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ESR1 GENE EDITING AND ITS IMPACT ON S6K1 SIGNALING AND CELL BEHAVIOR

Aim. *The aim of this study was to validate the link between the expression of estrogen receptor alpha (ESR1) isoforms and the regulation of S6K1-dependent signaling using the previously generated MCF-7 sublines with the CRISPR/Cas9 editing of the ESR1 gene.* ***Methods.*** *Western blotting, RT-PCR analysis, resazurine cell proliferation assay, and wound healing assay.* ***Results.*** *The presented study reveals a complex and multifaceted relationship between ESR1 isoforms, the S6K1 signaling pathway, and key aspects of MCF-7 cell behavior. It was demonstrated that complete inactivation of full-length p66 ESR1 leads to a significant decrease in S6K1 expression, which, in turn, negatively impacts cell proliferation and motility. Interestingly, the increased expression of the p46 ESR1 isoform, despite maintaining overall S6K1 levels, disrupts its activation dynamics, suggesting a possible regulatory role for p46 ESR1 in modulating cellular responses to growth factors.* ***Conclusions.*** *These findings are crucial for understanding the mechanisms by which different estrogen receptor isoforms can influence the growth, aggressiveness, and metastatic potential of breast cancer cells, particularly through their effect on S6K1 expression and activity. Further research is needed to elucidate the precise molecular mechanisms by which p46 ESR1 modulates S6K1 activation.*

Keywords: *ESR1, CRISPR/Cas9, MCF-7 cells, S6K1, epithelial-mesenchymal transition.*

Introduction

According to WHO estimates, breast cancer is the most common cancer worldwide, especially among women. A primary reason for the high mortality rate in patients with this type of cancer is its ability to metastasize and develop resistance to anticancer therapy. Most primary carcinomas are

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not life-threatening and can be easily removed surgically. However, when breast cancer cells metastasize, they affect vital organs like the bones, brain, lungs, and liver [1]. In such cases, surgical intervention becomes very complex and life-threatening. Therefore, research into the molecular mechanisms underlying breast tumor carcinogenesis is extremely important for developing effective diagnostic systems and improving therapy.

In clinical practice, the primary diagnosis and prognosis evaluation of breast cancer utilize four main biomarkers: estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER-2), and Ki-67 protein. Among them, the estrogen receptor alpha (ERα) is a primary therapeutic target in 80% of all ER-positive cancers in which it is expressed. These tumors are classified as estrogen receptor-positive (ER+). They are non-aggressive, grow slowly, have a good prognosis and a low risk of recurrence, and are responsive to hormonal therapy aimed at reducing estrogen levels or blocking the estrogen receptor. Estrogen receptor-negative (ER-) breast cancer is more aggressive, grows rapidly, does not respond to hormonal therapy, and has a high risk of recurrence within a short period after remission [2].

The estrogen receptor alpha belongs to the superfamily of nuclear receptors, which are transcription factors, and its activity is primarily regulated through binding with estrogen/estradiol (E2). Estradiol plays an indispensable role in the growth, development, reproduction, and maintenance of the functioning of many physiological systems in mammals. Prolonged exposure to estrogen strongly stimulates the development and progression of breast cancer [3]. E2 affects normal and cancerous breast tissue through a ligand-induced transcriptional program mediated by ERα, as well as through the initiation of rapid cytoplasmic signaling cascades [4].

Estrogens exert their biological role through the genomic and non-genomic activation of ERα. The genomic action of estrogens is associated with the regulation of gene transcription via ERα. Ligand binding activates ERα, followed by conformational changes: dimerization, translocation to the nucleus, and binding to estrogen response elements (EREs) on the promoters of target genes [5]. Subsequently, ERα recruits transcriptional coregulators to form a transcriptional regulatory complex, and transcription of the target gene occurs. In the absence of ligand, ERα exists as a monomer bound to the heat shock protein Hsp90 [3]. The non-genomic mechanism is not associated with transcription and occurs outside the nucleus. This pathway begins with the binding of estrogen to G-protein-associated ERα. Such complexes activate the Ras-Raf-MEK-MAPK and PI3K-mTOR-AKT-S6K signaling pathways. Activation of the MAPK signaling pathway results in the phosphorylation of transcription factors c-Jun and c-Fos, which regulate the transcription of target genes responsible for vital cellular functions [6, 7]. In turn, PI3K-mTOR-AKT-S6K signaling is involved in the regulation of cell growth, survival, motility, and the regulation of metabolic processes critical for malignant transformation. Hyperactivation of these cascade pathways is involved in the carcinogenesis of ER-positive breast cancer, as well as in the development of resistance to endocrine therapy [6]. On the other hand, the signaling cascades are involved in the ligand-independent activation of ERα through phosphorylation of Ser118 and Ser167, respectively, which allows ERα to influence the transcription of target genes even in the absence of ligand and leads to the development of cancer resistance to hormonal therapy [8]. Ribosomal protein S6 kinase (S6K1) is considered one of the most likely kinases responsible for Ser167 phosphorylation. Conversely, the literature data suggest that ERα, as a transcription factor, can influence the transcription of the gene encoding S6K1 and its expression [9].

Current anticancer therapies aimed at blocking ERα require improvement due to the development of resistance, metastasis, and recurrent relapses. Therefore, research on ERα signaling in carcinogenesis and the development of alternative treatment methods is currently very relevant, which is complicated by the existence of several

ERα isoforms, the role of which in the development of breast cancer requires further study. Thus, the aim of this study was to investigate a possible association between changes in the expression of ESR1 isoforms and the status of S6K1-dependent signaling.

Materials and Methods

Cell Culture

The MCF-7 cell sublines (clones #9 and #12) with altered ESR1 isoform expression, mediated by CRISPR/Cas9 ESR1 gene editing [10, 11], were used in this study. Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) (Lonza, USA), supplemented with 10% fetal calf serum (FCS, HyClone, USA), 4 mM glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. Studies were performed at different cell growth conditions: regular, as described above (intact cells); 18 h growth in medium with 1% FBS, with or without further 1h stimulation by addition of FBS up to 10%

Real-Time qPCR

RNA isolation, reverse transcription, and qPCR have been performed as described in [10]. Primer sequences for quantitative real-time PCR analysis of S6K1 splice mRNAs coding for all three isoforms, or only p60-S6K1, were provided in Table.

Wound healing assay

Migration speed was measured using a modified scratch assay. The cells were trypsinized, counted, and seeded onto a 24-well plate at 300,000 cells per well in 1 mL DMEM with 10% FBS to achieve 90—100% confluence by the next day. The following day, the medium was replaced with 1 mL of DMEM containing 1% FBS for 24 hours. Next, a "hole" or "scratch" was created in the center of the cell monolayer by aspiration with a 200 μL pipette tip. The wells were then washed twice with PBS, and 1 mL of DMEM with 1% FBS was added. At 0, 24, and 48 hours of incubation, cells were examined under the Leica DM 1000 microscope, and images of the wound were captured with a digital camera. Six technical replicates were performed for each cell line. For each well, the area of the hole was measured at each time point, and the radius was calculated. The migration distance was determined by subtracting the "hole" radius at 24 and 48 hours from the radius at 0 hours time point. Migration speed was calculated by dividing the migration distance by the elapsed time.

Proliferation Assay

Proliferation was assessed using a resazurin salt assay. The corresponding MCF-7 sublines were trypsinized, counted, and seeded on a 96-well plate at a concentration of 5,000 cells per well (in 200 μl DMEM with 10% FBS). The next day, a 10% resazurin solution was added for 2 hours to each well of cells representing the 0-time point and to a well with only growth media as blank. Then, the signal from resorufin was measured using a fluorimeter at an emission of 520 nm and an excitation of 590 nm. Six technical replicates were tested for each cell subline at multiple time points (24h, 48h, 72h, 96h). Background-corrected fluorescence

Primers used for qPCR analysis

Gene	Forward primer	Reverse primer
p70/p85/p60-S6K1	5'-TGCACAGGAGCCAAGAGTGAA-3'	5'-CACATCACAGCTCCCCACCA-3'
p60-S6K1	5'-GTCGAGGGAAAAATAGGCTG-3'	5'-GCCGAGAGCTACACGTTCAC-3'
TBP	5'-TGCACAGGAGCCAAGAGTGAA-3'	5'-CACATCACAGCTCCCCACCA-3'

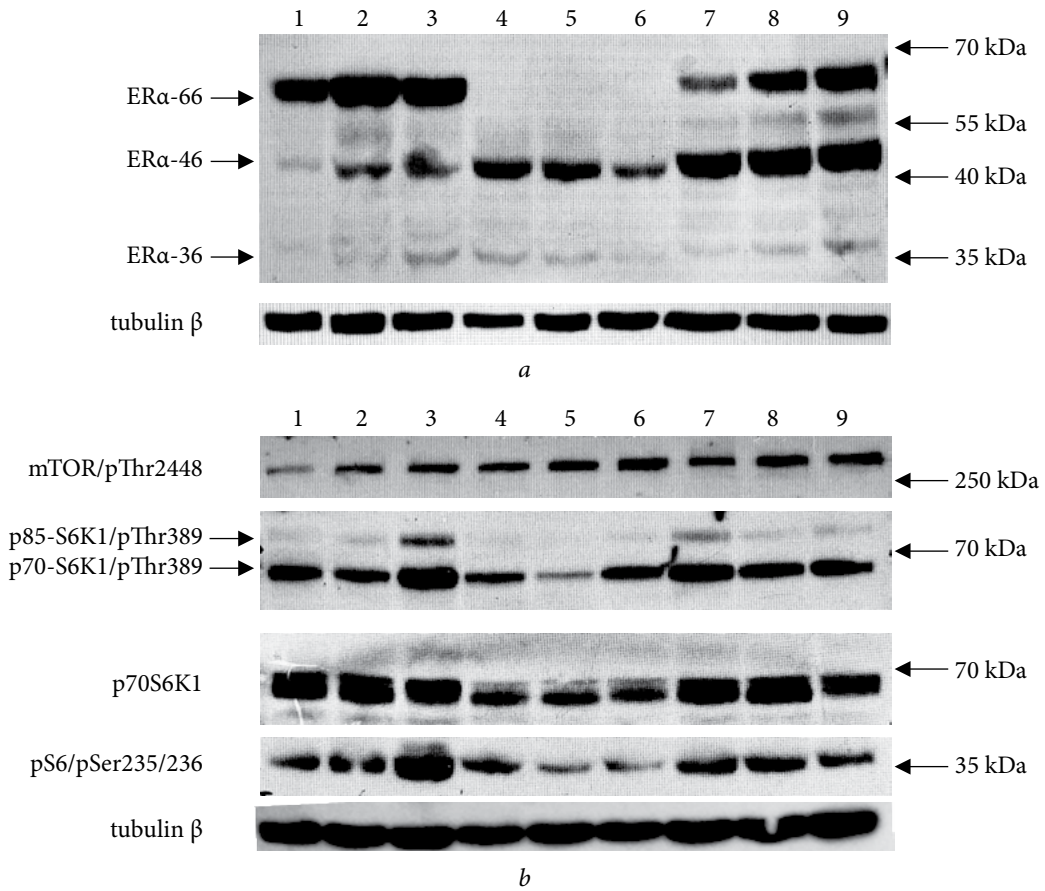

Fig. 1. Analysis of ESR1 impact on mTOR/S6K1-dependent signaling in MCF-7 sublines, with CRISPR/Cas9-mediated ESR1 gene editing, under different cell growth conditions. Western blot analysis of ESR1 expression (*a*) as well as S6K1 expression, S6K1, S6, and mTOR phosphorylation at indicated sites (*b*) in MCF-7 sublines. β-tubulin expression has been used as a loading control. Lines: 1, 4, 7 — intact cells growing in media with 10% FBS; 2, 5, 8 — cells after 18 h growth in FBS-depleted (1%) medium; 3, 6, 9 — cells after 18 h growth in FBS-depleted (1%) medium with the subsequent 1 h stimulation by FBS (10%). MCF-7 wt — lines 1—3. MCF-7 subline #9 — lines 4—6. MCF-7 subline #12 — lines 7—9

values were log-transformed. Linear regression of log-transformed means versus time yielded proliferation rates (slopes) with standard errors. Proliferation rates were compared across cell lines using histogram plots with error bars representing standard errors of the slopes.

Data Visualization and Code

All code was run in Python 3.12.9. Python libraries were installed via Anaconda (v 24.9.2). Plots and diagrams were generated using Excel. Our analyses utilized NumPy (v2.0.1) for data manipulation and SciPy (v1.15.3) for statistical tests.

Western Blot Analysis

Lysates of the MCF-7 cell sublines were analyzed by Western blotting as previously described [10], using specific rabbit anti-ERα monoclonal antibodies directed against the C-terminal region of ERα (Cell Signaling Technology #8644), anti-phospho Thr389

S6K1 (Cell Signaling Technology #9234), anti-phospho Ser235/236 S6 (Cell Signaling Technology #4858), anti-tubulin β (Abcam #7291), anti-S6K1, C-terminal (generated in Institute of Molecular Biology and Genetics NASU), and anti-CD326, anti-CD66e, anti-cytokeratin 18 (provided by R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology of the NASU).

Results

In our previous studies, we reported the creation of MCF-7 cell sublines with disrupted expression of the ESR1 gene by CRISPR/Cas9 gene editing [11]. Briefly, we developed two MCF-7 sublines with complete and partial downregulation of the full-length p66 ESR1 isoform (clones #9 and #12, respectively). Importantly, the MCF-7 subline clone #12 additionally demonstrated a significant increase of the p46 ESR1 isoform, providing a valuable model for analyzing the impact of p46 ESR1 upregulation on cell behavior. Considering the existing crosstalk between ESR1 activity and S6K1, we aimed to analyze how alterations in ESR1 isoform expression affect S6K1-dependent signaling, including S6K1 expression.

The data presented in Fig. 1 demonstrate that downregulation of the p66 ESR1 isoform impaired S6K1 expression in MCF-7 cells (clone #9), as detected by anti-S6K1 antibodies. This appears to be the main reason why the phosphorylation level of both S6K1 isoforms (p70 and p85) is also proportionally decreased. In the case of partial p66 ESR1 downregulation and upregulation of p46 isoform expression in clone #12, S6K1 expression level and its overall phosphorylation status are unchanged.

Subsequent studies demonstrated that the S6K1 decrease at the protein level correlates with the downregulation of its mRNA expression. According to the data of qPCR analysis (Fig. 2), the expression of two main S6K1 splice mRNAs responsible for the translation of all three S6K1 isoforms (p60, p70, p85) or only p60 was downregulated.

At the same time, S6K1 activation dynamics in the control MCF-7 and its subline (clone #9) were

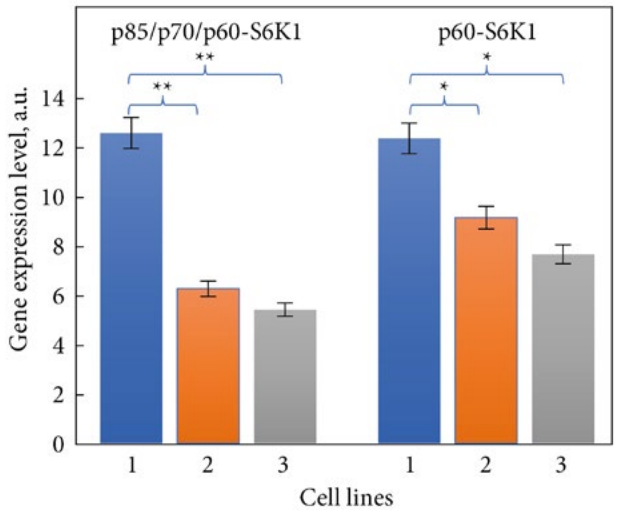

Fig. 2. qPCR analysis of S6K1 isoforms expression (p70/p85/p60 and p60) in MCF-7 cells and MCF-7 sublines with disrupted ESR1 expression. 1 — MCF-7 wt; 2 — MCF-7 subline #9; 3 — MCF-7 subline #12. (*) — $p < 0.05$. (**) — $p < 0.01$

quite similar and, as expected, demonstrated an increase in the phosphorylation level of one of the key activity sites (Thr389) after FBS stimulation compared to intact cells. Notably, in clone #12 of the MCF-7 subline with up-regulated expression of p46 ESR1, we detected retardation in the dynamics of S6K1 activation under the conditions of cell stimulation by FBS after growth in FBS-depleted media: Even after 1 hour of FBS stimulation, the phosphorylation level was lower than in intact cells. It is worth noting that at the same time, the dynamics of ribosomal protein S6 phosphorylation, which is the most studied S6K1 substrate, were affected similarly in both sublines, demonstrating delayed activation after FBS stimulation.

Considering that mTOR is a key regulator of S6K1 phosphorylation at Thr389, we analyzed how changes in ESR1 expression affect mTOR activity. According to the data in Fig. 1, no significant differences in mTOR phosphorylation were found between MCF-7 wt and MCF-7 sublines #9 and #12.

Further studies revealed that a decrease in S6K1 expression and its overall phosphorylation at

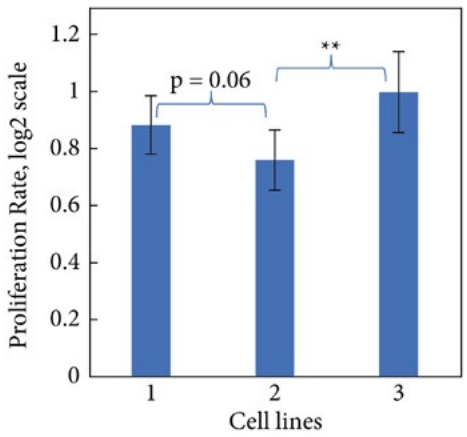

Fig. 3. Evaluation of MCF-7 cell sublines' proliferation rate. 1 — MCF-7 wt; 2 — MCF-7 subline #9; 3 — MCF-7 subline #12. The proliferation rate is based on the slopes of the linear log2 proliferation scale of corresponding cell lines at different time points (0h, 24h, 48h, 72h) and represents their index of doubling time. The resazurin assay has been used for th e estimation of viable cells. The proliferation rate coefficients represent the index of doubling time of the corresponding cell line. (**) — $p < 0.01$

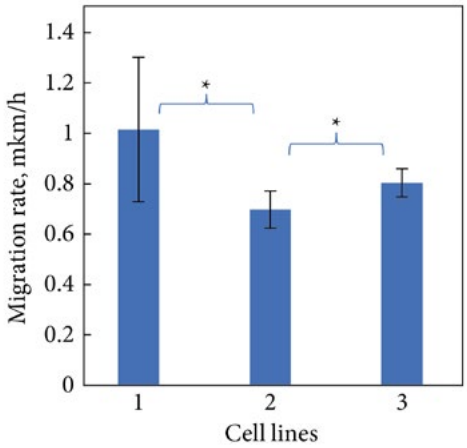

Fig. 4. Evaluation of MCF-7 cell sublines' migration rate by wound healing assay. 1 — MCF-7 wt; 2 — MCF-7 subline #9; 3 — MCF-7 subline #12; (*) — $p < 0.05$

Thr389 in the MCF-7 subline #9 negatively affected cell proliferation. However, a statistically significant difference was demonstrated only between sublines #12 and #9 (Fig. 3). At the same time, alterations in ESR1 expression negatively affected the migration of both clones, with a statistically significant difference between wt and clone #9, suggesting the implication of p66 ESR1 in this process (Fig. 4).

According to our previous studies, alterations in the S6K1 isoform expression may completely abolish ESR1 expression and initiate EMT transition, which was confirmed by alterations in the expression of the main EMT markers [10]. However, in our further studies, we demonstrated that direct downregulation of ESR1 by CRISPR/Cas9 gene editing is not sufficient for EMT initiation [11], but it may indeed affect the expression of genes related to EMT. In the present study, we analyzed the impact of ESR1 expression on Carcinoembryonic Antigen (CEA/CD66e) and Epithelial Cell Adhesion Molecule (EpCAM/CD326) expression and found a strong decrease in CEA in MCF-7 subline #9 and a moderate decrease in EpCAM in both sublines (Fig. 5).

Discussion

In this study, we continued to analyze the impact of ESR1 gene expression modifications on MCF-7 cells, utilizing previously established the MCF-7 cell sublines exhibiting altered ESR1 isoform expression [11]. Given the well-known crosstalk between ESR1 activity and S6K1 [12, 13], we aimed to analyze how the alterations in ESR1 isoform expression affect S6K1-dependent signaling and how this may impact EMT initiation.

Our findings (Figs. 1, 2) demonstrate that the inactivation of the full-length p66 ESR1 isoform in MCF-7 cells leads to a significant reduction in the S6K1 expression at both the mRNA and protein levels. According to our data, the two main S6K1 mRNAs responsible for the translation of S6K1 isoforms (p60, p70, and p85), or mRNA coding for only the p60S6K1 isoform, were downregulated. This result is consistent with the established function of ESR1 as a ligand-activated nuclear receptor that directly or indirectly regulates the transcription of numerous genes involved in breast cancer cell proliferation and survival [14, 15]. Our data

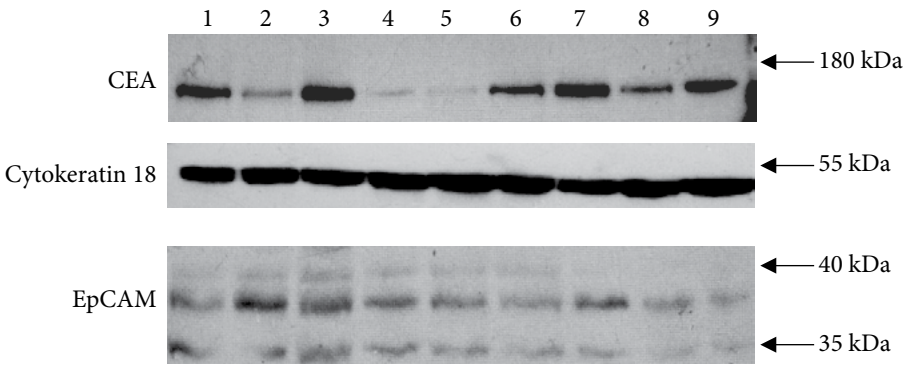

Fig. 5. Analysis of ESR1 impact on the cell markers expression in MCF-7 cells with CRISPR/Cas9-mediated ESR1 gene editing. Western blot analysis of CEA, EpCAM, and cytokeratin-18 in MCF-7 cells with disrupted ESR1 expression. Lines: 1, 4, 7 — intact cells growing in media with 10% FBS; 2, 5, 8 — cells after 24 h growth in FBS-depleted (1%) medium; 3, 6, 9 — cells after 18h growth in FBS-depleted (1%) medium with subsequent 1h stimulation by FBS (10%); MCF-7 wt — 1—3. MCF-7 subline #9 — 4—6. MCF-7 subline #12

provide additional evidence that ESR1 can directly impact the S6K1 expression at transcriptional level. The observed decrease in S6K1 level and its phosphorylation, a key component of the mTOR signaling pathway responsible for regulating protein biosynthesis and cell growth [16], may explain the changes in cell proliferation and migration observed in this study for the MCF-7 subline #9.

In contrast, in the subline #12, which is characterized by a partial reduction in p66 ESR1 and an increased expression of the p46 ESR1 isoform, S6K1 expression remained intact, with no significant alteration in its overall phosphorylation status. This suggests that the p46 ESR1 isoform either does not affect the basal expression level of S6K1 or is capable of compensating for the deficiency of p66 ESR1 in this regard. The p46 ESR1 isoform, which may originate from an internal translation initiation site, is often associated with distinct functional properties compared to p66, including the absence of a ligand-binding domain and the potential for ligand-independent signaling, which may influence proliferation and therapeutic response [17, 18].

Despite the intact S6K1 expression in the MCF-7 subline #12, we detected significant alterations in the dynamics of S6K1 activation in response to FBS stimulation after culturing in FBS-depleted media. Phosphorylation at Thr389 is a direct and widely used indicator of S6K1 activation by mTORC1 [16, 19]. This result is fascinating, as it suggests that the p46 ESR1 isoform may affect the sensitivity or efficiency of S6K1 activation by upstream pathways, despite maintaining overall S6K1 levels, and thereby influence S6K1 signaling. This could be related to a disruption in the integration of signals from growth factor receptors or an alteration in intracellular mechanisms regulating S6K1 activation, potentially through changes in the kinetics of interaction with mTOR or other regulatory proteins.

We also examined how the changes in ESR1 expression affect mTOR activity, as mTOR is a key regulator of S6K1 phosphorylation at Thr389 [16, 19]. According to the data in Fig. 1, there were no significant differences in mTOR phosphorylation at Ser2448, which indicates its activity, between wild-type MCF-7 cells and their sublines. This suggests that the impairments in S6K1 activation observed in the subline #12 are likely not due to direct changes in mTOR kinase activity itself. Instead, they may reflect alterations in S6K1's accessibility for mTOR phosphorylation, changes in protein localization, or other post-translational modifications that influence S6K1's interaction with mTOR or other regulators. Early research initially identified Akt as the

main candidate for phosphorylating Ser2448; however, later, more detailed studies established that p70S6 kinase (S6K1) is the primary kinase in this process [20]. Therefore, our current findings are somewhat surprising, as they did not reveal a correlation between S6K1 suppression and mTOR phosphorylation at Ser2448. Instead, our data suggest that under certain conditions, Akt may compensate by phosphorylating mTOR at Ser2448. Additionally, some studies indicate that other kinases, such as ERK1/2 (MAPK), could be involved in Ser2448 phosphorylation, especially in specific contexts or in response to particular stimuli [19, 21].

The decrease in S6K1 expression and its overall phosphorylation at Thr389 in the MCF-7 subline #9 was linked to slower cell movement and reduced cell growth (Figs. 3, 4). This highlights the important role of S6K1 in these key processes in breast cancer, which is well supported by previous research [22].

According to our data [10], the changes in S6K1 isoform expression associated with the downregulation of ESR1 may trigger the epithelial-mesenchymal transition (EMT), which was confirmed by alterations in the expression of key EMT markers [10]. In further studies, we showed that simply knocking down ESR1 using CRISPR/Cas9 gene editing might not be enough to initiate EMT [11], as we did not observe expression of vimentin and Cadherin N, nor the downregulation of Cadherin E. In this study, we examined how ESR1 influences CEA and EpCAM, well-known epithelial cell markers, whose reduced expression often indicates a loss of epithelial traits, increased invasiveness, and tumor progression [23, 24]. We observed a decrease in CEA in the MCF-7 subline #9 and a reduction in EpCAM in both sublines (Fig. 5). These findings further support that ESR1 alterations can impact EMT via expression of the genes involved in the regulation of cell adhesion, which is a crucial factor in metastatic potential, and that different ESR1 isoforms may exert distinct effects.

Conclusion

Our study confirmed a complex and multifaceted relationship between ESR1 isoforms, the S6K1 signaling pathway, and key aspects of MCF-7 cell behavior. The complete inactivation of p66 ESR1 leads to a significant decrease in S6K1 expression, which, in turn, negatively affects cell proliferation and motility. Interestingly, the increased expression of the p46 ESR1 isoform, despite maintaining overall S6K1 levels, disrupts its activation dynamics, suggesting a possible regulatory role for p46 ESR1 in modulating cellular responses to growth factors. These findings are important for understanding how different estrogen receptor isoforms influence the growth, aggressiveness, and metastatic potential of breast cancer cells, by affecting some aspects of EMT. Further research is needed to clarify the precise molecular mechanisms by which ESR1 isoforms modulate S6K1 activity and affect EMT.

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РЕДАГУВАННЯ ГЕНА *ESR1* ТА ЙОГО ВПЛИВ
НА S6K1-ЗАЛЕЖНЕ СИГНАЛЮВАННЯ ТА ПОВЕДІНКУ КЛІТИН

Мета. Дослідження мало на меті проаналізувати зв'язок між експресією ізоформ естрогенного рецептора альфа (ESR1) та регуляцією S6K1-залежного сигналювання, з використанням попередньо створених субліній

MCF-7 з використанням системи CRISPR/Cas9 для редагування експресії гена *ESR1*. ***Методи.*** Вестерн-блоттинг, RT-PCR аналіз, тест на проліферацію клітин з використанням резазурину, тест ранової поверхні. ***Результати.*** Представлене дослідження виявляє складний зв›язок між активністю ізоформ ESR1, S6K1-залежним сигнальним шляхом та ключовими аспектами поведінки клітин MCF-7. Було продемонстровано, що повна інактивація повнорозмірної p66 ізоформи ESR1 призводить до значного зниження експресії S6K1, що, у свою чергу, негативно впливає на проліферацію та рухливість клітин. Цікаво, що підвищення експресії p46 ізоформи ESR1 не впливає на загальний вміст S6K, однак уповільнює динаміку її активації FBS, що свідчить про можливу регуляторну роль p46 ESR1 у модуляції клітинних відповідей на фактори росту. ***Висновки.*** Ці результати є важливими для розуміння того, як різні ізоформи рецепторів естрогену впливають на ріст, агресивність та метастатичний потенціал клітин раку молочної залози, особливо через їхній вплив на експресію та активність S6K1. Необхідні подальші дослідження для з'ясування точних молекулярних механізмів, за допомогою яких p46 ESR1 модулює активацію S6K1.

***Ключові слова*:** ESR1, CRISPR/Cas9, клітини MCF-7, S6K1, епітеліально-мезенхимальний перехід.