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Alterations in S6K1 isoforms expression induce Epithelial to Mesenchymal Transition and Estrogen Receptor 1 Silencing in human breast adenocarcinoma MCF-7 cells

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Aim. To evaluate an impact of different S6K1 isoforms expression in MCF-7 cells on initiation of Epithelial to Mesenchymal Transition (EMT). **Methods.** Immunocytochemical analysis, Real-Time PCR, Western blot. **Results.** We have demonstrated that unbalanced expression of p60, p70 and p85-S6K1 isoforms in MCF-7, namely downregulation of p70 and p85, and basal p60 expression, mediated by the CRISPR-Cas9 gene editing resulted in altered morphology and increased cell motility. Such changes were associated with the expression of genes whose products are involved in the regulation of cell motility, interaction with the extracellular matrix and loss of cellular adhesion demonstrating their increased potential for invasion and metastatic activity. qPCR analysis confirmed increased expression of a whole spectrum of genes associated with mesenchymal characteristics and loss of epithelial specific markers. Additionally, we observed a complete repression of the estrogen receptor 1 (*ESR1*) expression and downregulation of HER2/NEU *(ERBB2)*. **Conclusions.** Our data demonstrate for the first time the implication of S6K1 isoforms in the regulation of EMT in MCF-7 cells by triggering for the concomitant onset of a series of possibly parallel events that changes the cell from an epithelial to a mesenchymal type and has a strong negative impact on *ESR1* expression. At the tumor level that can mean breast tumor transition to a more aggressive most probably triple negative molecular subtype.

Keywords: S6K1 isoforms, breast cancer, MCF-7 cells, EMT, *ESR1*.

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Introduction

Ribosomal protein S6 kinase 1 (S6K1) functions as a component of the mTOR/S6K1-dependent signaling pathway, that is a central integrator of a wide range of extracellular signals. S6K1 modulates a number of cellular processes, including gene expression, protein synthesis, lipid and energy metabolism, and thus regulates cell growth, proliferation and migration through the action of its kinase activity on a large number of downstream mediators [1].

In mammals, the S6K1 gene encodes several isoforms that are responsible for all mentioned functions [2, 3].

Most of the earlier studies were focused on the p70-S6K1 and p85-S6K1 isoforms, which are translated from the common mRNA but using alternative translation starts [1–3]. Additionally, one splice variant of S6K1, called p31-S6K1, which is truncated from the C-terminus [4] and has no kinase activity, but exhibits oncogenic properties has been characterized.

Recently, we proved the existence of a putative p60-S6K1 isoform that can be translated either from an mRNA transcript common to p70 and p85 S6K1 isoforms using a third translation start, or from novel spliced mRNA [5, 6].

At the protein level, all three mentioned isoforms differ from each other only in the length of their N-terminal regulatory regions due to the implementation of alternative mRNA translation starts [2, 3, 5, 7], which, in turn, may affect their cellular functions and regulation mode.

Numerous studies indicate an important role of S6K1 in cancer progression. Deregulation of S6K1 signaling caused by kinase overexpression and overactivation has been found in various malignancies, including breast, lung, thyroid, brain, and esophageal cancer, which very often correlates with poor disease prognosis [8–15] and therefore makes the kinase a promising therapeutic target for cancer treatment [16].

Unfortunately, the exact role of each S6K1 isoform in the regulation of vital cellular functions, as well as their implication in carcinogenesis, is poorly understood.

To extend our knowledge about the functions of S6K1 isoforms and especially the less studied p60-S6K1 in the cell, we generated HEK 293 cells with differential expression of S6K1 isoforms. According to our data, the cells with CRISPR-Cas9-mediated knockout of all S6K1 isoforms showed the inhibition of cell proliferation and migration [17]. However, down-regulation of only the p70 and p85 S6K1 isoforms with intact p60-S6K1 expression had only minor effects on the cell behavior [2]. In all cases, deregulation of S6K1 isoforms expression does not affect phosphorylation of the S6K1 substrates analyzed that could be explained by the existence of homologous S6K2 that shares most of the substrates with S6K1.

Further analysis revealed that p60-S6K1, unlike p70 and p85 isoforms of S6K1, does not undergo mitogen-stimulated phosphorylation at PDK1- and mTORC1-dependent sites critical for the S6K1 activity (Thr 229 and Thr 389, respectively). At the same time, analysis of the p60-S6K1 kinase activity *in vitro* confirmed its ability to phosphorylate the well-known S6K1 substrate ribosomal protein S6 (rpS6) with almost the same efficiency as p70- and p85-S6K1. Noteworthy, the p60-S6K1

activity happened to be rapamycin-independent suggesting the existence of mTORC1-independent mechanism of the p60-S6K1 regulation in HEK-293 cells. Moreover, the modulation of the p60-S6K1 activity via the PI3K/mTORC1 signaling appears to be cell-type specific, since in the breast cancer cell line MCF-7 the p60-S6K1 isoform undergoes PDK1- and mTORC1-mediated phosphorylation, suggesting its tissue specific activity and thus the different function of p60-S6K1 in normal and tumor cells.

Considering the important role of S6K1 in the breast tumor progression, we aimed to evaluate the activity of S6K1 isoforms in breast carcinogenesis. To this end we applied CRISPR-Cas9-mediated differential knockout of p85, p70 and p60 isoforms of S6K1 in the breast adenocarcinoma MCF-7 cell line and demonstrated that these isoforms affect the cancer cells behavior in a specific manner [18].

The most profound effect was observed for the cells with knocked out expression of p70 and p85 isoforms of S6K1 and intact expression of p60-S6K1. These cells demonstrated significant acceleration of the locomotor activity and acquisition of a fibroblast like phenotype that may suggest induction of EMT transition, which is a hallmark of the metastatic and most aggressive tumor types.

In the present study, we analyzed the expression of a number of EMT-related genes in the cell sublines expressing different S6K1 isoforms, and confirmed EMT in the MCF-7 cells that express only p60-S6K1.

Materials and Methods

Cell culture. Human breast adenocarcinoma cell line MCF-7 derived from the ATCC (American Type Culture Collection, USA) was maintained at 37 °C in a humidified atmosphere of 5 % CO_2 in DMEM (Gibco, USA) supplemented with 10 % fetal calf serum (FCS, HyClone, USA), 4 mM glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin. MCF-7 sublines with differential expression of S6K1 isoforms were generated using the CRISPR-Cas9 gene edition system as previously described [18, 19].

Isolation of RNA. Total RNA was extracted from MCF-7 cell sublines using “GeneJET RNA purification kit” (“Thermo Fisher Scientific”, USA) according to the recommendation of manufacturer. Purified RNA was quantified spectrophotometrically using a NanoDrop (Thermo Scientific, Wilmington, United States).

Reverse transcription. Before cDNA synthesis, total RNA was treated with DNase I (Thermo Fisher Scientific, USA). The reaction mixture (10 μL) that contained total RNA (1 μg), reaction buffer and DNase I (1 U) was incubated for 30 min at 37 °C. To inactivate DNase I, 1 μL of 50 mM EDTA was added followed by 10 min incubation at 65 °C. Total RNA samples were used for cDNA synthesis. The reaction mixture (20 μL) contained 11 μL of total RNA treated with DNase I, 1 μL of Oligo(dT)18 primers (0.5 μg), 4 μL of 5X reaction buffer, 0.5 μL of RNase inhibitor RiboLock, 2 μL of 10 mM dNTP and 1 μL of reverse transcriptase RevertAid (Thermo Fisher Scientific, USA). Before adding reverse transcriptase, RNA was denatured at 65 °C for 5 min. For cDNA synthesis, the complete reaction mixture was incubated for one hour at 42 °C, followed by termination of the reaction at 70 °C for 10 min.

PCR. PCR analysis was applied for the detection of *ESR1* and *ESR2* expression in different sublines of MCF-7 cells. The reaction mixture for PCR (25 μL) contained 1 μL of cDNA, 1 μL of "DreamTaq DNA polymerase" ("Thermo Fisher Scientific", USA), 2.5 μl of 10X "DreamTaq" buffer, 0.5 μl of 10 mM dNTP, 2 μl of 5 μM primers and 18 μl of nuclease free water. For the amplification of DNA fragments of *ESR1, ESR2* and *TBP* the following temperature regime was applied: initial denaturation at 95 °C for 10 min with following 30 cycles of amplification — denaturation at 95 °C for 15s; hybridization and elongation at 60 °C for 4 min. The Primer sequences for PCR detection of transcripts are presented in Table 1.

Real-time qPCR. For real-time PCR we used a Maxima SYBR Green fluorescein qPCR mix kit (Thermo Fisher Scientific, USA) and a DNA amplifier Thermal Cycler CF X96 Real-Time System (Bio-Rad, Singapore). The reaction mixture for quantitative PCR (20 μL) contained 1 μL of cDNA, 10 μL of 2X Master Mix, 0.12 μL of 50 μM forward primer, 0.12 μL of 50 μM reverse primer, and 8.76 μL of nucleases free H_2O. For the amplification the following temperature regime was applied: initial denaturation at 95 °C for 10 min with following 40 cycles of amplification: denaturation at

Table 1. **Primers used for qPCR analysis**

Gene	Forward primer	Reverse primer
TBP	5'-TGCACAGGAGCCAAGAGTGAA-3'	5'-CACATCACAGCTCCCCACCA-3'
CDH1	5'-GTCGAGGGAAAAATAGGCTG-3'	5'-GCCGAGAGCTACACGTTCAC-3'
CDH2	5'-AGCTTCTCACGGCATACACC-3'	5'-GTGCATGAAGGACAGCCTCT-3'
VIM	5'-GCAAAGATTCCACTTTGCGT-3'	5'-GAAATTGCAGGAGGAGATGC-3'
MMP1	5'-CACAAACCCCAAAAGCGTGT-3'	5'-TCGGCAAATTCGTAAGCAGC-3'
MMP2	5'-AAGAAGTAGCTGTGACCGCC-3'	5'-TTGCTGGAGACAAATTCTGG-3'
MMP9	5'-CTCAGGGCACTGCAGGAT-3'	5'-CGACGTCTTCCAGTACCGA-3'
MMP14	5'-CCCCTTTAACTCCAAGCCGA-3'	5'-GGGAGGCAGGTAGCCATATT-3'
CD44	5'-CCTCCGTCTTAGGTCACTGTTTT-3'	5'-TGGAATACACCTGCAAAGCGG-3'
CD24	5'-GGCGCGGACTTTTCTTTTG-3'	5'-CCTTGGTGGTGGCATTAGTTG-3'
ESR1	5'-AGGTGGACCTGATCATGGAG-3'	5'-AAGCTTCGATGATGGGCTTA-3'
ESR2	5'-ACCAAAGCATCGGTCACG-3'	5'-CATGATCCTGCTCAATTCCA-3'
TWIST1	5'-TACGCCTTCTCGGTCTGGAG-3'	5'-CGTCTGGGAATCACTGTCCA-3'
SNAI1	5'-ACCACTATGCCGCGCTCTT-3'	5'-GGTCGTAGGGCTGCTGGAA-3'
SNAI2	5'-ACGCCTCCAAAAAGCCAAAC-3'	5'-ACTCACTCGCCCCAAAGATG-3'
ZEB1	5'-TTCTCATTGTGGAGAGATGACTT-3'	5'-CTGCTCCTCCCTGGTAACAC-3'
ZEB2	5'-CCCAGGAGGAAAAACGTGGT-3'	5'-CTGGACCATCTACAGAGGCTT-3'
CTSD	5'-GCGACAAGTCCAGCACCTAC-3'	5'-CTCTCCACTTTGACACCGCC-3'
TJP1	5'-GGGACAAGATGAAGTACCAGA-3'	5'-GGATTTTCAGAGGATGGCGT-3'
ERBB2	5′-TGTGTGGGAGCTGATGACTT-3′	5′-TCTTGGCCGACATTCAGAGT-3′

95 °C for 15s; hybridization and elongation at 60 °C for 1 min. Expression levels were normalized to housekeeping gene (TBP) for each time point. The ΔCt calculation method was used to measure relative gene expression. Three technical replicates were performed for each reaction. The primer sequences for quantitative real-time PCR are presented in Table 1.

Immunofluorescence analysis of MCF-7 cell lines. MCF-7 cells and MCF-7 sublines seeded onto cover glasses and cultured for 48 hours were fixed with 10 % formalin for 15 min at room temperature (RT). After that, the cells were permeabilized by treatment with 0.2 % Triton X-100 in PBS for 10 min. To reduce autofluorescence, the cells were incubated with 10 mM cupric sulphate and 50 mM ammonium acetate (pH 5.0) for 30 min at RT. Nonspecific binding was blocked by incubation for 30 min at 37 °C in PBS supplemented with 10 % foetal calf serum in a humidifying chamber. For vimentin detection the samples were incubated overnight with anti-vimentin mAbs (Invitrogen, MA5–11883) in dilution 1:1000 in blocking buffer at +4 °C. The secondary (FITC)-AffiniPure Goat Anti-Mouse IgG antibodies (Jackson ImmunoResearch Labs, 115–095–003) were applied in dilution 1:400 for 45 min at 37 °C in a humidified chamber. Samples were embedded into Mowiol medium (Sigma-Aldrich, USA) containing 2.5 % DABCO (Sigma-Aldrich), 0.5 % DAPI (Sigma-Aldrich). Microscopy image acquisition was performed using Zeiss LSM 510 META laser scanning confocal microscope (Carl Zeiss Microscopy GmbH, Germany). Fluorescence images were analyzed using Fiji/ImageJ v1.52b.

Western blot analysis. MCF-7 cells cultured on Petri dish were washed with ice-cold PBS and protein extraction was performed by incubation with lysis buffer, containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 % Triton X-100, supplemented with protease (Termo Scientifc Halt Protease Inhibitor Cocktail) and phosphatase (Halt Phosphatase Inhibitor Cocktail) inhibitors for 15 min at +4 °C. Cell lysates were centrifuged at 13000 rpm for 20 min at 4 °C and the supernatant was collected. 25 μg of extracted proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Immunoreactive protein bands were detected using antibodies directed against: tubulin (1:1000, Invitrogen, A11126); vimentin (1:1000, Invitrogen, MA5–11883); CD326 (1:1000, R.E. Kavetsky IEPOR, NAS of Ukraine); CD227 (1:1000, R.E. Kavetsky IEPOR, NAS of Ukraine; CD66e (1:1000, R.E. Kavetsky IEPOR, NAS of Ukraine); Pan-cytokeratin (clone C11, 1:1000, R.E.Kavetsky IEPOR NAS of Ukraine), Cytokeratin 18 (clone C-04, 1:1000, R.E.Ka—vetsky IEPOR NAS of Ukraine). As secondary antibody we used HRP-AffiniPure Goat Anti-Mouse IgG (1:10000, Jackson ImmunoResearch, 115–035–003) according to the standard protocol.

Results

As we demonstrated in our previous work [18], differential expression of S6K1 isoforms had different impact on the behavior of breast adenocarcinoma MCF-7 cells. Down-regulation of all S6K1 isoforms expression or only p85-S6K1 had the minor effect on cell motility. Instead, MCF-7 cells with $p85^{-}/p70^{-}/ p60^{+}$ phenotype demonstrated significant (3-fold) acceleration of cell migration rate. In addition, these cells were characterized by the appearance of cells with an elongated shape and ar-

chitecture of actin cytoskeleton, which is inherent to mesenchymal cells like fibroblasts and are the hallmarks of migrating cells. Such changes could suggest the possibility of EMT initiation in this subline of MCF-7 cells.

It is known that MCF-7 cell line was initially obtained from human breast tumor of the luminal A subtype, which is the most effectively amenable to treatment and has the most favorable prognosis regarding the course of the disease. However, the imbalance in the expression of S6K1 isoforms could cause the initiation of EMT and the acquisition of MCF-7 cells with a phenotype characteristic of more aggressive subtypes of tumors with signs of invasion and metastasis. To verify this assumption, we analyzed the expression of a number of epithelial and mesenchymal markers in the generated MCF-7 subclones. The immunofluorescent analysis of cells with differential expression of S6K1 isoforms revealed cytoskeletal protein vimentin, a marker of mesenchymal phenotype, only in $p85^-/p70^-/p60^+$S6K1/MCF-7 cells (Fig. 1).

At the same time, this was accompanied by a decrease in the expression of markers of epithelial cells, such as cytokeratins (Fig. 2A).

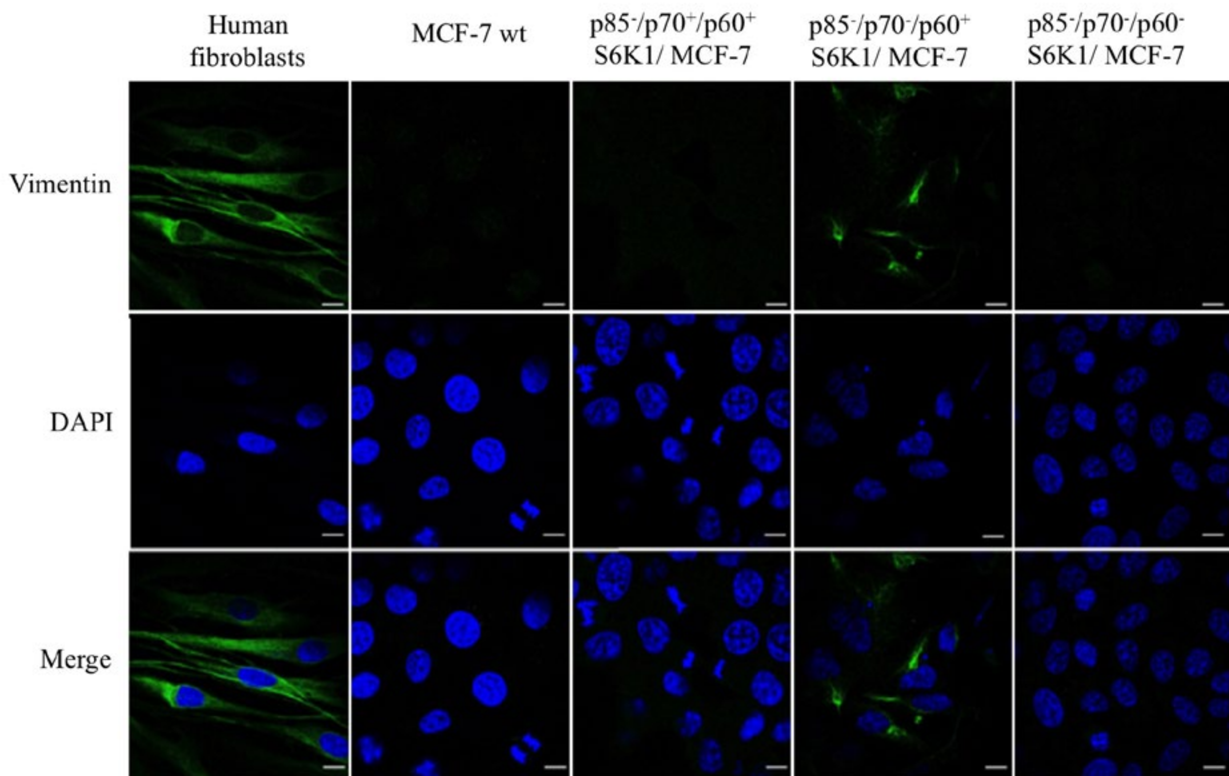

Fig. 1. Vimentin expression in MCF-7 cells depends on the expression of S6K1 isoforms and was detected only in $p85^-/p70^-/p60^+$S6K1/MCF-7 cells. Immunofluorescence staining of vimentin (green) in MCF-7 cell sublines with differential expression of S6K1 isoforms. Human fibroblasts were used as a positive control of vimentin expression. Nuclei were stained with Hoechst 33342 (blue). Scale 20 μm

Noteworthy, the expression of another cytoskeletal protein, actin, was also affected without affecting the tubulin content. In these cells, other epithelial cell markers such as adhesion molecule CD326 (EpCAM), CD66e (CEACAM5) and CD227 (Muc1) were also demonstrated to be down-regulated at the protein level (Fig. 2B).

Further transcriptional profiling in model cell lines with differential expression of S6K1 isoforms by qPCR helped us to detect significant changes in the EMT-related gene expression. In particular, in addition to the immunofluorescence data of the cells expressing only the p60-S6K1 isoform, we confirmed the expression of *VIM*, which was not expressed at all in parental MCF-7 cells. *q*PCR analysis revealed also strong down- and up-regulation of cadherin E and N genes *(CDH1* and *CDH2*, respectively). Such a switch in the expression of homologous cell adhesion molecules is one of the main signs of EMT. Supporting the induction of EMT in the studied cells was the fact that the expression of the main transcription factors *ZEB1*, *ZEB2*, *TWIST1*, *SNAI2*/ (S*LUG*) responsible for the initiation of EMT was significantly increased in the range of 5- to 10^4-fold, with the exception of *SNAI1* that was slightly down-regulated (Fig. 3).

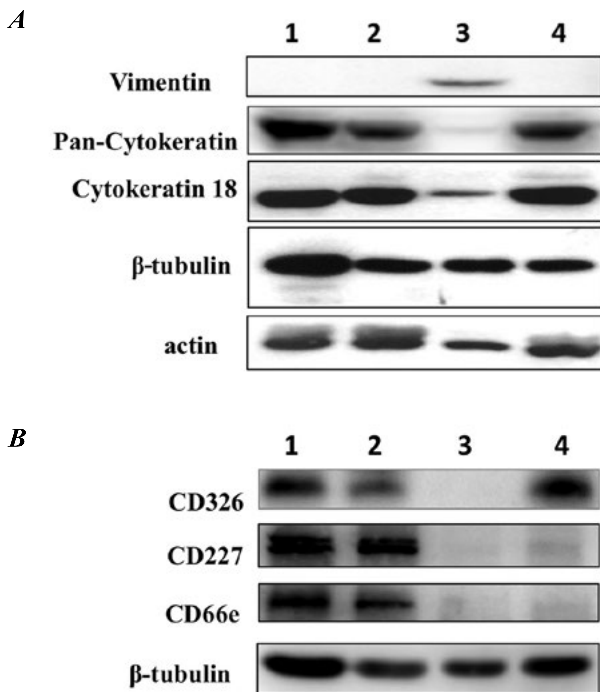

Fig. 2. Expression patterns of cytoskeletal proteins and adhesion molecules in $p85^-/p70^-/p60^+$S6K1/MCF-7 cells are characteristic of EMT. WB analysis of vimentin and cytokeratins (A), CD 326, CD227 and CD66e (B) in lysates of MCF-7 wt (1), $p85^-/p70^+/p60^+$S6K1/MCF-7 (2); $p85^-/p70^-/p60^+$S6K1/MCF-7 (3); $p85^-/p70^-/p60^-$S6K1/MCF-7 (4)

Noteworthy, down-regulation of all or only p85-S6K1 isoform had only a minor effect on the expression of above-mentioned genes, which, moreover, was in most cases even opposite to that in $p85^-/p70^-/ p60^+$S6K1/MCF-7 cells.

It is generally accepted that EMT, which has a major impact on tumor invasion and metastasis, is accompanied by the increased expression and activity of matrix metalloproteinases (MMPs), which are responsible for the remodeling of extracellular matrix and thus support cell migration. Indeed, we detected changes in *MMPs* expression, but again only in $p85^-/p70^-/p60^+$S6K1/MCF-7 cells. According to our data, among the several *MMPs* genes analyzed, we found a significant increase in *MMP2*, *MMP14* (up to 25-fold) and a decrease in *MMP9* (up to 5-fold) without sufficient changes in the expression of *MMP1* (Fig. 3). Expression of another proteinase, cathepsin D *(CTSD)*, involved in ECM degradation was down-regulated similarly to *MMP9*.

The ability to form metastases is associated with the acquisition of characteristics of cancer stem cells (CSCs), namely the ability to survive

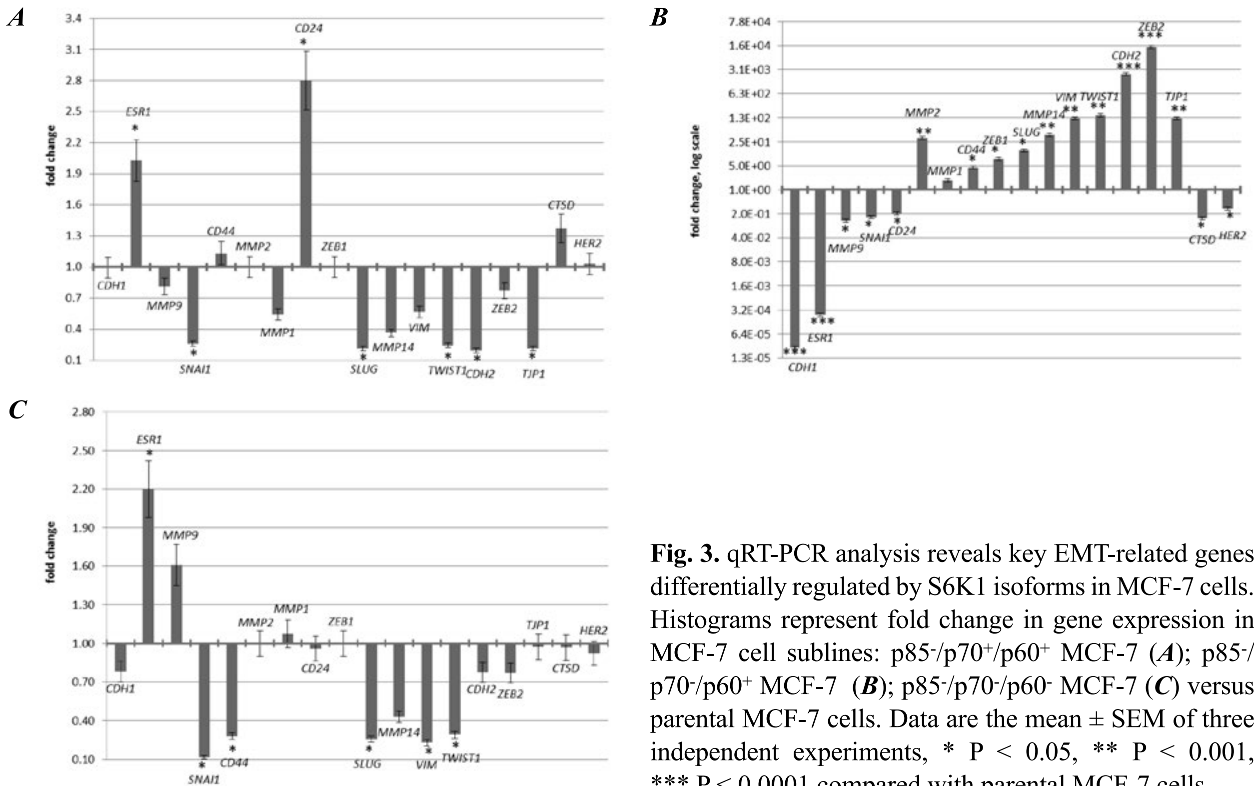

Fig. 3. qRT-PCR analysis reveals key EMT-related genes differentially regulated by S6K1 isoforms in MCF-7 cells. Histograms represent fold change in gene expression in MCF-7 cell sublines: p85$^-$/p70$^+$/p60$^+$ MCF-7 (***A***); p85$^-$/p70$^-$/p60$^+$ MCF-7 (***B***); p85$^-$/p70$^-$/p60$^-$ MCF-7 (***C***) versus parental MCF-7 cells. Data are the mean ± SEM of three independent experiments, * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$ compared with parental MCF-7 cells.

in an unattached state, activation of signaling pathways characteristic of embryonic pluripotent cells, and resistance to anticancer therapy. Overexpression of EMT and stem cell markers in breast tumors has been shown to correlate with poor overall survival [20]. To reveal the effect of S6K1 isoforms expression on the induction of cancer stem cell phenotype in MCF-7 cells, we analyzed the expression of *CD24* and *CD44* CSCs markers in MCF-7 subclones studied. According to our data, the switch from CD24$^+$/CD44$^+$ in parental MCF-7 cells to a CD24$^-$/CD44$^+$ phenotype in p85$^-$/p70$^-$/p60$^+$S6K1/MCF-7 cells was mediated by down-regulation of *CD24* and instead up-regulation of *CD44*, which is characteristic of the cancer stem cells (Fig. 3).

Based on the above data, we hypothesize that changes in the expression of S6K1 isoforms in MCF-7 cells may cause an epithelial to mesenchymal transition causing increased aggressiveness and metastatic potential of breast tumors.

Molecular subtyping of breast tumors [21] supports the histological observations showing so-called luminal A/B and HER2-enriched cancers as those maintaining a more epithelial phenotype; while non-luminal cancers, including triple-negative or basal-like cancers, are characterized by more mesenchymal features [20, 21]. These observations can generally be clinically correlated with the findings that basal-like cancers are constitutively more in-

vasive than their non-basal counterparts [22]. Furthermore, since breast cancer is driven by aberrant hormone-dependent pathways, the hypothesis that loss of estrogen receptor function results in a trans-differentiation from an epithelial to a mesenchymal phenotype, with increased aggressiveness and metastatic potential, has been explored by several authors in recent years [22]. For example, the siRNA-mediated silencing of *ESR1* in the cell lines like MCF-7 resulted in the cells with altered morphology, increased motility and a transition from keratin/actin to a vimentin-based cytoskeleton [22].

Taking into account the above data, we analyzed an impact of S6K1 isoforms expression on *ESR1* status and found a complete repression of *ESR1* expression at the mRNA and protein level in subline of MCF-7 cells with strong signs of EMT (p85$^-$/p70$^-$/p60$^+$ S6K1) (Fig. 4A). No changes in *ESR2* expression were detected (Fig. 4B).

The results obtained support our idea that the reprogramming of S6K1 isoforms expression may be involved in the trans-differentiation of breast tumors from luminal to a more aggressive non-luminal type. Along with the complete suppression of *ESR1* expression, in the same MCF-7 subline we found down-regulation of HER2/NEU *(ERBB2)* (Fig. 3), suggesting the belonging of these cells to the triple negative molecular subtype of breast tumor.

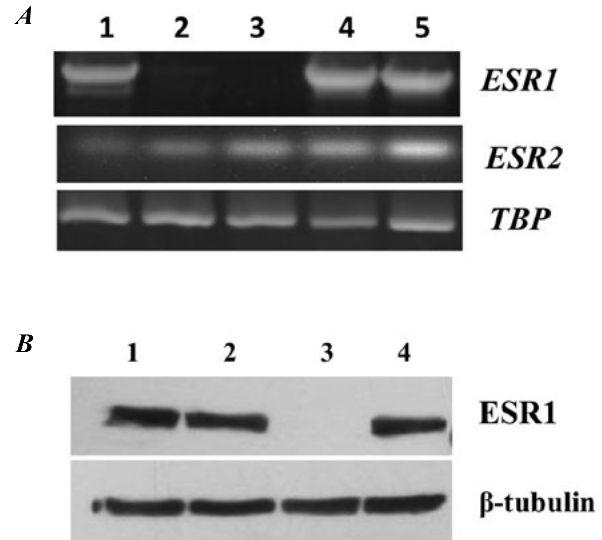

Fig. 4. *ESR1* gene expression is suppressed in p85$^-$/p70$^-$/p60$^+$S6K1/MCF-7 cells at both the mRNA and protein levels. (A) PCR analysis of *ESRs* expression in MCF-7 cell sublines: MCF-7 (1), p85$^-$/p70$^-$/p60$^+$S6K1/MCF-7 (2), MDA-MB-231 (3), p85$^-$/p70$^+$/p60$^+$S6K1/MCF-7 (4), p85$^-$/p70$^-$/p60$^-$S6K1/MCF-7 (5). (B) WB analysis of ESR2 expression in MCF-7 (1), p85$^-$/p70$^+$/p60$^+$S6K1/MCF-7 (2), p85$^-$/p70$^-$/p60$^+$S6K1/MCF-7 (3), p85$^-$/p70$^-$/p60$^-$ S6K1/MCF-7 (4).

Discussion

In present study, we demonstrate that the MCF-7 breast adenocarcinoma cells with imbalanced expression of S6K1 isoforms, namely down-regulation of p70-S6K1 and p85-S6K1 and unchanged expression of p60-S6K1, by CRISPR-Cas9-mediated editing of S6K1 gene, display a novel phenotype that has features of mesenchymal cells. The cytoskeletal redistribution and loss of intercellular adhesion affects their interaction with the ECM and promotes increased locomotive activity that is pre-requisite for tumor cells with high invasive potential. Transcriptome profiling by qPCR convincingly confirmed the induction of EMT in p85$^-$/p70$^-$/ p60$^+$S6K1/MCF-7 cells based on the tremendous increase in mRNA expression of the main EMT-activating transcription factors, SNAIL2, TWIST1, ZEB1 and ZEB2 (EMT-TFs). In addition, increased expression

of a set of metalloproteinases was found in these cells, which ensures the degradation of the basement membrane and ECM, thus facilitating the invasion of tumor cells. Notably, in the cells with reduced expression of all three S6K1 isoforms or only the p85-S6K1 isoform and without signs of EMT, the EMT-TFs expression was reduced, suggesting that p85-S6K1 may play a positive regulatory role in signaling pathways that control transcription initiation of corresponding genes. Instead, p70-S6K1 may function as a negative regulator, as its down-regulation causes increased expression of EMT-TFs. Noteworthy, expression of the p60-S6K1 isoform is a necessary requirement for the initiation of EMT, as $p85^-/p70^-/p60^-$ S6K1/MCF-7 cells with repression of all three S6K1 isoforms showed no signs of EMT.

Taken together, our data demonstrate that the induction of EMT may be somehow associated with the functional activity of p60-S6K1 and is also highly dependent on the expression level of p70-S6K1 or even p85-S6K1, suggesting a regulatory interdependence of S6K1 isoforms in the realization of their effects. Thus, dynamic alterations in the S6K1 isoforms expression in the course of carcinogenesis may induce trans-differentiation of epithelial tumor cells to a more aggressive basal-like or triple negative subtype. A huge reduction in the *ESR1* expression and down-regulation of *ERBB2* in $p85^-/p70^-/p60^+$S6K1/MCF-7 subline suggest that this molecular phenotype may most likely be a triple negative molecular subtype of breast tumor.

However, to date, there is no data in the literature indicating that S6K1 may affect directly or indirectly the expression of any of the EMT-related genes. Thus, based on our data, we can assume that the induction of EMT is linked to the regulation of one of the known or possibly still unknown S6K1 substrates, which in turn may participate in the initiation of a signaling cascades leading to EMT-related transcriptome reprogramming.

As a part of mTOR-dependent signaling, S6K1 is known to be involved in the regulation of various cellular functions by phosphorylating a number of specific substrates, including IRS1, SKAR, FMRP, S6, BAD, GSK3, p21, Cyclin D1 with the transcription factors CREMt and ESR1 among them [17, 23, 24]. Phosphorylation of ESR1 by S6K1 in the cytoplasm is also known to be required for the ESR1 transcriptional activity, as it initiates its translocation to the nucleus [25]. Thus, it cannot be excluded that a differential knockdown of S6K1 isoforms may have a negative effect on the activity and expression of ESR1. More intriguingly, there is an evidence that siRNA-mediated knockdown of *ESR1* in MCF-7 cells resulted in estrogen/tamoxifen-resistant cells with altered morphology, increased motility, a shift from a keratin/actin-based cytoskeleton to a vimentin-based cytoskeleton, and the ability to invade the simulated components of the extracellular matrix [22]. Therefore, it can be hypothesized that inhibition of the ESR1 activity associated with down-regulation of S6K1 isoforms expression may have the same effect. However, in our MCF-7 subline, we dealt with the down-regulation of ESR1 expression at the transcriptional level. So far, there is no evidence in the literature to support a role for S6K1 in the regulation of *ESR1* transcription, but based on the data obtained, we can predict that a key regulator(s) of the *ESR1* transcription may be under the control of S6K1 isoforms.

Overall, our data demonstrate for the first time the involvement of S6K1 isoforms in the regulation of EMT in MCF-7 cells by triggering the co-initiation of a series of sequential or possibly parallel events that switch the cell from an epithelial to a mesenchymal type and cause a complete repression of the *ESR1* expression. At the tumor level, this may indicate the progression of the breast tumor to a more aggressive, most likely triple negative molecular subtype. Inhibiting this transition by targeting specific mediators may offer a useful additional strategy to circumvent the consequences of endocrine loss.

We believe that in the initiation of EMT induced by the changes in S6K1 isoforms expression, the driving factor is not the expression of a certain isoform, but rather a change in the balance of all isoform's expression, which may determine the final regulatory output in the cancer cell. Further evaluation of the effect of different S6K1 isoforms on the induction of EMT and so on tumor invasion and metastasis using established MCF-7 cell sublines will be very valuable for potential new treatment solutions for the most aggressive types of breast tumors.

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REFERENCES

1. *Saxton RA, Sabatini DM.* mTOR Signaling in Growth, Metabolism, and Disease. *Cell.* 2017; **169**(2):361–71.
2. *Grove JR, Banerjee P, Balasubramanyam A, et al., and Woodgett JR.* Cloning and expression of two human p70 S6 kinase polypeptides differing only at their amino termini. *Mol Cell Biol.* 1991; **11**(11):5541–50.
3. *Tavares MR, Pavan IC, Amaral CL, et al., and Simabuco FM.* The S6K protein family in health and disease. *Life Sci.* 2015; **131**:1–10.
4. *Karni R, de Stanchina E, Lowe SW, et al., and Krainer AR.* The gene encoding the splicing factor SF2/ASF is a proto-oncogene. *Nat Struct Mol Biol.* 2007; **14**(3):185–93.
5. *Zaiets IV, Sivchenko AS, Khoruzhenko AI, et al., and Filonenko VV.* The P60-S6K1 isoform of ribosomal protein S6 kinase 1 is a product of alternative mRNA translation. *Ukr Biochem J.* 2018; **90**(4):25–35.
6. *Zaiets IV, Holiar VV, Sivchenko AS, et al., and Filonenko VV.* p60-S6K1 represents a novel kinase active isoform with the mode of regulation distinct from p70/p85-S6K1 isoforms. *Ukr Biochem J.* 2019; **91**(4):17–25.
7. *Magnuson B, Ekim B, Fingar DC.* Regulation and function of ribosomal protein S6 kinase (S6K) within mTOR signalling networks. *Biochem J.* 2012; **441**(1):1–21.
8. *Filonenko VV, Tytarenko R, Azatjan SK, et al., and Lyzogubov VV.* Immunohistochemical analysis of S6K1 and S6K2 localization in human breast tumors. *Exp Oncol.* 2004; **26**(4):294–9.
9. *Lyzogubov V, Khozhaenko Y, Usenko V, et al., and Filonenko V.* Immunohistochemical analysis of Ki-67, PCNA and S6K1/2 expression in human breast cancer. *Exp Oncol.* 2005; **27**(2):141–4.
10. *van der Hage JA, van den Broek LJ, Legrand C, et al., and van de Vijver MJ.* Overexpression of P70 S6 kinase protein is associated with increased risk of locoregional recurrence in node-negative premenopausal early breast cancer patients. *Br J Cancer.* 2004; **90**(8):1543–50.
11. *Chen B, Yang L, Zhang R, et al., and Tang H.* Hyperphosphorylation of RPS6KB1, rather than overexpression, predicts worse prognosis in non-small cell lung cancer patients. *PLoS One.* 2017; **12**(8):e0182891.
12. *Lyzogubov VV, Usenko VS, Khojaenko YuS, et al., and Pogribniy PV.* Immunohistochemical analysis of p70S6 kinase α in human thyroid tissue upon pathology. *Exp Oncol.* 2003; **25**(4):304–6.
13. *Ismail HM.* Overexpression of s6 kinase 1 in brain tumours is associated with induction of hypoxia-

responsive genes and predicts patients' survival. *J Oncol.* 2012; **2012**:416927.
14. *Fan W, Wang W, Mao X, et al., and Fan S.* Elevated levels of p-Mnk1, p-eIF4E and p-p70S6K proteins are associated with tumor recurrence and poor prognosis in astrocytomas. *J Neurooncol.* 2017; **131**(3):485–93.
15. *Li SH, Chen CH, Lu HI, et al., and Lin WC.* Phosphorylated p70S6K expression is an independent prognosticator for patients with esophageal squamous cell carcinoma. *Surgery.* 2015; **157**(3):570–80.
16. *Sridhar J, Komati R, Kumar S.* Targeting RPS6K1 for Refractory Breast Cancer Therapy. In: Mayrovitz HN, editor. Breast Cancer [Internet]. Brisbane (AU): Exon Publications; 2022 Aug 6. Chapter 11.
17. *Zaiets IV, Sivchenko AS, Khoruzhenko AI, Filonenko VV.* Generation of HEK-293 stable cell lines with disrupted expression of ribosomal protein S6 kinase (S6K1) isoforms using the CRISPR/Cas9 genome editing system. *Biopolym Cell.* 2017; **33**(5):356–66.
18. *Kosach VR, Hotsuliak NYa, Zaiets IV, et al., and Filonenko VV.* Alterations in expression of S6K1 isoforms in MCF7 cells have a strong impact on the locomotor activity as well as on S6K1 and Akt signaling. *Biopolym Cell.* 2020; **36**(2):110–21.
19. *Holiar VV, Gotsulyak NYa, Khoruzhenko AI, Zaiets IV.* Generation and characterization of the MCF-7 cell line with a knockout of a p85-S6K1 isoform of the ribosomal protein S6 kinase 1. *Biopolym Cell.* 2019; **35**(4):303–12.
20. *Pomp V, Leo C, Mauracher A, et al., and Varga Z.* Differential expression of epithelial-mesenchymal transition and stem cell markers in intrinsic subtypes of breast cancer. *Breast Cancer Res Treat.* 2015; **154**(1):45–55.
21. *Prat A, Perou CM.* Deconstructing the molecular portraits of breast cancer. *Mol Oncol.* 2011; **5**(1):5–23.
22. *Al Saleh S, Al Mulla F, Luqmani YA.* Estrogen receptor silencing induces epithelial to mesenchymal transition in human breast cancer cells. *PLoS One.* 2011; **6**(6):e20610.
23. *Zhou H, Huang S.* Role of mTOR signaling in tumor cell motility, invasion and metastasis. *Curr Protein Pept Sci.* 2011; **12**(1):30–42.
24. *Sridharan S, Basu A.* Distinct Roles of mTOR Targets S6K1 and S6K2 in Breast Cancer. *Int J Mol Sci.* 2020; **21**(4):1199.
25. *Paplomata E, O'Regan R.* The PI3K/AKT/mTOR pathway in breast cancer: targets, trials and biomarkers. *Ther Adv Med Oncol.* 2014; **6**(4):154–66.

Зміни в експресії ізоформ S6K1 індукують епітелійно-мезенхимний перехід та призводять до пригнічення експресії естрогенового рецептора 1 в клітинах MCF-7 раку грудної залози людини

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Мета. Оцінити вплив експресії різних ізоформ S6K1 у клітинах MCF-7 на ініціацію ЕМТ. **Методи.** Імуноцитохімічний аналіз, ПЛР у реальному часі, Вестерн-блот. **Результати.** Встановлено, що незбалансована експресія ізоформ p60, p70 і p85 S6K1 в клітинах MCF-7, а саме пригнічення p70 і p85 та базальна експресія p60, спричинене CRISPR-Cas9 опосередкованим редагуванням гена, призвело до утворення клітин зі зміненою морфологією та підвищеною рухливістю. Такі зміни були пов'язані з експресією генів, продукти яких беруть участь у рухливості клітин, втраті клітинної адгезії та взаємодії з позаклітинним матриксом, демонструючи їх підвищений потенціал для інвазії та метастатичної активності. Кількісний ПЛР аналізом виявлено підвищену експресію широкого спектру генів, зазвичай пов'язаних із характеристиками мезенхімних клітин і відповідно пригнічення експресії епітеліальних маркерів. Крім того, ми спостерігали повне пригнічення експресії рецептора естрогену 1 (ESR1) і зниження регуляції HER2neu. **Висновки.** Наші дані вперше вказують на роль ізоформ S6K1 у регуляції ЕМТ у клітинах MCF-7 шляхом запуску можливо паралельних подій, які змінюють клітину з епітеліального на мезенхімальний тип, а також впливають на експресію ESR1. На рівні пухлини це може означати перехід пухлини грудної залози до більш агресивного, ймовірно, потрійно негативного молекулярного підтипу.

Ключові слова: ізоформи S6K1, рак грудної залози, клітини MCF-7, ЕМТ.

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