^+$ ), F2 ( $p85^-/p70^-/p60^+$ ), F3 ( $p85^-/p70^-/p60^-$ ). May-Grunwald Giemsa staining (A). Morphological analysis of multicellular spheroids formed by the cells of MCF-7 wild-type (wt) cells and stable cell lines F1 ( $p85^+/p70^+/p60^+$ ), F2 ( $p85^-/p70^-/p60^+$ ), F3 ( $p85^-/p70^-/p60^-$ ). Transmitted light microscopy (B).

tion level of focal adhesion kinase (FAK) (Tyr925) as an indicator site of cell locomotor capacity was determined in studied cells. FAK is a cytoplasmic protein tyrosine kinase that is overexpressed and activated in several cancers: for example, prostate or breast cancer [30]. Also, the inhibition of FAK activity arrested breast carcinoma growth as spheroids and as orthotopic tumors [31].

The result of a Western blot analysis of phosphorylated FAK content revealed that the highest signal was observed in clone F2. The level of FAK phosphorylation in clones F1 and F3 was comparable to that in wild-type MCF-7 cells. (Fig. 3). Since the cell migration is ensured by coordinated phases of FAK activation and integrin beta 1 (CD29) functioning, the content of the latter in the cells under study

was evaluated [32]. The result of Western blot analysis revealed that the signal for CD29 was significantly reduced in F1 and F3 while in F2 it was at the level of control parental MCF-7 cells. The similar situation was observed for CD44 (**Fig. 3**), a glycoprotein involved in the formation of intercellular contacts and regulation of cell adhesion.

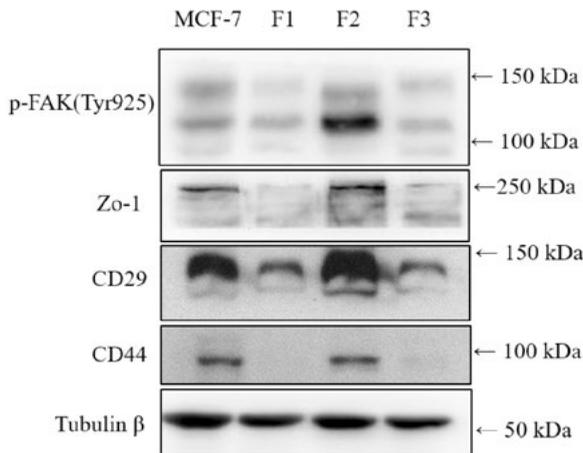
Since morphological analysis revealed the differences in the intercellular contacts of migrating cells of the studied MCF-7 clones, the expression level and localization of ZO-1 protein were also analyzed. ZO-1 is one of the most common proteins of epithelial tight junctions localized on the apical membrane. Previous studies have shown that in podocytes, the cells responsible for blood filtration in the kidney, mTOR pathway signaling impaired barrier function due to the internalization of the protein TJ (tight junction) adapter ZO-1. Besides, along with the loss of epithelial mark-

ers of E-cadherin and ZO-1, the bladder tissue acquired the features of epithelial-mesenchymal transition characteristic of oncotransformed cells [33].

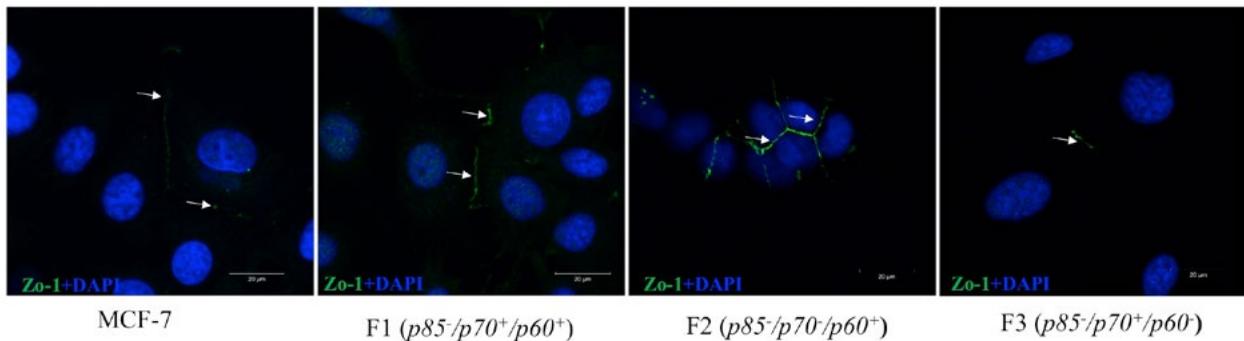
Western blot analysis showed a reduced expression of ZO-1 in clones F1 and F3, and despite the active pattern of cell migration, an increased expression in F2 compared with wild-type cells. These results prompted the immunofluorescence analysis to determine the content and localization of the ZO-1 protein (**Fig. 4**). For detection of intercellular contacts, the cells were cultured for several days, and the groups of contacting cells were analyzed. The data obtained showed a similar reaction. In the MCF-7 cells wild type, F1 ( $p85^-/p70^+/p60^+$ ) and F3 ( $p85^-/p70^-/p60^-$ ), a weak immunofluorescence response of ZO-1 was observed. Moreover, tight contacts were found only between some cells, and not between all. Instead, the cells of clone F2 ( $p85^-/p70^-/p60^+$ ) showed a strong positive reaction between all cells in contact.

*Analysis of the influence of fibroblast-produced factors on locomotor properties of tumor cells with altered expression of S6K1 isoforms.* To determine, which of S6K1 isoforms of tumor cell may be the most sensitive or resistant to the modulating effect of the tumor microenvironment, the mentioned clones were tested for phosphorylation of rpS6 and focal adhesion kinase FAK. For this purpose, Western blot analysis was performed on the cell samples that were pre-incubated with human dermal fibroblast conditioned medium.

Ribosomal protein S6 (rpS6) is considered as a classic target of S6K1 and its phosphorylation level indicates the activity of S6K1. The phosphorylation of this protein at Ser235/236



**Fig. 3.** Western blot analysis of FAK kinase phosphorylation level at tyrosine 925, content of Zo-1, CD29, CD44 in MCF-7 wild-type (wt) cells and stable cell lines F1 ( $p85^-/p70^+/p60^+$ ), F2 ( $p85^-/p70^-/p60^+$ ), F3 ( $p85^-/p70^-/p60^-$ ).



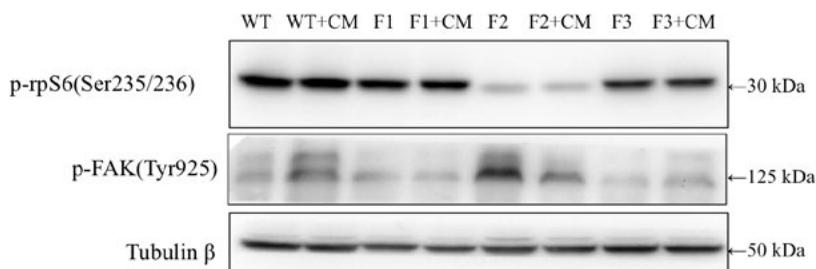
**Fig. 4.** Immunofluorescence analysis of ZO-1 (green) tight junction protein subcellular localization in wt MCF-7 cells and stable cell lines F1 ( $p85^-/p70^+/p60^+$ ), F2 ( $p85^-/p70^-/p60^+$ ), F3 ( $p85^-/p70^+/p60^-$ ). Nuclei counterstained with Hoechst 33342. Arrows point out Zo-1 positive intercellular contacts. Bar corresponds to 20  $\mu$ m.

can be initiated by several kinases, primarily S6K1 and S6K2, p90S6K, and protein kinase A [34]. An analysis of wild-type MCF-7 cells and cells with altered S6K1 expression for rpS6 phosphorylation confirmed this statement (**Fig. 5**). The lowest phosphorylation level of rpS6(Ser235/236) was detected in clone F2 ( $p85^-/p70^-/p60^+$ ), with completely knocked out p70 and p85 S6K1 isoform.

The data obtained indicate that p60S6K1 is not able to phosphorylate the ribosomal protein rpS6. On the other hand, it is difficult to explain why knock out of all three S6K1 isoforms even partially rescues the rpS6 phosphorylation. It seems that p60 S6K1 may have a negative effect on other rpS6 effectors, for

example S6K2. The fibroblast conditioned medium does not have any effect on the ability to phosphorylate the rpS6 in either the wild-type cells or the cells with altered S6K1 expression.

The literature data suggest that FAK promotes tumor progression and metastasis [35]. Usually, high FAK expression is associated with significant tumor growth, increased invasive phenotype, and poor prognosis of anticancer therapy [36, 37]. Besides, it was revealed that FAK phosphorylation including Tyr925 is not only the index of cancer cell motility but epithelial-mesenchymal transition as well [38]. Therefore, the level of FAK (Tyr925) phosphorylation was verified in MCF-7 cells and



**Fig. 5.** Western blot analysis of p-rpS6 (Ser235/236) and p-FAK (Tyr925) in (wt) MCF-7 cells and stable cell lines F1 ( $p85^-/p70^+/p60^+$ ), F2 ( $p85^-/p70^-/p60^+$ ), F3 ( $p85^-/p70^+/p60^-$ ) under the standard culture conditions and with the addition of fibroblast conditioned medium (CM).

derivates under the influence of fibroblast conditioned medium (**Fig. 5**). In case of MCF-7 wild-type cells, the addition of fibroblast conditioned medium resulted in a significant elevation of the level of FAK phosphorylation at Tyr925. However, under regular cell growth conditions the highest p-FAK (Tyr925) content was detected in cells of clone F2 (*p85<sup>-</sup>/p70<sup>-</sup>/p60<sup>+</sup>*). Instead, the addition of fibroblast conditioned media significantly reduced the level of FAK phosphorylation. So, a high level of FAK phosphorylation in wild-type MCF-7 cells under the influence of fibroblast produced factors confirmed that tumor microenvironment had a tumor-stimulating effect. On the other hand, the paracrine effect of fibroblasts demonstrated a negative feedback on FAK phosphorylation in cells with selective expression of p60S6K1.

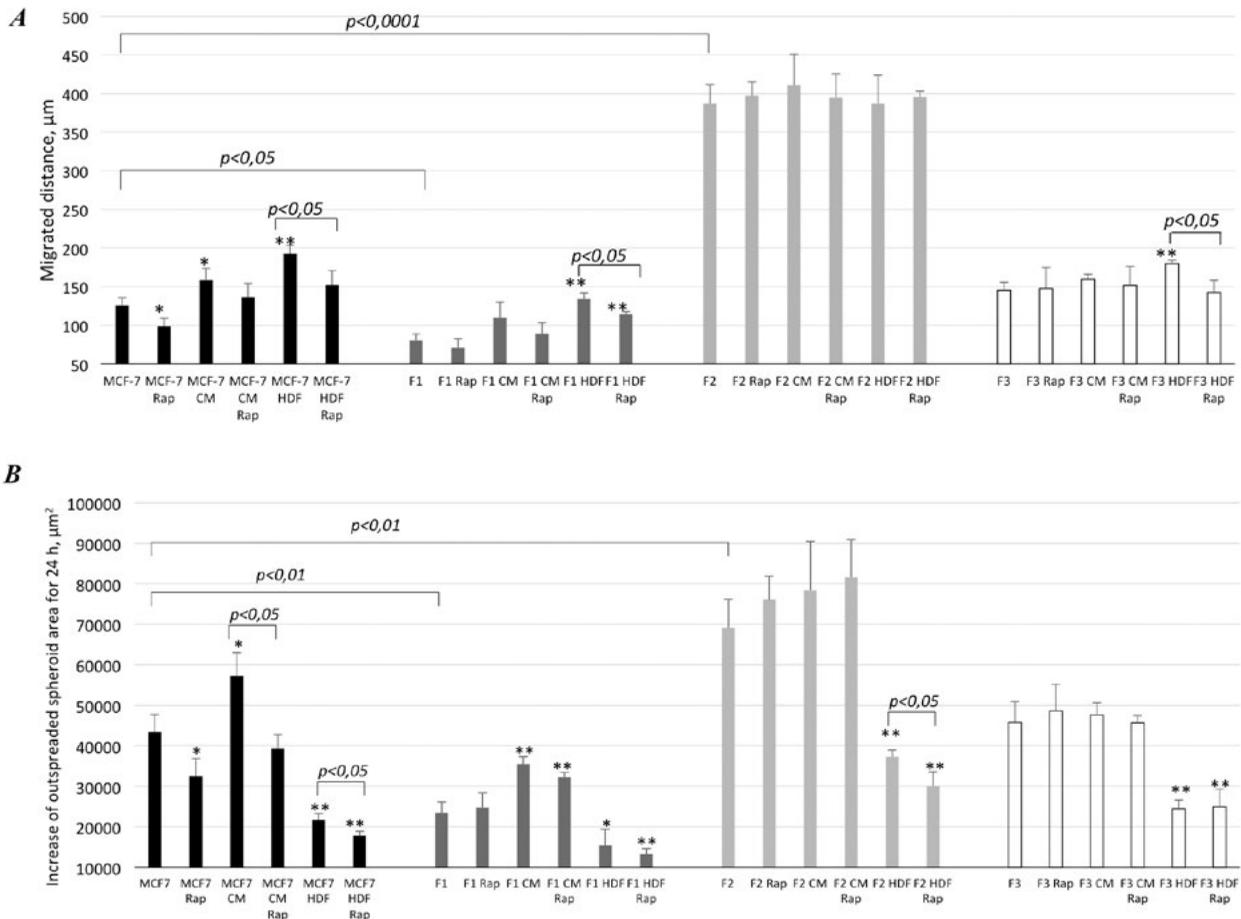
Thus, the obtained data evoked the evaluation of locomotor properties of cells with altered S6K1 expression under the influence of tumor microenvironment factors. Scratch test was applied to the cells cultured under the following conditions: intact cells, cells under the influence of rapamycin, cells under the influence of medium conditioned by fibroblasts, a combination of fibroblast conditioned medium and rapamycin, tumor cells co-cultivation with fibroblasts located around the perimeter of a Petri dish, and the effect of rapamycin on tumor cells co-cultured with fibroblasts. The analysis of the migration activity of wild-type MCF-7 cells showed that inhibition of the mTOR/S6K signaling network led to a decrease in the rate of tumor cell migration (**Fig. 6.A**). The presence of the factors produced by fibroblasts in the growth medium accelerated the movement of these cells, and the addition of rapamycin

returned the migratory activity of the investigated cells to the control level. Noteworthy, co-cultivation with fibroblasts was the most powerful enhancer of the migration potential of wild-type MCF-7 cells in our experiment. In this case, the addition of rapamycin led to inhibition of mTOR/S6K signaling in both tumor cells and fibroblasts (which was more similar to the conditions of the body), and reduced cell motility to the level of the control cells. Thus, the activating effect of fibroblasts, both the conditioned medium and co-culturing, on the tumor cell motility was found as well as the inhibitory effect of rapamycin in the presence or absence of fibroblasts in the culture test system.

It has also been demonstrated that knockdown of the p85S6K1 isoform resulted in a significant decrease in the locomotor capacity of F1 clone (*p85<sup>-</sup>/p70<sup>+</sup>/p60<sup>+</sup>*) cells. However, the effect of all factors studied was quite similar to that observed in wt MCF-7 suggesting the main role of p60 or p70 isoform in such cell response.

A dramatic increase of migration capacity of MCF-7 cells with knockdown of p85S6K1 and p70S6K1, namely clone F2 (*p85<sup>-</sup>/p70<sup>-</sup>/p60<sup>+</sup>*) [14], was confirmed. Interestingly, the cells that expressed only p60S6K1 were insensitive to either rapamycin, fibroblast conditioned media, or to co-cultivation with fibroblasts.

The knockdown of all three isoforms of p85S6K1, p70S6K1, and p60S6K1 in our experiment resulted in a slight but statistically unreliable increase in the tumor cell motility of clone F3 (*p85<sup>-</sup>/p70<sup>-</sup>/p60<sup>-</sup>*). The cells of this clone remained insensitive to the effects of rapamycin and fibroblast conditioned media. Only the direct co-cultivation with fibroblasts



**Fig. 6.** Estimation of the motility of MCF-7 cells with edited expression of S6K1 under the effect of the factors of local tumor environment. **(A)** Analysis of locomotor activity of wtMCF-7 cells and stable cell lines F1 ( $p85^-/p70^+/p60^+$ ), F2 ( $p85^-/p70^-/p60^+$ ), F3 ( $p85^-/p70^-/p60^-$ ) under experimental conditions: intact cells; treatment with 10nM rapamycin (Rap); adding 20% of the medium conditioned by human dermal fibroblasts (CM); combination of conditioned medium and rapamycin (CM Rap); direct co-culture of tumor cells and human dermal fibroblasts (HDF); direct co-culture of tumor cells and fibroblasts in the presence of rapamycin (HDF Rap) in scratch test. **(B)** Analysis of migratory capacity in the test of multicellular spheroid outspreading of wild-type MCF-7 cells and stable cell lines F1 ( $p85^-/p70^+/p60^+$ ), F2 ( $p85^-/p70^-/p60^+$ ), F3 ( $p85^-/p70^-/p60^-$ ) under the experimental conditions: intact spheroids; treatment with 10nM rapamycin (Rap); addition of 20% of the medium conditioned by human dermal fibroblasts (CM); combination of conditioned medium and rapamycin (CM Rap); outspreading of tumor cell spheroids on the monolayer of human dermal fibroblasts (HDF); outspreading of tumor cell spheroids on the monolayer of human dermal fibroblasts in the presence of rapamycin (HDF Rap). Data are expressed as mean  $\pm$  SD, and analysed using unpaired Student's t-test (GraphPadPrism 6.001), \*  $p < 0.05$ , \*\*  $p < 0.01$  in comparison to corresponding intact cells.

enhanced the migration properties of these cells. Noteworthy, the data obtained do not confirm the preliminary data on increased locomotor properties of this clone [14]. A possible explanation may be that the scratch test was applied to these cells at the later cultivation passages. We hypothesize that, by analogy with the animal model [39], knockdown of all S6K1 isoforms may lead to the compensatory overexpression of S6K2, which may partially take over the functions of knockout S6K1.

So, according to our data, knockdown of p85S6K1 and p70S6K1 in clone F2 led to a significant increase in the cell motility. The knockdown of all three isoforms had no significant effect on the migration capacity of MCF-7 cells. An increased FAK phosphorylation, and CD29 and CD44 content in clone F2 could shed light on its high migration potential. It was detected an increase of tight junction protein content in cells of this clone on one hand, and changing the morphology of their multicellular spheroids on the other hand. Therefore, to evaluate the tumorigenic potential of cells with edited expression of S6K1 isoforms a three-dimensional cell culture model was applied as well. For this purpose, it was used a method that combined the cell adhesion to the growth surface with the subsequent cell dissemination, namely, the transfer of multicellular spheroids onto the growth surface and their outspreading. For this test, the following experimental conditions were applied: spreading of tumor cell spheroids on culture plastic, addition of rapamycin or fibroblast conditioned medium, or a combination of fibroblast conditioned medium with rapamycin, as well as spreading tumor spheroids on the fibroblast monolayer with or without rapamycin. First of

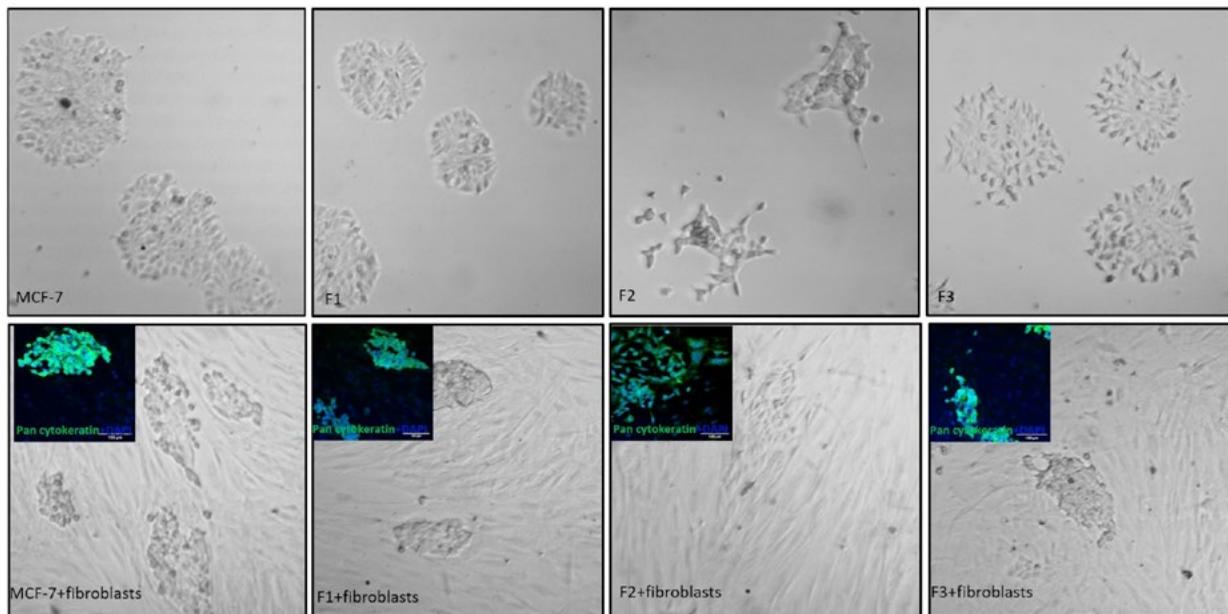
all, it is important to indicate the significant modulating effect of fibroblast monolayer on the morphology of outspreading spheroids (**Fig. 7**). As indicated previously (**Fig. 2.C**), cells migrated from spheroids onto culture plastic predominantly formed monolayer colonies of round shape. Instead, in direct co-culture of tumor spheroids with the fibroblast monolayer the tumor cells migrated along the fibroblast growth lines (**Fig. 7**).

To assess the ability of cells to propagate from a three-dimensional spheroid to the growth surface, the difference between the colony area after 24 hours of migration and the initial spheroid just adhered to the substrate was calculated (**Fig. 6.B**). An analysis of wild-type MCF-7 cells revealed that rapamycin statistically significantly suppressed the cell distribution. Instead, the addition of fibroblast conditioned media facilitated the rapid spread of cells. Rapamycin statistically significant suppressed the effect of the conditioned medium. Notable, the effect of direct co-cultivation of tumor spheroids with monolayer of fibroblasts was completely opposite to the conditioned medium effect. In this case, there was a significant inhibition of tumor cell dissemination. Rapamycin, acting on both tumor cells and the monolayer of fibroblasts, also statistically significant suppressed also the outspreading of tumor spheroids. The knockdown of p85S6K1 led to a considerable decrease of the tumor spheroid outspreading (**Fig. 6.B**). The cells of clone F1 under these experimental conditions were insensitive to rapamycin. As wild-type cells, the spheroids formed by clone F1 cells propagated much more intensely under the influence of the paracrine factors of fibroblast conditioned medium.

Rapamycin slightly reduced the effect of the conditioned medium, but not statistically significant. Direct co-cultivation with fibroblast monolayer caused lowering ability of tumor spheroid to spread. The delaying effect of fibroblasts was slightly enhanced when rapamycin was added to the F1 tumor spheroid-fibroblast co-culture.

Similarly to the increased locomotor activity of F2 clone with selective expression of p60S6K1 in the monolayer Scratch test, the cells demonstrated active dissemination of the growth surface from multicellular spheroid (**Fig. 6.B**). However, noteworthy the pattern of these cells propagation. Outspreading spheroids of wild-type cells, the clones F1 and F3 formed colonies of contacting cells (**Fig. 2**,

**Fig. 7**). Instead, the cells of clone F2 multicellular spheroids being transformed in the monolayer cell colony visually substantially lost their intercellular contacts, and expanded through the groups of cells, and sometimes single cells, although, in monolayer culture the tight junctions Zo-1 protein content in the clone F2 cells was higher than in wild-type MCF-7 cells and clones F1 and F3 (**Fig. 3**). Therefore, to assess the ability of these cells to disseminate the growth surface, we determined the area covered by large and small groups of cells that migrated from one spheroid. Like in the case of the scratch test, the outspreading spheroids of clone F2 were the most mobile and insensitive to either rapamycin or growth factors of fibroblast conditioned



**Fig. 7.** Assay of migrating cell morphology, 24 h after initiation of migration of wtMCF-7 cells and stable cell lines F1 ( $p85\text{-}/p70\text{+}/p60\text{+}$ ), F2 ( $p85\text{-}/p70\text{-}/p60\text{+}$ ), F3 ( $p85\text{-}/p70\text{-}/p60\text{-}$ ) from the multicellular spheroids onto the growth surface and onto the fibroblast monolayer. Transmitted microscopy oc.10x, ob.10. Confocal microscopy: epithelial cells revealed by anti-pan cytokeratin antibody (green), nuclei counterstained with Hoechst 33342 (blue), bar corresponds 100  $\mu\text{m}$ .

media. Additionally, the reduction of propagation velocity of F2 clone cells was statistically significant when these spheroids outspreaded on the fibroblast monolayer. Rapamycin, which affected the both tumor cells and fibroblast monolayer in co-culture conditions, enhanced the effect of fibroblasts.

Besides, there was no statistically significant difference in the ability of tumor multicellular spheroids to scatter on the growth surface between the MCF-7 wild-type cells and the F3 clone with inhibited expression of p85S6K1, p70S6K1, and p60S6K1. These cells also lost the sensitivity to the effects of rapamycin and fibroblast conditioned media. At the same time direct co-cultivation with the monolayer of fibroblasts led to the inhibition of their ability to migrate from the multicellular spheroid.

So, our results indicate the importance not only of the presence but also of the ratio of different isoforms of the S6K1 molecule for cell behavior. Thus, the highest level of FAK phosphorylation was detected in the cells of clone F2 (*p85/p70/p60*<sup>+</sup>) with selective expression of p60S6K1, however, this isoform is expressed together with p70S6K1 in the cells of clone F1 (*p85/p70*<sup>+</sup>/*p60*<sup>+</sup>), but the level of FAK phosphorylation in these cells is much lower. Moreover, in wild-type cells that express all the isoforms under study, the level of FAK phosphorylation is higher than in F1 but lower than in F2. Therefore, if p60S6K1 has a direct or indirect positive effect on FAK phosphorylation, then it can be assumed that p70S6K1 negates this effect. The same analogies can be made concerning the determination of tight junction protein Zo-1 content in different clones.

Although the scratch test for assessing the cell migration activity is not a direct analogue

of the test for determining the ability of cells to spread from the multicellular spheroid to the growth surface, they both characterize the metastatic potential of tumor cells associated with the mobility of the latter. Scratch test refers to two-dimensional cultivation systems, whereas spheroids - to three-dimensional one. The difference in tumor cell behavior under two- and three-dimensional culture conditions has been described in a number of studies [40]. Using the second approach, we initiated the adhesion and migration of tumor cells by transferring multicellular spheroids from the non-adhesive surface to the adhesive one, which resulted in the transformation of a three-dimensional spheroid into a two-dimensional cell colony. However, in both tests we observed similar cell behavior, F1 clone cells were less mobile than wild-type cells, whereas no statistically significant difference in the motility of F3 clone cells and wild-type cells was observed. Instead, the cells of clone F2 migrated several times faster under two-dimensional conditions as well as after transfer of spheroids onto the adhesive substrate. Noteworthy, under two-dimensional test conditions, co-cultivation with fibroblasts stimulated the locomotor properties of cells more strongly than the addition of conditioned medium. However, in contrast to the fibroblast conditioned medium, direct contact of tumor spheroids with a monolayer of fibroblasts led to a significant slowdown of spheroid spreading.

Taking into the consideration the significance of S6K signaling for cell vital function and considerable differences of S6K signaling at cancer progression and normal tissue [41, 42] there is still a strong need for an in-depth comprehensive analysis of this signal link.

Thus, the presented work compared the behavior of breast cancer cells with the editable expression of different S6K1 isoforms *in vitro* and revealed a modulating effect of tumor microenvironment on the manifestation of the malignant phenotype of these cells. The results obtained provide a basis for further analysis of the investigated isoforms in the carcinogenesis and the role of tumor stroma in the regulation of this process.

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#### **Відповідь пухлинних клітин із редагованою експресією S6K1 на вплив пухлинного мікрооточення.**

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**Мета.** Дослідити участь mTOR/S6K1 сигнальної мережі пухлинних клітин у їхній відповіді на регуляторний вплив фібробластів. **Методи.** Культура клітин, включаючи спів-культивування фібробластів та пухлинних клітин, імунофлуоресцентний аналіз, вестерн-блот-аналіз, оцінка міграційної активності пухлинних клітин методом подряпин та перетворення багатоклітинного сфероїду в одношарову клітинну колонію. **Результати.** Представлена робота продемонструвала позитивний вплив стромальних клітин на рівень фосфорилування ланок mTOR/S6K1

сигнального каскаду: p85S6K1, p70S6K1 та mTOR пухлинних клітин лінії MCF-7. Щоб визначити, яка з ізоформ S6K1: p85S6K1, p70S6K1 чи p60S6K1 може бути найбільш чутливою до дії позаклітинного середовища, були використані стабільні клітинні лінії на основі клітин MCF-7 з редагованою експресією ізоформ S6K1. Таким чином, було встановлено, що селективна експресія p60S6K1 призводила до зміни морфологічних особливостей пухлинних клітин за умов дво- та тривимірної культури. Ці клітини також демонстрували високий рівень фосфорилування кінази фокальної адгезії та високий вміст білків Zo-1, CD29, CD44, що пояснює їх високий міграційний потенціал в тесті на подряпини. Крім того, клітини, що селективно експресують p60S6K1, були стійкими як до факторів, що продукують фібробласти так і до рапаміцину. Також, було продемонстровано, що фібробласти підвищують рухливість пухлинних клітин в тесті на подряпини та розпластуванні сфероїдів в умовах спів-культивування паракриновим шляхом, тоді як безпосередній контакт пухлинних сфероїдів з моношаром фібробластів значно знижував швидкість розпластування сфероїдів. **Висновки.** Аналіз поведінки пухлинних клітин із зміненою експресією ізоформ S6K1 виявив, що для життєдіяльності клітин та їхньої відповіді на вплив найближчого оточення важливі не тільки експресія певних ізоформ, а й їх співвідношення в клітині.

**Ключові слова:** S6K1, міграція, пухлинне мікрооточення.

#### **Ответ опухолевых клеток с редактированной экспрессией S6K1 на воздействие опухолевого микроокружения.**

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**Цель.** Исследовать участие mTOR/S6K1 сигнальной сети опухолевых клеток в их ответе на регуляторное влияние фибробластов. **Методы.** Культура клеток, включая со-культивирование фибробластов и опухолевых клеток, иммунофлуоресцентный анализ, вестерн-блот-анализ, оценка миграционной активности опухолевых клеток методом царапин и преобразования многоклеточного сфероида в монослойную клеточную колонию. **Результаты.** Представленная работа продемонстрировала положительное влияние стромальных

клеток на уровень фосфорилирования звеньев mTOR/S6K1 сигнального каскада: p85S6K1, p70S6K1 и mTOR опухолевых клеток линии MCF-7. Чтобы определить, какая из изоформ S6K1: p85S6K1, p70S6K1 или p60S6K1 может быть наиболее чувствительной к действию внеклеточного окружения, были использованы стабильные клеточные линии на основе клеток MCF-7 с редактированной экспрессией изоформ S6K1. Таким образом, было установлено, что селективная экспрессия p60S6K1 приводила к изменению морфологических особенностей опухолевых клеток в условиях двух- и трехмерной культуры. Эти клетки также демонстрировали высокий уровень фосфорилирования киназы фокальной адгезии и высокое содержание белков Zo-1, CD29, CD44, что объясняет их высокий миграционный потенциал в тесте на царапины. Кроме того, клетки, селективно экспрессирующие p60S6K1, были устойчивыми как к факторам, которые продуци-

руют фибробласты так и к рапамицину. Также, было продемонстрировано, что фибробласты повышают подвижность опухолевых клеток в тесте на царапины и распластывание сфероидов в условиях со-культивирования паракринным путем, тогда как непосредственный контакт опухолевых сфероидов с монослоем фибробластов значительно снижал скорость распластывание сфероидов. **Выводы.** Анализ поведения опухолевых клеток с измененной экспрессией изоформ S6K1 обнаружил, что для жизнедеятельности клеток и их ответа на факторы микроокружения важны не только экспрессия определенных изоформ, но и их соотношение в клетке.

**Ключевые слова:** S6K1, миграция, опухолевое микроокружение.

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