EXPERIMENTAL WORKS

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GENERATION OF THE MCF-7 CELL SUBLINES WITH CRISPR/Cas9 MEDIATED DISRUPTION OF ESTROGEN RECEPTOR ALFA (ESR1) EXPRESSION

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Supported by the literature, our initial hypothesis was that Estrogen Receptor alfa (ESR1) may function as a master regulator by influencing the expression of epithelial-to-mesenchymal transition (EMT)-related genes in cancer cells. To explore this further, we used the CRISPR/Cas9 gene editing system to create MCF-7 sublines with down-regulated ESR1 expression and analyzed its impact on EMT initiation. By applying two distinct types of gRNA for gene editing, we established six MCF-7 cell sublines with either nearly complete or partial down-regulation of the ESR1 isoforms. Unexpectedly, the data obtained revealed no discernible impact of ESR1 down-regulation on EMT manifestation as Western blot and Real-Time qPCR analysis of selected clones revealed no changes in EMT markers expression. We suggested that those of the ESR1 isoforms, the expression of which was not affected by gene editing, could be crucial for the initiation of EMT. The obtained cell models will be used further to evaluate the activity of ESR1 isoforms.

K e y w o r d s: estrogen receptor alfa, CRISPR/Cas9, MCF-7 cells, epithelial-to-mesenchymal transition.

Ribosomal protein S6 kinase 1 (S6K1) is an integral component of the mTOR/S6K1-dependent signaling pathway, acting as a key integrator of numerous extracellular signals that influence cellular responses to nutrient availability and mitogenic stimuli [1]. With over 20 identified substrates exhibiting diverse functional activities, S6K1 plays a role in the regulation of protein synthesis, energy metabolism, and gene expression, and is implicated in the initiation and progression of various diseases, including cancer, cardiovascular, and metabolic disorders [2-9]. Overexpression or hyperactivation of S6K1 has been observed in multiple human malignancies, such as breast, thyroid, endometrial, lung, brain, and esophageal cancers [2-9]. Consequently, S6K1 is widely recognized as a promising target for developing specific anticancer therapies for clinical application [10].

The functioning of S6K1 is based on the expression and activity of three main isoforms (p85, p70 and p60) translated from the common mRNA through the using of alternative translation starts (p85 and p70) or alternative splicing (p60) [11-14]. Recently, we demonstrated that alterations in S6K1 isoforms expression in MCF-7 cells, induced by CRISPR/Cas9-mediated targeting of different S6K1 translation start sites, can initiate epithelial-to-mesenchymal transition (EMT), a hallmark of highly invasive and metastatic tumors [15]. Along with up- and down-regulation of a broad set of EMT-related genes, we observed a knockdown of *ESR1* gene expression, suggesting a potential transition of MCF-7 cells from the luminal A to a possibly triple-negative breast cancer molecular subtype.

The key question to address is identifying the primary target affected by changes in S6K1 isoform

expression, which may trigger essential alterations in EMT-related genes expression and initiate EMT. Supported by the literature, our initial hypothesis was that ESR1 may function as a master regulator by influencing the expression of EMT-related genes. To explore this further, we used the *CRISPR/Cas9* gene editing system to create MCF-7 sublines with down-regulated ESR1 expression and analyzed its impact on EMT initiation.

Materials and Methods

Cell culture. The MCF-7 cell line, derived from human breast adenocarcinoma of the luminal A molecular subtype (ATCC #HTB-22), and its sublines 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.

Generation of MCF-7 cell lines with disrupted ESR1 expression using CRISPR/Cas9 genome editing. To create MCF-7 cell sublines with ESR1 knockout, we employed the same approach previously described in [16]. gRNA design was conducted using the web tool at http://crispr.mit.edu/. Two pairs of oligonucleotides targeting the first exon region of ESR1 are presented in Table 1.

Western blot analysis. Lysates of MCF-7 cell subclones with CRISPR/Cas9-disrupted ESR1 expression were analyzed by Western blotting as previously described, using specific rabbit anti-ERα monoclonal antibodies (Abcam, MA5-14501) directed against the C-terminal region of ERα [15].

Real-Time qPCR. RNA isolation, reverse transcription, and qPCR were performed as described in [15]. Primer sequences for quantitative real-time PCR analysis of selected genes are provided in Table 2.

Results

To generate MCF-7 cell sublines, we introduced the CRISPR/Cas9 gene editing system using two sets of gRNA primers (individually and in combination) targeting the Ex1 region of the *ESR1* gene. The design of gRNAs, cloning into the pSpCas9(BB)-2A-Puro (PX459) V2.0 vector, MCF-7 cell transfection, and selection of positive clones were performed as described in the Materials and Methods section. The efficiency of *ESR1* disruption in selected clones was analyzed by Western blotting using mAbs specific to the C-terminal region of the ESR1 protein. As shown in Fig. 1, we selected MCF-7 sublines with varying degrees of ESR1 down-regulation. MDA-MB-231 cells and MCF-7 sublines with S6K1/p85-/p70-/p60+ phenotype, which do not express ESR1 and exhibit strong signs of EMT, were used as control cells. Among the six selected MCF-7 cell sublines, expression of the full-length ESR1 isoform (p66) was significantly decreased in only two sublines (#7 and #9), created using gRNA1. In the remaining selected sublines, generated with gRNA2 (#1, #14) or a combination of gRNA1 and gRNA2 (#12, #13), only partial down-regulation of p66 ESR1 was observed. Additionally, the anti-ESR1 mAbs detected other ESR1 isoforms possessing C-terminal regions, specifically ESR1-p46 and ESR1 isoform with an apparent molecular weight of approximately 36 kDa

Table 1. Primers used for gRNA cloning

	Top strand	Bottom strand
gRNA1	CACCGCGCGGCGTTGAACTCGTAGG	AAACCCTACGAGTTCAACGCCGCGC
gRNA2	CACCGGCCGTGTACAACTACCCCGA	AAACTCGGGGTAGTTGTACACGGCC

Table 2. 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′
ESR1	5′-AGGTGGACCTGATCATGGAG-3′	5′-AAGCTTCGATGATGGGCTTA-3′

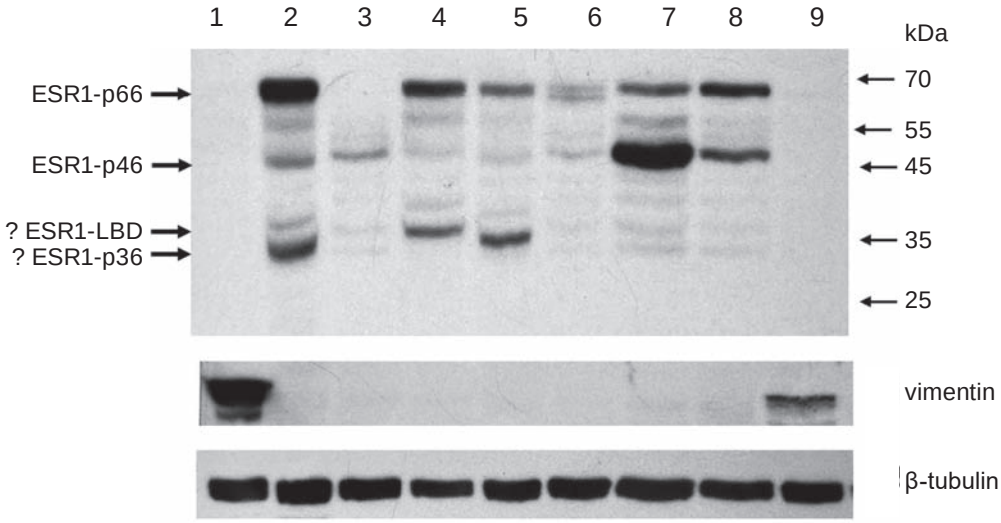

Fig. 1. Establishment of MCF-7 cells sublines with CRISPR/Cas9 disrupted expression of ESR1. Western blotting analysis of ESR1 expression in different cell lines and MCF-7 sublines with CRISPR/Cas9 mediated ESR1 editing: MDA MB 231 (1), MCF-7 (2), MCF-7/9 (3), MCF-7/14 (4), MCF-7/1 (5), MCF-7/7 (6), MCF-7/12 (7), MCF-7/13 (8) cells subclones with CRISPR/Cas9 mediated ESR1 disruption (3-8), MCF-7 subline with S6K1/p85-/p70-/p60+ phenotype (9) using anti-ESR1 mAbs specific to its C-terminal region (Upper panel). Middle panel – vimentin expression. Lower panel – β-tubulin expression. MCF-7 subclones generated using: gRNA #1 – 9 and 7; gRNA#2 – 1 and 14; combination of gRNA #1 and #2 – 12 and 13

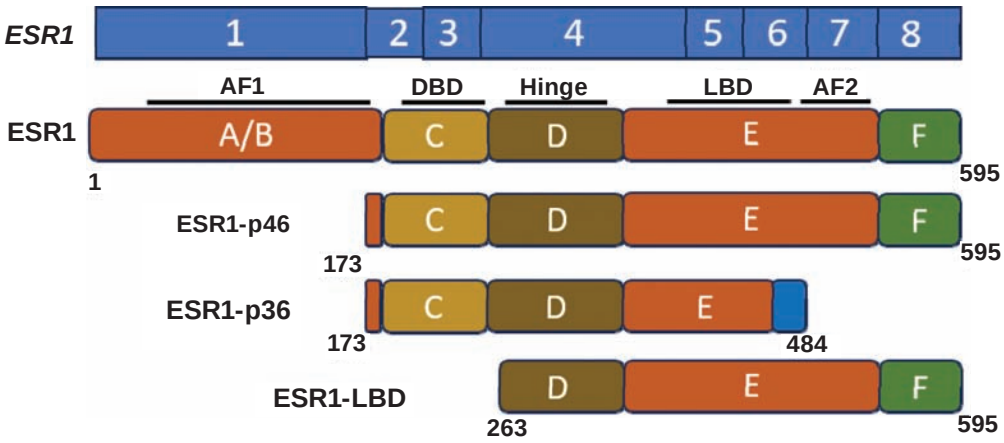

Fig. 2. Estrogen receptor isoforms. The 8 exons of the ESR1 (blue) that encode the wild type ESR1 are illustrated as numbered boxes. Protein domains are denoted as A to F with numbering denoting amino acid sequence number based on full-length protein. Full length ESR1 is 595 amino acids in length. p46 ESR1 isoform results from an alternative start codon and lacks exon 1 resulting in a truncated form of the receptor that is missing the first 173 amino acids of the full-length sequence. ERa-36 is generated from a promoter located in the first intron and lacks exon 1 but also lacks the last 138 amino acids encoded by exons 7 and 8 which are replaced by 27 amino acids at the C-terminus. ESR1-LBD isoform results from an alternative start codon in exon 4 and lacks exon 1-3 resulting in a truncated form of the receptor that is missing

(ESR1-p36 or ESR1-LBD), which are very similar in size (Fig. 1, 2).

The ESR1 36kDa isoform was preserved in sublines #1 and #14 (created with gRNA2) but was completely abolished in sublines created using gRNA1 (#9, #7) or the combination of both gRNAs (#12, #13). The expression of the p46 ESR1 isoform was slightly reduced in most clones; however, when

both gRNA1 and gRNA2 were applied, the content of this isoform in cells increased several-fold. These findings demonstrate that different gRNAs targeting nearby regions of Ex1 have varied impacts on ESR1 isoform expression. Further qPCR analysis of ESR1 expression in MCF-7 sublines #9 and #12, which differed significantly in terms of p66 and p46 expression, confirmed a substantial up-regulation of ESR1 in subline #12, presumably due to increased expression of the p46 ESR1 mRNA isoform.

The primary goal of this study was to assess the impact of ESR1 knockout in MCF-7 breast adenocarcinoma cells on EMT initiation. To achieve this, we analyzed the expression of several EMT markers in clones #9 and #12, which represent the main changes in ESR1 expression. First, we assessed the content of the cytoskeletal protein vimentin (Fig. 1, middle panel), which replaces cytokeratins in the cell during EMT. According to the data obtained, vimentin was detected only in control cell lines, MDA-MB-231 and MCF-7/S6K1/p85-/p70-/p60+, with known EMT status. Vimentin was not detected in any of the selected sublines, a finding that was further confirmed by qPCR analysis, of *Vim* expression in the newly established sublines.

Additionally, qPCR analysis showed that the expression of two other EMT markers, E-cadherin and N-cadherin encoded by *CDH1* and *CDH2* respectively, remained unchanged (Fig. 3). We did not observe the characteristic *CDH1* down-regulation and *CDH2* up-regulation typically associated with EMT. Instead, expression of the epithelial cell marker *CDH1* was even increased.

Discussion

The ESR1-dependent signaling pathway plays a critical role in the progression of human breast cancer and is a primary therapeutic target for ESR1-positive tumors, which constitute approximately 75% of breast cancer cases. ESR1 has multiple splice isoforms that, due to various combinations of functional domains, may perform distinct cellular functions [18]. The ESR1 p66 isoform functions as a transcription factor regulating the expression of numerous genes involved in carcinogenesis in a ligand-dependent manner. The recently discovered ESR1-LBD isoform has been shown to promote breast cancer proliferation and confer endocrine resistance. Additionally, the p36 and p46 splice isoforms of ESR1 are involved in signal transduction via the mTOR/S6K1 pathway [18]. Literature also suggests that ESR1 may affect ribosomal protein S6 kinase expression, which, in turn, regulates ESR1 activity through phosphorylation, leading to transcriptional activation of ESR1 [19, 20].

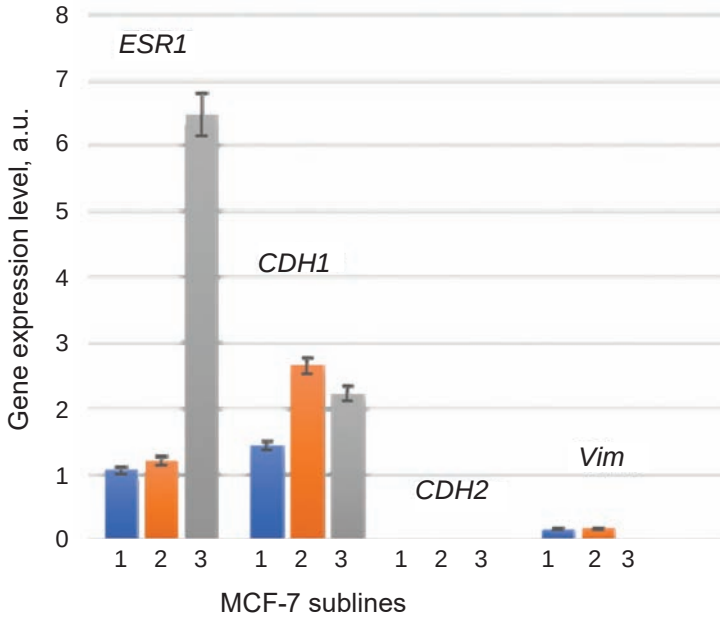

Fig. 3. qPCR analysis of EMT related gens (CDH1, CDH2 and Vim) expression in MCF-7 cells and MCF-7 sublines with disrupted expression of ESR1. 1 – MCF-7; 2 – MCF-7 subline #9; 3 – MCF-7 subline #12

Previously, we demonstrated that altering the balance of S6K1 isoforms expression, specifically p85, p70, and p60, via CRISPR/Cas9-mediated targeting of alternative S6K1 translation starts in the MCF-7 breast adenocarcinoma cell line, has a pronounced negative effect on *ESR1* gene expression. This alteration led to the knockout of ESR1 isoforms recognized by C-terminal anti-ESR1 antibodies, which was accompanied by EMT initiation [15, 16]. Our findings are partially supported by other studies showing that EMT can be initiated by the expression of activated S6K1 in breast and ovarian cancer cells [21-23]. Notably, independent research also suggests that siRNA-mediated ESR1 silencing may initiate EMT in the same breast cancer cell lines [24, 25]. However, neither report focused on changes in ESR1 isoform expression.

In this study, we generated MCF-7 cell sublines with disrupted ESR1 expression using the *CRISPR/Cas9* gene editing system. By applying two distinct gRNAs targeting sequences within Ex1, we established cell lines with either nearly complete or partial down-regulation of the p66 ESR1 isoform. Interestingly, in cases of strong inhibition of ESR1-p66 expression, splice variants recognized by C-terminal antibodies (36 and 46 kDa) were also disrupted. In cases of partial p66 decrease, likely due to the targeting of only one allele, the 36 kDa ESR1 isoform remained intact, while the p46 ESR1 isoform was significantly up-regulated, as demonstrated by both Western blotting and qPCR. The up-regulation of the p46 isoform was also correlated with a decrease in the 36 kDa ESR1 isoform. These findings suggest that the p66 ESR1 isoform may influence ESR1 mRNA splicing and transcription, as shown by qPCR analysis using an oligonucleotide probe covering the junction region of Ex4 and Ex5, present in both p66 and p46 mRNA isoforms (Fig. 2). We anticipate that using an oligonucleotide targeting the junction of Ex1 and Ex2 of ESR1 mRNA, that is not present in mRNA of p46kDa ESR1 isoform, will not show up-regulation of ESR1 expression in subline #12.

Unexpectedly, our analysis of the effect of ESR1 down-regulation on EMT manifestation revealed no discernible impact on EMT marker expression, contrary to previous claims [24, 25]. This discrepancy with the literature could potentially be explained by the use of different techniques for ESR1 disruption in this study – *CRISPR/Cas9* gene editing instead of shRNA-mediated mRNA degradation. Given that the 46 kDa and 36 kDa ESR1 isoforms mRNA translation starts are located outside of Ex1 (Fig. 2), this may explain why their expression is less sensitive to gene editing than that of p66. It is possible that any of the detected ESR1 isoforms, the expression of which was not affected by gene editing, could be crucial for the initiation of EMT, although this hypothesis requires further confirmation.

Conclusion. Overall, we have successfully created MCF-7 cell sublines with differentially disrupted expression of ESR1 isoforms. These cell models will be used further to evaluate the activity of ESR1 isoforms, particularly in the context of EMT regulation and their functional link with S6K1 isoforms.

Conflict of interest. The authors have completed the Unified Conflicts of Interest form at http://ukrbiochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

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СТВОРЕННЯ СУБЛІНІЙ КЛІТИН MCF-7 З CRISPR/CAS9 ІНДУКОВАНИМ ПОРУШЕННЯМ ЕКСПРЕСІЇ ЕСТРОГЕНОВОГО РЕЦЕПТОРА АЛЬФА (ESR1)

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Наша початкова гіпотеза, що узгоджувалася з даними літератури, полягала в тому, що естрогеновий рецептор альфа (ESR1) може функціонувати як головний регулятор експресії генів, пов'язаних з епітеліально-мезенхімальним переходом (EMT) у ракових клітинах. Щоб дослідити це, ми використали систему редагування генів *CRISPR/Cas9* для створення сублінй MCF-7 із зниженою експресією ESR1 та проаналізували її вплив на ініціацію EMT в клітинах. Застосовуючи два різних типи

gRNA для редагування гену, ми створили шість сублінійMCF-7 клітин із майже повним або частковим зниженням вмісту ізоформ ESR1. Несподівано, нами не було виявлено помітного впливу зниження експресії ESR1 на прояв EMT, оскільки ні Вестерн-блот ні ПЛР аналіз в реальному часі обраних клонів не виявили змін у експресії маркерів EMT. Ми припустили, що ті ізоформи ESR1, на експресію яких не вплинуло редагування гена, можуть мати вирішальне значення в ініціації EMT. Отримані моделі клітин будуть використані надалі для оцінки активності ізоформ ESR1.

Ключові слова: естрогеновий рецептор альфа, CRISPR/Cas9, лінія клітин MCF-7, епітеліально-мезенхімальний перехід.

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