**Overexpression of adaptor protein Ruk/CIN85 in mouse breast adenocarcinoma 4T1 cells induces an increased migration rate and invasion potential**

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**Aim.** To study the effect of adaptor protein Ruk/CIN85 overexpression on the dynamics of migration and Matrigel invasion as well as transendothelial migration of murine 4T1 breast adenocarcinoma cells. **Methods.** Dynamics of 4T1 cells migration/invasion was monitored in real time using the xCELLigence Real-Time Cell Analyzer (RTCA) DP Instrument equipped with a CIM-plate 16. Transendothelial migration (TEM) of 4T1 cells was performed through the layer of primary mouse lung endothelial cells seeded on gelatin-coated 24-well transwell inserts (8-μm pores). The two-tailed Student’s t-test for unequal variances was used for statistical analysis. **Results.** Ruk/CIN85-overexpression in 4T1 cells are indices a significantly increased motility, Matrigel invasiveness and migration through endothelial cells layer. **Conclusions.** The Ruk/CIN85 adaptor protein may play a potential role in the control of metastasis *in vivo*.

**Key words:** tumor cell migration, invasion, 4T1 cells, adaptor protein Ruk/CIN85.

Acquisition of increased motility rate and invasion potential is a key prerequisite for metastatic dissemination of malignantly transformed cells followed by their maintenance in the blood stream, arrest in the distant organ, extravasation, homing and distant organ colonization [1]. This extremely complex and multistage process is orchestrated by cellular plasticity associated with adaptation of tumor cells to dynamic changes in extracellular milieu, which, in its turn, modulate the functional state of signaling networks resulting in epigenetic changes, induction or repression of specific genes and miRNAs expression patterns [2, 3].

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Adaptor proteins are essential components of signaling complexes. They not only assemble the multimolecular complexes but also direct and coordinate intracellular signaling in order to fine-tune cellular behavior [4, 5, 6]. One of such adaptor proteins is Ruk/CIN85, ubiquitously expressed in a variety of normal and cancer tissues in mammals (including human), birds, reptiles, amphibians, bony fishes and even insects (according to NCBI Gene tool). Ruk/CIN85 consists of three SH3 domains, proline- and serine-enriched regions, and coiled-coil domain [7]. These binding domains and motifs as well as sites for post-translational modifications make Ruk/CIN85 a binding partner for more than 200 proteins involved in growth factors signaling, apoptosis, cell shape signaling, cell adhesion, motility and invasion [8, 9, 10]. Ruk/CIN85 was demonstrated to be overexpressed in many human cancers, such as gliomas [11], breast [12] and colon cancers [13], head and neck squamous cell carcinomas [14] in comparison to conditionally normal surrounding tissues. Using tissue samples from breast cancer patients, we previously demonstrated that the highest levels of Ruk/CIN85 content was observed in the lymph node metastases and intravascular tumor emboli [12] that suggests its important role in the metastatic process. In the present study we analyzed the effect of Ruk/CIN85 overexpression in highly invasive mouse breast adenocarcinoma 4T1 cells on the cellular features, required for efficient metastasis: motility, invasiveness, and transendothelial migration.

Materials and Methods

Cells and transfection. Murine breast adenocarcinoma 4T1 cells were cultured in RPMI-1640 medium supplemented with 10 % fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin in a humidified atmosphere containing 5 % CO₂ at 37°C. Ruk/CIN85-overexpressing subline RukUp and corresponding control subline were obtained by Ca-phosphate transfection of 4T1 cells with pRc/CMV2-Ruk or empty vector, respectively [12]. Transfected cells were selected with 1 mg/ml geneticin (G418) followed by subcloning.

Dynamics of cell migration/invasion. Monitoring of 4T1 cells migration and invasion in real time was performed using the xCELLigence Real-Time Cell Analyzer (RTCA) DP Instrument equipped with a CIM-plate 16 (Roche, Indianapolis, IN) as described previously [16]. For invasion experiments, the membranes of CIM-plate 16 wells were coated with Matrigel and for migration experiments they were left uncoated. 4T1 cells (7.5×10⁴) were added to the upper chamber of the plate well in serum-free medium, and the lower chamber was filled with 10 % FCS medium. Also, as negative control we used wells without FCS in the lower chamber (SF). Migration/invasion was monitored every 10 or 15 min for several hours. For quantification, the cell index at indicated time points was averaged from at least three independent measurements.

Transendothelial migration assay. Transendothelial migration (TEM) assay was performed as described previously [17]. Briefly, primary mouse lung endothelial cells (2.5×10⁴) were seeded on gelatin-coated 24-well transwell inserts (8-µm pores; BD Biosciences) and were grown for two days to reach the confluence. 4T1 cells of both anal-
ysed sublines \((2.5 \times 10^4)\), pre-stained with 0.4 \(\mu\)M Calcein AM, were seeded into transwell inserts with monocytes \((1 \times 10^5)\) purified from bone marrow in 3 % FCS/RPMI. 10 % FCS/RPMI was added into the bottom chamber. As a negative control, we seeded mock-transfected 4T1 cells alone (without monocytes). After 24 hours transwell membranes were fixed with 2 % PFA and nuclei were stained with DAPI. Migrated 4T1 cells were counted using fluorescent microscopy in at least 20 consecutive viewfields.

**Statistical analysis.** All experiments were performed at least in triplicates, and the data were presented as Mean ± SD. For statistical analysis we used two-tailed Student’s t-test for unequal variances and difference between groups was suggested to be significant at \(p < 0.05\).

**Results and Discussion**

In order to assess the effect of adaptor protein Ruk/CIN85 on the motility and invasiveness of 4T1 breast adenocarcinoma cells we used 4T1 cells with stable overexpression of full-length Ruk/CIN85 isoform (RukUp subline) and mock-transfected 4T1 cells as a control.

First, we analysed the dynamics of migration and Matrigel invasion of both RukUp and control cells. For this purpose we used xCEL-Ligence RTCA DP instrument that has integrated Boyden chamber (CIM-Plate 16) and allowed us to monitor motility and invasiveness in real time as a function of the impeded electron flow through the membrane [18].

On the graph representing the dependence of cell index on the time (Fig. 1), for both analyzed sublines we observed a small peak of cell index during first hour in migration and invasion experiments, and this peak refers to the attachment of cells to the membrane. Then, approximately at 12th hour, RukUp and control cells started to migrate/invade through the membrane. It was demonstrated that Ruk/CIN85-overexpressing 4T1 cells had significantly higher cell index rate in comparison to control cells in both migration and Matrigel invasion experiments (Fig. 1 A, C). For migration rate, these differences were statistically significant \((p < 0.05)\) at 12, 16, 20 and 24 hrs time points (Fig. 1 B) while, for Matrigel invasion rate, – at 36, 48, 60 and 72 hrs time points (Fig. 1 D).

Next, we compared the ability of RukUp and control 4T1 cells to transmigrate through the layer of endothelial cells \textit{in vitro} using this approach as a surrogate model of extravasation process \textit{in vivo}. After mouse lung endothelial cells (ECs) seeded on the Boyden chamber membrane reached the confluence, the inserts were filled with tumor cells \((2.5 \times 10^4)\) together with monocytes \((1 \times 10^5)\) allowing them to migrate through the layer of ECs for 24 hrs. As can be seen from Fig. 2, the efficiency of TEM was about 18 times higher for Ruk/CIN85-overexpressing 4T1 cells comparing to the parental control cells.

Taken together, our findings evidence that overexpression of adaptor protein Ruk/CIN85 in highly aggressive 4T1 cells led to further increase of traits associated with breast cancer progression and metastasis \textit{in vivo}, especially motility, Matrigel invasion and transendothelial migration. These data were in agreement with our previous results showing that Ruk/CIN85-overexpressing human breast adenocarcinoma MCF-7 cells acquired increased motil-
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Fig. 1. Ruk/CIN85 affects the dynamics of migration and Matrigel invasion of 4T1 cells. A — Real-time migration of 4T1 RukUp and control cells; SF — negative control with serum-free medium in lower chamber of CIM-Plate. B — Cell migration index of 4T1 RukUp and control cells at time points 12, 16, 20 and 24 hours. * — p < 0.05 in comparison to control. C — Real-time Matrigel invasion of 4T1 RukUp and control cells; SF — negative control. D — Cell invasion index of 4T1 RukUp and control cells at time points 12, 24, 36, 48, 60 and 72 hours. * — p < 0.05 comparing to control.

Importantly, treatment of these cells with the Src inhibitor PP2 and the PI3K inhibitor LY294002 abolished the Ruk/CIN85-dependent changes in cell motility [12]. The contribution of Ruk/CIN85 to breast cancer malignancy was further supported by the fact that reverse silenc-
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The binding of the adaptor protein in MCF-7 cells was followed by inhibition of their motility [19]. Moreover, Cascio et al. showed that Ruk/CIN85 promoted invasiveness and metastatic potential of mouse B16 melanoma cells [20]. When we divided breast cancer cell lines from a GSE44552 dataset (GEO) into TEM+ (cell lines with high efficiency of transendothelial migration) and TEM- (cell lines with low efficiency of transendothelial migration) (according to [21]), it was found that every TEM+ cell line expresses higher levels of SH3KBP1 mRNA compared to TEM- cell lines (Fig. 3). These data indicate that Ruk/CIN85 might be involved in breast cancer metastatic cascade, possibly by affecting interactions of tumor cells with microvasculature.

An increasing number of reports have indicated that in different types of human carcinomas acquisition of invasive and migratory properties can be driven by an epithelial-to-mesenchymal transition (EMT). This process implies lack of apical-basal polarity, intercellular contacts and adhesive properties, but acquisition of front–rear polarity, changes in the cell shape, organization of the cytoskeleton, ability to degrade the extracellular matrix (ECM) [22, 23]. EMT can be induced by a number of transcription factors, known as “master regulators” of EMT, such as Twist, Snail, Slug, Zeb 1/2 etc. These transcription factors induce expression of mesenchymal markers (vimentin, N-cadherin, fibronectin) and repress expression of epithelial markers (E-cadherin, claudins, β-catenin) [24, 25]. The changes in expression levels and activity of EMT-inducing transcription factors may occur via signaling dependent on TGFβ receptor, tyrosine kinase receptors (such as EGFR, VEGFR, FGFR), Wnt, Notch, Hedgehog, integrins, inflammation or hypoxia [22, 26]. There are data suggesting a possible involvement of adaptor protein Ruk/CIN85 in the

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**Fig. 2.** Adaptor protein Ruk/CIN85 enhances transendothelial migration of 4T1 cells. * — p < 0.05 comparing to control cells.

![Fig. 2](image1)

**Fig. 3.** Expression of SH3KBP1 gene in TEM+ and TEM- breast cancer cell lines.

![Fig. 3](image2)
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EMT regulation. First, Yakymovych and co-authors [27] demonstrated that Ruk/CIN85 stimulates the presentation of TGFβ receptor on the cell plasma membrane and thus regulates TGFβ-dependent signaling. Also, Ruk/CIN85 was shown to be involved in endocytosis and sorting of receptor tyrosine kinases, the well-known regulators of EMT process, including EGFR [28, 29], Met [30] and VEGFR-1[31]. Finally, Ruk/CIN85 is a binding partner of several proteins, involved in migration, adhesion and cell shape regulation, such as c-Cbl, endophilins, dynamins, FAK, Src, and F-actin [9, 28, 32, 33]. All these features of adaptor protein Ruk/CIN85 can contribute to the control of tumor cell invasiveness and metastatic potential.

In conclusion, to suppress the Ruk/CIN85-induced malignant properties of tumor cells revealed in our study, future investigations of molecular mechanisms of its action are required.

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Associate with diverse cytoskeletal elements, including FAKs, and modulate cell adhesion. J Cell Sci. 2003;116(Pt 14):2845–55.

Надекспресія адаптерного протеїну Ruk/CIN85 в клітинах аденокарциноми молочної залози миші лінії 4Т1 супроводжується зростанням їх рухливості та інвазивного потенціалу

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Мета. Дослідити вплив надекспресії адаптерного протеїну Ruk/CIN85 на динаміку міграції й інвазії через Матригель, а також на ефективність трансендотеліальної міграції клітин аденокарциноми молочної залози миші лінії 4Т1. Методи. Динаміку міграції/інвазії клітин 4Т1 аналізували в режимі реального часу за допомогою приладу XCELLigence Real-Time Cell Analyzer (RTCA) DP Instrument, оснащеного імпедансним планшетом CIM-plate 16. Трансендотеліальну міграцію (TEM) клітин 4Т1 здійснювали через шар первинних ендотеліоцитів легені миші, висіяних на мембрану (розмір пор 8 μм) камери Бойдена. Для статистичного аналізу використовували двовибірковий t-тест Стьюдента для незалежних вибірок з нерівними дисперсіями. Результати. Установлено, що надекспресія Ruk/CIN85 у клітинах лінії 4Т1 супроводжується значним зростанням рухливості, здатності до інвазії через Матригель та шар ендотеліальних клітин. Висновки. Отримані результати вказують на потенційну роль адаптерного протеїну Ruk/CIN85 у контролі метастазування in vivo.

Ключові слова: міграція пухлинних клітин, інвазія, клітини 4Т1, адаптерний протеїн Ruk/CIN85.

Сверхэкспрессия адаптерного белка Ruk/CIN85 в клетках аденокарциномы молочной железы мыши линии 4T1 сопровождается увеличением их подвижности и инвазивного потенциала

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Цель. Исследовать влияние сверхэкспрессии адаптерного протеина Ruk/CIN85 на динамику миграции и инвазии через Матригель, а также на эффективность трансэндотелиальной миграции клеток аденокарциномы молочной железы мыши линии 4Т1. Методы. Динамику миграции/инвазии клеток 4Т1 анализировали в режиме реального времени с помощью прибора XCELLigence Real-Time Cell Analyzer (RTCA) DP Instrument, оснащенного импедансным планшетом CIM-plate 16. Трансэндотелиальную миграцию (ТЕМ) клеток 4Т1 осуществляли через слой первичных эндоцитов легкого мыши, высеянных на мембрану (размер пор 8 μм) камеры Бойдена. Для статистического анализа использовали двухвыборочный t-тест Стьюдента для независимых выборок с неравными дисперсиями. Результаты. Установлено, что сверхэкспрессия адаптерного протеина Ruk/CIN85 в клетках линии 4Т1 сопровождается значительным ростом подвижности, способности к инвазии через Матригель и слой эндотелиальных клеток. Выводы. Полученные результаты указывают на потенциальную роль адаптерного протеина Ruk/CIN85 в контроле метастазирования in vivo.

Ключевые слова: миграция опухолевых клеток, инвазия, клетки 4Т1, адаптерный протеин Ruk/CIN85.

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