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ALTERATIONS IN S6K1 ISOFORM EXPRESSION IN MCF-7 CELLS THAT INDUCE EMT, AFFECT AKT1-, AMPK-, PKC-, AND PKA-RELATED CELL SIGNALING

Aim. *This study aimed to evaluate how S6K1 isoform expression affects the activities of Akt1, AMPK, PKC, and PKA, as well as their related kinase signaling pathways.* ***Methods.*** *MCF-7 cells and a subline with disrupted p70 and p85 S6K1 isoform expression (MCF7/p60$^+$/p70$^-$/p85$^-$) were cultured under different growth conditions. Western blot analysis was performed on cell lysates using antibodies specific to phosphorylated substrates of Akt1, AMPK, PKC, PKA, and GSK-3β/pSer9.* ***Results.*** *The phosphorylation pattern of kinase substrates showed that changes in S6K1 isoform expression influenced either the activity of Akt1, AMPK, PKC, PKA, or their substrate specificity. Unexpectedly, in the MCF-7 subline exhibiting EMT features, GSK-3β kinase — an Akt1 substrate and EMT inhibitor — was not downregulated by phosphorylation of Ser9.* ***Conclusions.*** *S6K1, a ribosomal protein kinase involved in EMT regulation, can modulate the activity of Akt1, AMPK, PKC, PKA, and GSK-3β, as well as their substrate specificity; therefore, all these kinases may be involved in EMT regulation. GSK-3β could also have a bifunctional role in EMT progression initiated by changes in S6K1 isoform expression that modulate its activity.*

Keywords: *EMT, MCF7 cell line, S6K1, Akt1, AMPK, PKC, PKA, GSK-3β.*

Introduction

Epithelial-mesenchymal transition (EMT) is a complex and vital biological process where epithelial cells lose their defining features — such as cell-cell adhesion and apical-basal polarity — and acquire a more motile and invasive mesenchymal phenotype. This process is essential for embryonic development, tissue repair, and for the progression of diseases like cancer and fibrosis. The initiation of EMT is tightly regulated by an intricate network of signaling pathways that respond to signals from the

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cellular microenvironment. These include Transforming Growth Factor-beta (TGF-β) [1], Wnt/β-catenin [2, 3], PI3K/mTOR/Akt [4, 5], and Ras/MAPK/ERK pathways [6].

The mechanism of TGF-β action involves initiating the phosphorylation of Smad proteins, which then translocate to the nucleus and directly increase the expression of key EMT-inducing transcription factors (EMT-TFs) such as Snail, Slug, and ZEB1/2. The Ras/MAPK/ERK cascade can also activate EMT-TFs like Snail and Slug, and its interaction with the TGF-β pathway is a well-known mechanism for EMT initiation, especially in cancer.

The role of the Wnt pathway, an essential component in cell proliferation and stem cell maintenance, in regulating EMT depends on its ability to inactivate GSK-3β kinase. This inactivation causes β-catenin to move to the nucleus, where it collaborates with LEF to activate the expression of various genes. This includes EMT-TFs like Snail and Twist, which then promote the changes in cell adhesion and shape typical of EMT.

The PI3K/mTOR/Akt pathway is a vital integrator of many external signals that influence the cells respond to nutrient levels and mitogenic signals, supporting cell survival and growth. As mentioned earlier, it can also lead to the inactivation of GSK-3β, which stabilizes β-catenin and promotes Wnt-driven EMT. Recently, we have demonstrated that changes in the expression of another key component of the PI3K/mTOR/Akt pathway, specifically isoforms of ribosomal protein S6 kinase (S6K1), in MCF-7 cells caused by CRISPR/Cas9 targeting, can initiate EMT [7, 8]. In addition to the altered expression of various EMT-related genes that encode transcription factors such as Snail, Slug, and ZEB1/2, adhesion molecules E- and N-cadherins, and vimentin — a major part of the cytoskeleton in mesenchymal cells — we observed a reduction in the ESR1 gene expression. This suggests a potential shift of MCF-7 cells from the luminal A to possibly a triple-negative breast cancer molecular subtype.

The selective inhibition of the p70 and p85 isoforms of S6K1, while maintaining the expression of the p60 isoform, led to a significant decrease in the phosphorylation of the kinase substrate, ribosomal protein S6 [8]. At the same time, we observed a reduction in Akt1 phosphorylation at S473, indicating downregulation of kinase activity, which notably affected the phosphorylation of Akt1 substrates [8]. These results suggest that a possible mechanism by which altered S6K1 expression influences EMT features is through its effect on Akt1 activity, which may regulate the EMT inhibitor GSK-3β kinase, a well-known substrate of Akt1. In this study, we aimed to investigate the impact of Akt1-dependent signaling as well as other signaling pathways related to protein kinase C (PKC), AMP-activated protein kinase (AMPK), and protein kinase A (PKA) in EMT initiation caused by changes in S6K1 isoform expression.

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 subline MCF7/$p60^{+}$/$p70^{-}$/$p85^{-}$, were maintained at 37 °C in a humidified environment with 5% CO_2 using 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 have been conducted under various cell growth conditions: regular growth with intact cells as described above; a 24-hour growth period in media depleted of FBS (starvation); an additional 0.5-hour growth after starvation in media supplemented with 10% FBS (restimulation); and restimulation in the presence of mTOR inhibitor rapamycin or PI3K inhibitor wortmannin. The MCF-7 subline MCF7/$p60^{+}$/$p70^{-}$/$p85^{-}$ was created using the CRISPR/Cas9 gene editing targeting the S6K1 gene, as described in [7].

Western Blot Analysis

Lysates of MCF-7 cells and their subline MCF7/$p60^{+}$/$p70^{-}$/$p85^{-}$ with CRISPR/Cas9-disrupted S6K1

expression were prepared as previously described [7] and analyzed by Western blot, using Phospho-(Ser/Thr) Kinase Substrate Antibody Sampler Kit with rabbit monoclonal antibodies specific for Akt, AMPK, PKC, and PKA phosphosubstrates (Cell Signaling #9920), GSK-3β/pSer9 (Cell Signaling #9323P), and β-tubulin (Abcam #ab7291).

Results and Discussion

As mentioned earlier, we have recently found that changes in the balance of S6K1 isoform expression in MCF-7 cells caused by CRISPR/Cas9 editing of the S6K1 gene lead to EMT initiation in cells, as detected through real-time PCR and Western blot analysis of EMT markers (CDH1, CDH2, VIM, SNAIL1, TWIST1, ZEB2) expression [8]. This effect was observed in MCF-7 cells with reduced expression of two S6K1 kinase isoforms — p70 and p85 (MCF7/p60$^+$/p70$^-$/p85$^-$) — while maintaining an intact p60 isoform. Notably, suppression of all isoforms did not produce this effect [8]. It was also demonstrated that in the MCF7/p60$^+$/p70$^-$/p85$^-$ subline, Akt1 kinase was negatively regulated, especially when cells were grown in FBS-depleted medium [8].

The analysis of Akt1 substrate phosphorylation in MCF-7 wild type and the MCF7/p60$^+$/p70$^-$/p85$^-$ subline, using specific antibodies, confirmed the changes in Akt1 activity (Fig. 1). Based on [7] and the data presented, alterations in S6K1 expression significantly affect the phosphorylation pattern of Akt1 substrates under various cell growth conditions. These changes mainly impact phosphorylation of proteins in the 35—55 kDa range (Fig. 1), with some substrates showing decreased phosphorylation (e.g., a ~35 kDa protein), and others showing increased phosphorylation (e.g., a ~50 kDa protein). Interestingly, the mTOR and PI3K inhibitors, rapamycin and wortmannin, respectively, influence Akt1 substrate phosphorylation differently across MCF-7 sublines. According to the data, the MCF7/p60$^+$/p70$^-$/p85$^-$ subline, which exhibits EMT features, was more sensitive to the PI3K inhibitor wortmannin but more resistant to the mTOR inhibitor rapamycin. It is known that the rapamicine, in contrast to the wortmannin, inhibits only mTORC1, but not mTORC2 complex. Thus, our data suggest that p60S6K1 can be under regulation of mTORC2, but not mTORC1 as the main p70-S6K1 isoform.

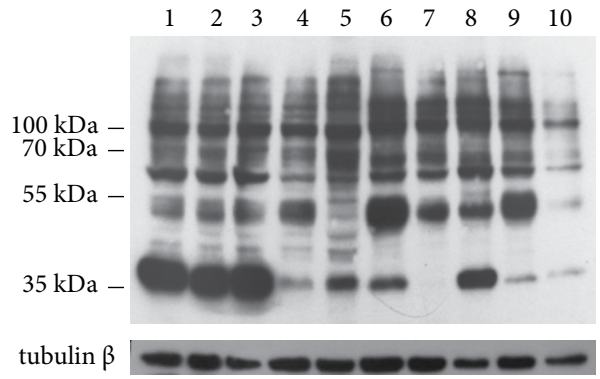

Fig. 1. Analysis of phosphorylated Akt1 substrates in MCF-7 wt and MCF7/p60$^+$/p70$^-$/p85$^-$ cells grown under different conditions. Western blot analysis of cell lysates using antibodies specific to phosphorylated Akt1 substrates. MCF-7: lines 1—5; MCF7/p60$^+$/p70$^-$/p85$^-$: lines 6—10. 1, 6 — intact cells; 2, 7 — starvation; 3, 8 — restimulation; 4, 9 — rapamycin treatment; 5, 10 — wortmannin treatment

Considering that one of the primary substrates of Akt1 is GSK-3β kinase, which, according to literature, plays a crucial role in EMT regulation and has a molecular mass of approximately 50 kDa — within the range where increased phosphorylation of Akt1 substrates has been observed — its phosphorylation status was examined in the specified cell samples using specific anti-GSK-3β/pSer9 antibodies. In addition, Ser9 is a phosphorylation site of GSK-3β that can be targeted by other kinases such as PKA, PKC, and RSK, which, similar to Akt1, can downregulate GSK-3β activity [9] that is important for EMT initiation [10]. Unexpectedly, however, the level of GSK-3β phosphorylation was significantly decreased in the MCF-7 subline showing signs of EMT (Fig. 2). Furthermore, unlike the MCF-7 wt, its phosphorylation was further reduced under serum starvation conditions, while in MCF-7 wt it was significantly increased. This

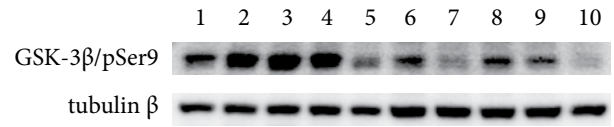

Fig. 2. Analysis of GSKβ phosphorylation in MCF-7 wt and MCF7/p60$^+$/p70$^-$/p85$^-$ cells grown under different conditions. Western blot analysis of cell lysates using antibodies specific to the phosphorylated GSK-3β/pSer9. MCF-7: lines 1—5; MCF7/p60$^+$/p70$^-$/p85$^-$: lines 6—10. 1, 6 — intact cells; 2, 7 — starvation; 3, 8 — restimulation; 4, 9 — rapamycin treatment; 5, 10 — wortmannin treatment

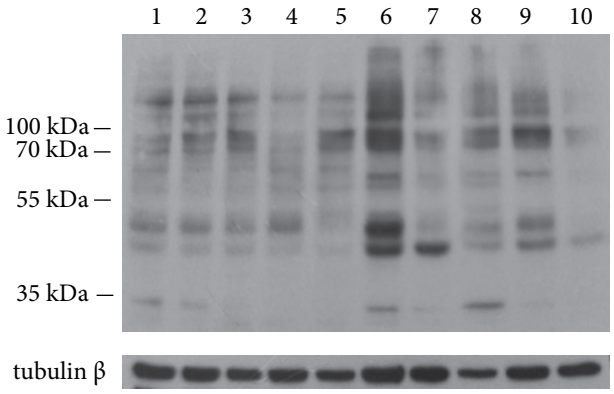

Fig. 3. Analysis of phosphorylated AMPK substrates in MCF-7 wt and MCF7/p60$^+$/p70$^-$/p85$^-$ cells grown under different conditions. Western blot analysis of cell lysates using antibodies specific to the phosphorylated AMPK substrates. MCF-7: lines 1—5; MCF7/p60$^+$/p70$^-$/p85$^-$: lines 6—10. 1, 6 — intact cells; 2, 7 — starvation; 3, 8 — restimulation; 4, 9 — rapamycin treatment; 5, 10 — wortmannin treatment

suggests that EMT initiation in the MCF7/p60$^+$/p70$^-$/p85$^-$ subline may occur independently of GSK-3β kinase activity, or it could involve GSK-3β in a manner that requires the kinase to be active, although its exact role remains unclear.

Thus, the Akt1 substrate with a molecular weight of 50 kDa, whose phosphorylation is increased in MCF7/p60$^+$/p70$^-$/p85$^-$ cells (Fig. 1), does not correspond to GSK-3β.

Given the connections among various cell signaling pathways, especially those involved in regulating similar cellular functions such as cell growth, proliferation, and migration, the phosphorylation patterns of substrates for several protein kinases — namely AMPK, PKC, and PKA — were examined. AMPK (AMP-activated protein kinase) plays a role in maintaining the balance among ATP, ADP, and AMP. It regulates glycolysis, the citric acid cycle, and fatty acid oxidation to produce energy [11, 12]. At the same time, AMPK inhibits fatty acid synthesis, protein synthesis, and overall energy consumption by inactivating mTORC1 [13]. When assessing the phosphorylation levels of AMPK substrates, it was observed that in wild-type MCF7 cells, the overall phosphorylation of these substrates was very low (Fig. 3). However, in MCF7/p60$^+$/p70$^-$/p85$^-$ cells, overall substrate phosphorylation was increased in intact cells, indicating AMPK activation. This likely leads to the inhibition of mTORC1 signaling and, through its connection with mTORC2 signals, may influence Akt1 activity. This aligns with our data on changes of Akt1 activity in MCF7/p60$^+$/p70$^-$/p85$^-$ cells (Fig. 1 and [7]).

PKC plays a multiple role in signaling pathways and controls cell proliferation, differentiation, survival, invasiveness, migration, and apoptosis [14]. Additionally, based on our previous data [15], there is a regulatory connection between PKC and S6K1, which involves the phosphorylation of the nuclear-cytoplasmic export site of S6K1 to regulate its cytoplasmic and nuclear pools.

When studying PKC phosphosubstrates, we also observed a difference in the phosphorylation spectrum of substrates between the two model cell lines (Fig. 4).

In wild-type MCF7 cells, a weak signal was observed around ~35 kDa under intact conditions and upon restimulation, which became stronger during cell starvation. In contrast, in MCF7/p60$^+$/p70$^-$/p85$^-$ cells, this signal was strong under intact conditions and upon restimulation but was significantly reduced during starvation. At the 50 kDa level, stable signals appeared in wild-type MCF7 cells, enhanced by both inhibitors. Conversely, in MCF7/p60$^+$/p70$^-$/p85$^-$ cells, the signals were much weaker and disappeared under starvation and inhibitor treatment. Overall, PKC in MCF7/p60$^+$/

p70⁻/p85⁻ was much more sensitive to cell starvation and PI3K/mTOR inhibitors, suggesting different regulatory mechanisms in these cell models.

Protein kinase A (PKA) is part of the serine/threonine kinase family, and its activity depends on cyclic AMP (cAMP). Protein kinase A plays a role in regulating gene expression, cell survival, and migration, and it can control actin polymerization, which is crucial for the migration and invasiveness of cancer cells [16].

Our data also show noticeable changes in the phosphorylation of PKA substrates associated with the expression of S6K1 isoforms in the studied cell sublines (Fig. 5).

The main differences in signals were observed in the ~35—42 kDa protein range. In wild-type MCF7 cells, a stable signal was detected at ~45 kDa, which did not change with cell growth conditions. In contrast, in MCF7/p60⁺/p70⁻/p85⁻ cells, this signal was also present but significantly weaker. At around 35 kDa, strong signals appeared in wild-type MCF7 cells, which remained unaffected by changes in growth conditions but disappeared when treated with both inhibitors. Meanwhile, in MCF7/p60⁺/p70⁻/p85⁻ cells, phosphorylation of this substrate was extremely sensitive to both growth factors in FBS and PI3K/mTOR/Akt inhibitors. After restimulation, the signal at ~35 kDa was even more pronounced in MCF7/p60⁺/p70⁻/p85⁻ cells than in wild-type MCF7 cells.

Thus, the data presented suggest that S6K1 may regulate the activity of Akt1, AMPK, PKC, PKA, and GSK-3β, triggering a series of changes in EMT-related signaling events. This idea is supported by the research data. For example, in the case of PI3K/AKT/mTOR, it has been shown that its role in EMT regulation can be mediated by stabilizing the SNAIL transcription factor [17] through Akt1-mediated phosphorylation and by negatively regulating GSK-3β, which then phosphorylates Snail at serine 104, 107, and 119, leading to its degradation via the ubiquitin-proteasome pathway [18]. Besides SNAIL, GSK-3β also phosphorylates and destabilizes β-catenin, another known EMT promoter. Therefore, phosphorylation of GSK-3β at serine 9 by AKT stabilizes two key EMT inducers simultaneously. However, our data show that GSK-3β remains active in MCF-7 cells undergoing EMT, indicating a different role for GSK-3β in EMT regulation in this MCF-7 subline if any.

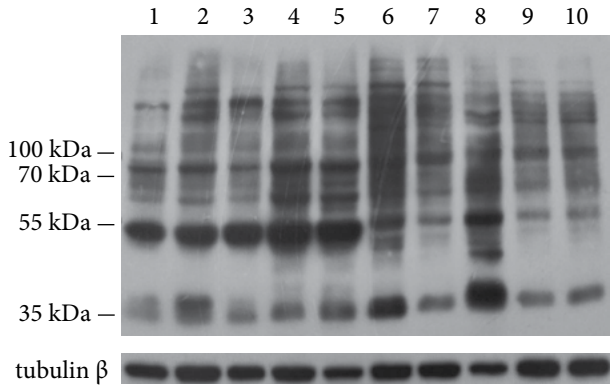

Fig. 4. Analysis of phosphorylated PKC substrates in MCF-7 wt and MCF7/p60⁺/p70⁻/p85⁻ cells grown under various conditions. Western blot analysis of cell lysates using antibodies specific to phosphorylated PKC substrates. MCF-7: lines 1—5; MCF7/p60⁺/p70⁻/p85⁻: lines 6—10. 1, 6 — intact cells; 2, 7 — starvation; 3, 8 — restimulation; 4, 9 — rapamycin treatment; 5, 10 — wortmannin treatment

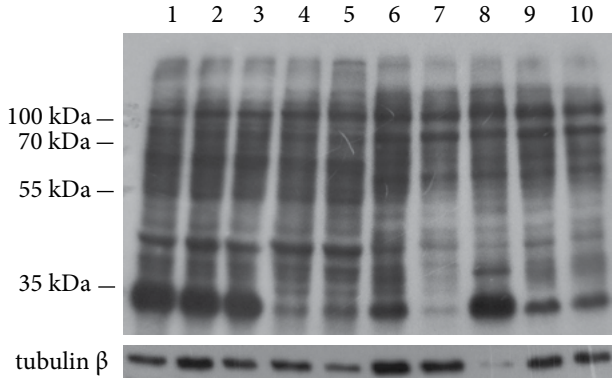

Fig. 5. Analysis of phosphorylated PKA substrates in MCF-7 wt and MCF7/p60⁺/p70⁻/p85⁻ cells grown under various conditions. Western blotting of cell lysates with antibodies specific to phosphorylated PKA substrates. MCF-7: lines 1—5; MCF-7/p60⁺/p70⁻/p85⁻: lines 6—10. 1, 6 — intact cells; 2, 7 — starvation; 3, 8 — restimulation; 4, 9 — rapamycin treatment; 5, 10 — wortmannin treatment

Besides the indirect interaction through GSK-3β, AKT can also influence another transcription factor that promotes EMT-Twist. When hepatocellular carcinoma cells are treated with SC-79, an AKT activator, the levels of vimentin and N-cadherin increase significantly, while E-cadherin decreases [19]. The PI3K/AKT signaling pathway is crucial in many cellular processes, including cell motility, and actively governs the cytoskeleton [20]. It is known that activated Akt co-localizes with actin, indicating its direct role in regulating cytoskeleton remodeling, which is vital for EMT [20, 21].

Thus, AKT can participate in EMT induction through several pathways. One of the most EMT-implicated kinases from the large PKC superfamily is PKCε. Overexpression of PKCε in MCF10A cells leads to signs of EMT, evidenced by a decrease in E-cadherin, ZO-1, and claudin 1, and an increase in vimentin. Additionally, evidence shows that PKCε positively regulates Snail at both the protein and mRNA levels, and its overexpression results in an increase in TGF-β levels [22, 23]. Another key kinase in EMT regulation is PKCα, whose increased expression is often seen in triple-negative breast cancer [24]. PKCα can interact with and activate ZEB1. Knocking out PKCα reduces ZEB1 protein and mRNA levels and inhibits EMT, cell migration, and invasiveness [25]. Furthermore, PKCα phosphorylates Twist1 at serine 144, which boosts Twist1 stability and prevents its ubiquitination and degradation by the proteasome [26].

Other important kinases are PKCδ and PKCι. PKCδ can phosphorylate E-cadherin at threonine 790. This phosphorylation is critically important because it decreases E-cadherin's interaction with β-catenin and disrupts interactions between the ectodomains of E-cadherin, thereby negatively regulating intercellular adhesion [27]. PKCι promotes EMT through the TGF-β/Par6/RhoA pathway and directly activates vimentin by phosphorylation at serine 39. Phosphorylation of vimentin is vital for organizing intracellular filaments and altering cell polarity [28].

Literature data show a contradictory role for AMPK in EMT, with the general trend being that if cells are already transformed, AMPK promotes EMT; if they are not malignant, AMPK, on the other hand, inhibits EMT. When the AMPK agonist A769662 is added to cancer cell lines MCF7, T47D, and A549, an increase in mesenchymal markers and a decrease in epithelial markers are observed. Conversely, when AMPK is knocked out, the level of epithelial markers increases, and mesenchymal markers, on the contrary, decrease [29].

PKA, like AMPK, has a dual effect on the epithelial-to-mesenchymal transition. PKA has been shown to promote both MET (Mesenchymal-to-Epithelial Transition) and EMT. PKA is involved in MET through epigenetic DNA modifications and the transcription of epithelial marker genes. The molecular mechanism of PKA action depends on its interaction with PHF2, a histone demethylase [30]. On the other hand, TGF-β can activate PKA not through the classical cAMP pathway, but by directly recruiting PKA to the Smad3/Smad4/PKA complex. This physical interaction is necessary for the TGF-β-induced increase in PKA activity, which, in turn, leads to the induction of EMT, invasion of pancreatic tumor cells, and regulation of tumor growth [31]. Meanwhile, other studies demonstrate that PKA negatively regulates EMT [32].

Thus, it can be concluded that knocking out two S6 kinase isoforms (p85 and p70) significantly affects the activity of the PI3K/mTOR/Akt pathway they are part of. Conversely, these changes influence other signaling pathways involved in EMT, that was not studied previously and such alterations may be essential in triggering EMT, which is initiated by an imbalance in the expression of S6K1 isoforms. Since the MCF7/$p60^{+}$/$p70^{-}$/$p85^{-}$ cell model might mirror the molecular events leading to the transition of tumors from Luminal A to triple-negative [8], the results broaden the search for new targets to improve therapy for the most aggressive tumors, which currently have limited options.

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ЗМІНИ ЕКСПРЕСІЇ ІЗОФОРМИ S6K1 У КЛІТИНАХ MCF-7,
ЩО ІНДУКУЮТЬ EMT, ВПЛИВАЮТЬ НА КЛІТИННУ СИГНАЛІЗАЦІЮ,
ПОВ'ЯЗАНУ З AKT1, AMPK, PKC ТА PKA

Мета. Метою цього дослідження було оцінити, як експресія ізоформи S6K1 впливає на активність Akt1, AMPK, PKC та PKA, а також на пов'язані з ними кіназні сигнальні шляхи. ***Методи.*** Клітини MCF-7 та сублінію з порушеною експресією ізоформи p70 та p85 S6K1 (MCF7/p60^{+}/p70^{-}/p85^{-}) культивували за різних умов росту. Вестерн-блот аналіз проводили на клітинних лізатах з використанням антитіл, специфічних до фосфорильованих субстратів Akt1, AMPK, PKC, PKA та GSK-3β/pSer9. ***Результати.*** Характер фосфорилювання кіназних субстратів показав, що зміни в експресії ізоформи S6K1 впливали або на активність Akt1, AMPK, PKC, PKA, або на їхню субстратну специфічність. Несподівано, у сублінії MCF-7, що проявляє ознаки EMT, GSK-3β-кіназа — субстрат Akt1 та інгібітор EMT — не була знижена фосфорилюванням Ser9. ***Висновки.*** S6K1, рибосомна протеїнкіназа, що бере участь у регуляції EMT, може модулювати активність Akt1, AMPK, PKC, PKA та GSK-3β, а також їхню субстратну специфічність; отже, всі ці кінази можуть бути задіяні в регуляції ЕМП. GSK-3β також може відігравати біфункціональну роль у прогресуванні ЕМП, ініційованому змінами експресії ізоформи S6K1, які модулюють її активність.

***Ключові слова*:** EMT, клітинна лінія MCF7, S6K1, Akt1, AMPK, PKC, PKA, GSK-3β.