RESEARCH ARTICLE

Nucleocytoplasmic distribution of S6K1 depends on the density and motility of MCF-7 cells *in vitro* [version 1; peer review: 1 approved, 1 approved with reservations]

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Abstract

Background: The ribosomal protein S6 kinase 1 (S6K1) is one of the main components of the mTOR/S6K signal transduction pathway, which controls cellular metabolism, autophagy, growth, and proliferation. Overexpression of S6K1 was detected in tumors of different origin including breast cancer, which was associated with a worse disease outcome. In addition, significant accumulation of S6K1 was found in the nuclei of breast carcinoma cells suggesting the implication of kinase nuclear substrates in tumor progression. However, this aspect of S6K1 functioning is poorly understood. The main aim of the present work was to study the subcellular localization of S6K1 in breast cancer cells with focus on cell migration.

Methods: Multicellular spheroids of MCF-7 cells were generated using agarose-coated Petri dishes. Cell migration was initiated by spheroids seeding onto growth surface and subsequent cultivation for 24 and 72 hours. S6K1 subcellular localization was studied in human breast cancer and normal tissue, 2D and 3D MCF-7 cell culture using immunofluorescence analysis and confocal microscopy.

Results: Analysis of histological sections of human breast cancer and normal tissue revealed predominantly nuclear localization of S6K1 in breast malignant cells and mainly cytoplasmic one in conditionally normal cells. *In vitro* studies of MCF-7 cells showed that the subcellular localization of S6K1 depends on the cell density in the monolayer culture. S6K1 relocation from the cytoplasm into the nucleus was detected in MCF-7 cells migrating from multicellular spheroids onto growth surface. Immunofluorescence analysis of S6K1 and immunoprecipitation assay revealed the colocalization and interaction between S6K1 and transcription factor TBR2 (T-box brain

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protein 2) in MCF-7 cells. Bioinformatical analysis revealed existence of several phosphorylation sites in TBR2 for S6K1 suggesting that TBR2 can be a target for phosphorylation and regulation by S6K1.

Conclusions: Subcellular localization of S6K1 depends on the density and locomotor activity of the MCF-7 cells.

Keywords

S6K1, subcellular localization, mTOR/S6K1 signaling pathway, breast cancer, TBR2, Eomesodermin, MCF-7 cell line

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Introduction

Ribosomal protein S6 kinase 1 (S6K1) belongs to the AGC family of serine/threonine protein kinases (Ruvinsky & Meyuhos, 2006). It is involved in the regulation of crucial physiological processes, such as protein synthesis, ribosomal biogenesis, the G1/S-phase transition of the cell cycle, mRNA splicing, differentiation of specific cell types, and apoptosis. A large number of cellular targets makes S6K1 a key regulator of cell size, growth, and proliferation (Magnuson *et al.*, 2012). S6K1 activity is under the control of the PI3K/Akt/mTOR signaling pathway, which is dysregulated in diverse human pathologies, including diabetes, obesity, neurodegenerative disorders, and cancer (Tavares *et al.*, 2015). Overexpression of S6K1 was found in several tumor types, including breast cancer, and this was associated with a worse disease outcome (Bostner *et al.*, 2015).

In mammalian cells, S6K1 is encoded by *RPS6KB1* gene located at chromosome 17. Several isoforms of the S6K1 protein are known: 85kDa S6K1 and 70kDa S6K1 (p85S6K1 and p70S6K1 respectively), which originate from alternative translation initiation sites, and hypothetical p60S6K1, suggested to be a product of alternate mRNA translation as well (Kim *et al.*, 2009). Recently, new 31kDa isoform of S6K1 (p31S6K1) encoded by mRNA splice variant was identified. It was demonstrated that translated p31S6K1 isoform does not have catalytic activity, but possesses oncogenic properties (Ben-Hur *et al.*, 2013; Song & Richard, 2015). The longer isoform p85S6K1 has an additional 23 amino acid extension at the N-terminus of the molecule where the nuclear localization signal is located. Earlier p85S6K1 was described as a predominantly nuclear kinase. However, recent studies revealed it in the cytoplasm of the breast cancer cells and in the primary human fibroblasts using nuclear-cytoplasmic fractionation (Kim *et al.*, 2009; Rosner & Hengstschläger, 2011). Another isoform of S6 kinase p70S6K1 was thought to localize predominantly in the cytoplasm. Treatment of cells with leptomycin B (the nuclear export inhibitor) led to the accumulation of the p70S6K1 in the nucleus. This finding allowed supposition that p70S6K1 shuttles between the cytoplasm and nucleus of the cell (Panasyuk *et al.*, 2006). To date, very little is known about p31S6K1 subcellular localization. It is thought to be nuclear in human normal fibroblasts (Rosner & Hengstschläger, 2011). Overall, S6K1 subcellular localization data have been based predominantly on subcellular fractionation assay or immunocytochemical analysis of recombinantly expressed kinase. Information about nucleocytoplasmic distribution of the endogenous S6K1 is still limited, and mechanisms of its regulation remain elusive.

Recent studies suggest that S6K1 subcellular localization and activation depends on physiological features of different tissues. Immunohistochemical analysis of breast tumors revealed prominent S6K accumulation in the nuclei of carcinoma cells (Filonenko, 2013; Filonenko *et al.*, 2004; Lyzogubov *et al.*, 2005). In other studies, it was shown that nuclear accumulation of S6K1 was indicative of a reduced tamoxifen effect in breast cancer patients, while cytoplasmic localization of S6K1 was associated with better prognosis (Bostner *et al.*, 2015).

Migration of the cancer cells is an important stage of cancer progression that usually lead to the tissue invasion and formation

of distant metastases. The recent data suggest that S6K1 could be involved in the regulation of the motility of normal and malignant cells. Knockdown of p70S6K1 or inhibition of S6K1 kinase activity causes a significant decrease in the migration properties of the prostate, breast, and ovarian cancer cells *in vitro* (Amaral *et al.*, 2016; Ip *et al.*, 2011). Moreover, activation of p70S6K1 in human ovarian carcinoma cells that occurs in response to stimulation by hepatocyte growth factor (HGF) led to increased expression of matrix metalloproteinase 9 (MMP9) and higher migration rate of these cells (Zhou & Wong, 2006). It was shown that p70S6K1 stimulated activation of Cdc42, Rac1, and PAK1 – the known regulators of actin cytoskeleton reorganization (Aslan *et al.*, 2011; Liu *et al.*, 2010). Besides, S6K1 colocalizes with the actin arches at the leading edge of moving mesothelioma cells. Treatment with rapamycin (specific mTOR inhibitor) complicated the formation of actin arches even when cells were stimulated with endothelial growth factor (EGF) (Berven *et al.*, 2004; Liu *et al.*, 2008). However, the link between subcellular localization of S6K1 and its functions in migrating cancer cells is not fully understood.

In the present research, we studied the subcellular localization of endogenous S6K1 in breast tumor and normal tissue, and in breast adenocarcinoma MCF-7 cells in monolayer culture, 3D multicellular spheroids, and in course of cancer cell migration. We found that nucleocytoplasmic distribution of S6K1 depends greatly on the density of the monolayer culture, and is different in 3D vs 2D cell culture. Moreover, we revealed that S6K1 relocates to the nucleus during migration of cancer cells from multicellular spheroids onto growth surface. In addition, we analyzed the possible interaction of S6K1 with a number of transcription factors, involved in the regulation of cell motility. For the first time, we described the colocalization and co-immunoprecipitation of S6K1 and TBR2 (T-box brain protein 2) in breast cancer cells. These data could indicate that during cell migration S6K1 possibly interacts with transcription factors in the cell nucleus, activating genes that are involved in the regulation of cell locomotor activity.

Methods

Cell culture

Human breast adenocarcinoma cell line MCF-7 was obtained from Bank of Cell Lines of the R. E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NASU (Ukraine). The cells were cultivated in DMEM culture medium (Gibco, USA) supplemented with 10% fetal calf serum (FCS, HyClone, USA), 4 mM glutamine, 50 units/ml penicillin, 50 µg/ml streptomycin at 37°C under 5% CO₂. The medium was changed every third day. For immunofluorescence analysis cells were seeded onto sterile glass coverslips.

To form multicellular spheroids, MCF-7 cells were detached and 1×10⁶ cells were seeded into 100 mm Petri dishes coated with 1% agarose (Sigma-Aldrich, A9045) for 72 h.

For initiation of spheroid-to-monolayer reversion and cell migration, multicellular spheroids were transferred onto growth surface (glass coverslip) and cultivated for 24 or 72 h. Then outspread spheroids were applied to immunofluorescence analysis.

Cellular and spheroid morphology was evaluated microscopically using transmitted light (CETI Versus inverted microscope, CETI, Belgium, and Leica DM 1000, Leica Microsystems, Germany).

Immunofluorescence analysis

Cultured MCF-7 cells were fixed with 10% formalin for 15 min at room temperature (RT). Thereafter, the cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min. Autofluorescence was decreased by incubation with 10 mM cupric sulphate and 50 mM ammonium acetate, pH 5.0 for 30 min at RT. Non-specific binding was blocked after incubation with 10% FCS in PBS for 30 min at 37°C in a humidifying chamber.

S6K1 subcellular localization was revealed using anti-S6K1-C-terminus rabbit polyclonal antibodies (generated and evaluated earlier (Savinska *et al.*, 2001; if you are interested in obtaining this antibody, please contact the corresponding author)) at 1:100, and anti-phospho-S6K1 (T389) rabbit polyclonal antibodies at 1:20 (Cell Signaling Technology Cat# 9205, RRID: AB_330944). The secondary Fluorescein (FITC)-AffiniPure Goat Anti-Rabbit IgG (H+L) antibody 1:400 (Jackson ImmunoResearch Labs Cat# 111-095-003, RRID:AB_2337972) were applied for 45 min at 37°C in a humidifying chamber.

Double immunofluorescence analysis was performed by addition of the primary antibody mix: anti-S6K1-C-terminal mouse monoclonal antibodies (generated earlier (Pogrebnoy *et al.*, 1999; if you are interested in obtaining this antibody, please contact the corresponding author) at 1:100 + anti-TBR2 rabbit polyclonal antibodies at 1:100 (Abcam Cat# ab23345, RRID:AB_778267), or + anti-ERG rabbit monoclonal antibodies at 1:50 (Dako, Cat#M7314), or anti-CDX2 rabbit monoclonal antibodies at 1:100 (Abcam Cat# ab76541, RRID:AB_1523334), overnight at +4°C in a humidifying chamber. The secondary Fluorescein (FITC)-AffiniPure Donkey Anti Mouse IgG (H+L) antibody (Jackson ImmunoResearch Labs Cat# 715-095-150, RRID: AB_2340792) at 1:400, and Rhodamine (TRITC)-AffiniPure Donkey Anti-Rabbit IgG (H+L) antibody (Jackson ImmunoResearch Labs Cat# 711-025-152, RRID:AB_2340588) at 1:400 were applied for 45 min at 37°C in a humidifying chamber. Samples were embedded into Mowiol medium (Sigma-Aldrich, USA) containing 2.5% DABCO (Sigma-Aldrich), 0.5 % DAPI (Sigma-Aldrich).

All microscopy studies were performed using Leica DM 1000 fluorescent microscope and Zeiss LSM 510 META microscope (Carl Zeiss Microscopy GmbH, Germany). Fluorescence images were analyzed with free software Fiji/ImageJ v1.52b (Fiji, RRID:SCR_002285; Schindelin *et al.*, 2012). Figures were generated with the FigureJ plugin (Mutterer & Zinck, 2013) in Fiji/ImageJ v1.52b.

For quantitative characterization of colocalization Pearson coefficient and Manders coefficients (M1 and M2) analysis was performed on background-subtracted images using JACoP plugin (Bolte & Cordelières, 2006) in Fiji/ImageJ v1.52b. Pearson coefficient (Rr) and Manders coefficients (M1 and M2) were expressed as mean value $\pm$ SD, the experiment was duplicated, n=5 for each condition. To validate and describe the obtained degree of colocalization pre-defined image sets from

Colocalization Benchmark Source were used. Obtained values of the colocalization coefficients were used to find the closest benchmark.

Immunohistochemical analysis

Histological samples of human mixed ductal/lobular carcinoma of the breast and surrounding conditionally normal tissue were obtained from 10 patients within the framework of the cooperation agreement between the National Cancer Institute and the Institute of Molecular Biology and Genetics of the National Academy of Sciences of Ukraine. This study has been approved by the Committee on Biological & Medical Ethics of the National Cancer Institute of Ukraine (approval number - № 67, 25.03.2015). Written informed consent was obtained from all patients for the use of their tissues in research.

Sections of human breast cancer and surrounding tissues or multicellular spheroids were deparaffinized in xylene and rehydrated in a series of graded alcohols. For the antigen retrieval, slides were placed in citrate buffer (10 mM citric acid, pH 6.0) and transferred to the microwave. Sections were boiled two times for 5–7 min. Then, sections were treated with 0.2% Triton X-100 for 10 min. Endogenous peroxidase was quenched with 3% H₂O₂ in PBS for 30 min. After blocking of non-specific staining with 10% FCS in PBS, sections were incubated with anti-S6K1-C-terminal rabbit polyclonal antibodies (1:100) overnight at +4°C, and thereafter they were incubated with peroxidase-conjugated secondary antibodies (1:100; Promega Cat# W4011, RRID:AB_430833) for 1 hour at 37°C. The reaction was developed with 3,3'-diaminobenzidine (Sigma-Aldrich) solution.

Bioinformatic analysis

Predicted sites of TBR2 phosphorylation by S6K1 were revealed using Group-based Prediction System v2.1 (Xue *et al.*, 2011; GPS, RRID:SCR_016374). The sequence of human TBR2 for this analysis was taken from the National Center for Biotechnology Information, NCBI Reference Sequence: NP_001265111.1.

Immunoprecipitation and immunoblot analysis

Anti-S6K1 mouse monoclonal antibodies (Pogrebnoy *et al.*, 1999) were immobilized on protein A/G PLUS Agarose beads (Santa Cruz Biotechnology) overnight at +4°C.

MCF-7 cells were washed with ice-cold phosphate-buffered saline and extracted with lysis buffer, containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 50 mM sodium fluoride, 5 mM β -glycerophosphate, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate and a mixture of protease inhibitors (Roche Molecular Diagnostics, France). Cell lysates were centrifuged at 13 000 rpm for 20 min at 4°C. Endogenous S6K1 was precipitated by adding 1000 μ g of total cell lysates to the immobilized antibodies and incubating overnight at 4°C. Immune complexes were washed three times with lysis buffer, boiled for 5 min in Laemmli sample buffer, and used for immunoblot analysis. As a control, protein A/G PLUS Agarose beads were incubated with monoclonal antibodies or cell lysates alone.

Samples were separated by 10% SDS-PAGE and transferred onto PVDF membrane (Millipore, Billerica, MA). The membrane was blocked with 5% skim milk in PBST (140 mM NaCl, 2.6 mM KCl, 10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 , 0.05% Tween-20, pH 7.4) for 1 h at RT, and then incubated with anti-TBR2/Eomes rabbit antibodies at 1:500 (Abcam Cat# ab23345, RRID:AB_778267) or anti-S6K1 C-terminal rabbit polyclonal antibodies 1:3000 overnight at 4°C. After washing three times with PBST, HRP-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Labs Cat# 111-035-144, RRID:AB_2307391) in 1:10 000 dilution were incubated with the membrane for 1 h at RT. Finally, the membrane was developed using an enhanced chemiluminescence system (Fluoro) and then exposed to Agfa X-ray film.

Results and discussion

Immunochemical detection of S6K1 subcellular localization in human breast cancer cells

There are several mediated and direct approaches to determine protein function in cells. The first includes cell models without direct interaction between the studied protein and the reporter marker. The function of a studied protein is evaluated as a correlation with a reporter marker, which in turn gives a characteristic to a particular cellular process (Nizheradze, 2006). The second group of approaches is based on closer interactions between the agent and the reporter marker. One of them consists of the determination of studied protein belonging to the specific subcellular compartment

and next revealing its protein-partners with known functions. The difference in subcellular localization of the protein in different cell types may suggest the realization of different function.

For the first step of the present study, subcellular distribution of S6K1 was analyzed in normal and cancer cells of a histological section of human breast cancer and normal tissues. Our results support previous data about preferential nuclear localization of S6K1 in breast malignant cells (Bostner *et al.*, 2015; Filonenko *et al.*, 2004) and mainly cytoplasmic one in conditionally normal surrounding tissues (Figure 1A, B).

As was shown earlier, 3D cell culture systems are preferable for tumor growth study (Bingel *et al.*, 2017). That's why we analyzed the intracellular distribution of S6K1 in multicellular spheroids of MCF-7 cells and in monolayer culture. We detected a bright signal of S6K1 in the cytoplasm and its absence in the nuclei of cells of the multicellular spheroids (Figure 1C). In contrast to a spheroid culture, S6K1 distribution in MCF-7 cells monolayer culture at 40–60% confluence revealed bright nuclear staining in interphase cells with the moderate cytoplasmic signal (Figure 1D).

Nucleocytoplasmic redistribution of S6K1 in MCF-7 cells at different cell density

A significant difference in S6K1 localization in monolayer and spheroid cultures can be explained by differences in cell growth conditions in two distinct cultures. First of all, the

Figure 1. S6K1 subcellular localization in breast cancer cells *in vivo* and *in vitro*. (A) Immunohistochemical detection of S6K1 in human conditionally normal breast tissue. Magnification 400x. (B) Immunohistochemical analysis of S6K1 subcellular localization in human breast cancer tissue. Magnification 400x. Arrows point out to the positive reaction in the nuclei of the cells. (C) Immunohistochemical detection of S6K1 in MCF-7 multicellular spheroids. Magnification 200x. (D) Immunofluorescence image of S6K1 subcellular distribution (green) in 40% confluent MCF-7 cell monolayer. Arrows point out to the positive reaction in the nuclei of the cells. DNA was counterstained with DAPI (blue). Scale bars correspond to 20 µm. The data are representative of three independent experiments.

reason for such alterations could be explained by a cascade of intracellular events induced by cell-matrix adhesion or intercellular interactions. One can assume that the S6K1 is involved in such intracellular rearrangement. To clarify this issue, S6K1 subcellular localization in MCF-7 cells cultured at different densities was analyzed by the present study. The immunofluorescence analysis revealed the displacement of S6K1 from the nucleus to the cytoplasm when the cell culture density increased (Figure 2A–E). At the lowest MCF-7 cell density, S6K1 was observed predominantly in the nuclei of cultured cells whereas at the highest density the main signal was concentrated in the cytoplasm.

Besides, to test the possible dependence of S6K1 subcellular localization on cell density the following approach was introduced. After reaching 90% confluence layer of MCF-7 cells, the monolayer was detached from growth surface by short treatment with trypsin (w/o EDTA), and replaced in fresh culture medium. After cultivation for 48 h such fragments of monolayer demonstrated high cell density in the center and spread out cells at the edges. Immunofluorescence analysis revealed that cells' spreading was accompanied by the alterations in S6K1 localization (Figure 2F). Densely packed cells in the center of the fragments demonstrated cytoplasmic reaction for S6K1, while spread out cells had preferentially nuclear staining.

Dataset 1. Unedited images that were used in Figure 1 and Figure 2, showing S6K1 subcellular localization in breast normal tissue, cancer tissue, and in MCF-7 cells monolayer

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Subcellular localization of S6K1 in migrating MCF-7 cells

Since cell spreading can be considered as a stage of migration, the previous data led to the hypothesis of a relation between the initiation of cell migration and the relocalization of S6K1. Among the variety of cell migration models, it is necessary to point out those that are based on 3D cell cultures (Metzger *et al.*, 2017). There are a lot of data concerning the similarity of multicellular spheroid organization and structure of solid malignant tissue (Rodrigues *et al.*, 2017). Besides, the transformation of 3D multicellular spheroids in 2D cell colonies can be realized only by activation of cultured cell migration. So, in the present study, S6K1 distribution was examined on migrating MCF-7 cells after replacing MCF-7 multicellular spheroids onto the growth surface (Figure 3). We applied the immunofluorescence analysis of cultured cells after 24 and 72 hours after initiation of cell migration from MCF-7 spheroids. Obtained data suggest that significant relocalization of S6K1 from the cytoplasm into the nuclei in course of locomotor function realization took place (Figure 4A, B). The cells, which were still in 3D condition, had positive cytoplasm and negative nuclei,

Figure 2. S6K1 re-localizes from the nucleus to the cytoplasm during formation of the confluent monolayer of MCF-7 cells. MCF-7 cells were seeded onto glass coverslips in the density 10 000 cells/well (A), 30 000 cells/well (B), 50 000 cells/well (C), 70 000 cells/well (D), 100 000 cells/well (E), and cultivated for 48 hours. Then cells were fixed and stained with anti-S6K1 (green). White arrows point to the prominent positive reaction in the nuclei of the cells, yellow arrows point to the decreased staining in the nuclei. Scale bars are 20 μ m. The images are representative of three independent experiments. (F) MCF-7 cells were cultivated to form a super-confluent monolayer. Then fragments of the monolayer were gently detached by short incubation with trypsin, transferred on the coverslip and left for 48 hours to grow. After it the fragments were fixed and stained with anti-S6K1 (green). White arrows point to the positive reaction in the nuclei of the cells at the leading edge of the monolayer fragment; yellow arrows point to the decreased staining in the nuclei of the cells at the center of the monolayer fragment. Magnification 400x.

Figure 3. Scheme of the multicellular spheroid formation and initiation of cell migration. To generate multicellular spheroids, MCF-7 cells were seeded in the Petri dishes coated with 1% agarose, and cultivated for 72 hours. To analyze cell migration, obtained spheroids were transferred onto glass coverslips and cultured for 24 or 72 hours. Microphotographs of the spheroids general view were taken by Leica DM1000 (Leica, Germany) in transmitted light. Magnification 200x.

Figure 4. S6K1 shuttles to the nuclei during spheroid-to-monolayer reversion. (A) Immunofluorescence analysis of S6K1 subcellular localization (green) in the MCF-7 spheroid reversed for 24 hours. Arrows point to the predominantly nuclear distribution of the S6K1 in the migrating cells. DNA was counterstained with DAPI (blue). Scale bars are 20 μ m. (B) Immunofluorescence analysis of S6K1 subcellular localization (green) in the MCF-7 cells at the leading age of spheroid reversed for 72 hours. Arrows point to the predominantly nuclear distribution of the S6K1 in the migrating cells. DNA was counterstained with DAPI (blue). Scale bars are 20 μ m. The images are representative of three independent experiments.

as well as cells of spheroid at histological sections regardless of their remoteness from the edge of the spheroid (Figure 1C). During MCF-7 spheroid transformation in monolayer, spreading cells demonstrated strong accumulation of S6K1 in the nuclei (Figure 4B).

Dataset 2. Unedited images from Figure 3

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The most often used marker of S6K1 activation is Thr389 phosphorylation mediated by mTOR (Romanelli *et al.*, 2002). Thus, we analyzed phosphorylation status of S6K1 in MCF-7 cells during spheroid transformation into monolayer by immunofluorescence analysis. Overall, the pattern of phospho-S6K1 distribution was similar to that observed for total S6K1 (Figure 5). Namely, in the central part of the spheroid, S6K1 was mainly observed in the cytoplasm (however some of

Figure 5. Immunofluorescence analysis of phospho-S6K1 (T389) subcellular localization. (A) Confocal image of MCF-7 spheroid-to-monolayer reversion model. Cells were stained with anti-phospho-S6K1 (T389) (green). DNA was counterstained with DAPI (blue). Scale bars correspond to 20 µm. Arrows point to the positive reaction in the nuclei of the migrating cells at the leading edge of the spheroid. (B) Confocal image of monolayer culture of the MCF-7 cells stained with anti-phospho-S6K1 (T389) (green). DNA was counterstained with DAPI (blue). Scale bars correspond to 20 µm. The images are representative of two independent experiments.

the nuclei were positive), whereas all cells of the leading edge demonstrated predominant nuclear kinase localization (Figure 5A). Also, strong nuclear localization of phospho-S6K1 (Thr389) was revealed in monolayer culture of MCF-7 cells (Figure 5B).

Dataset 3. Unedited images that were used in Figure 4 and Figure 5, showing S6K1 and phospho-S6K1 (T389) subcellular localization during MCF-7 cell migration

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S6K1 function in nuclei and cell migration

Obtained data suggested that activation of cell locomotor function is accompanied by cytoplasm/nuclear shuttling of S6K1; however, the biological sense of the event is not yet clear. The possible explanation could be the implication of S6K1 in the regulation of transcription factors affecting expression of genes that control cell migration.

As it was mentioned above, several transcription factors are known as targets of S6K, but their nuclear localization in relation to cell migration was not reported. That's why, we analyzed subcellular distribution of several transcription factors, which are mTOR/S6K signaling regulated and activated in migrating cells either in a cancer tissue or in the process of organism development. Among them the mammalian transcription factor CDX2, which plays a key role in intestinal development

and differentiation. It was determined that reduced expression of CDX2 is important in colon tumorigenesis through mTOR-mediated chromosomal instability (Aoki *et al.*, 2003). Fusion of another transcription factor ERG and androgen-responsive TMPRSS2 serine protease is an important feature of prostate cancer. A strong correlation has been revealed between TMPRSS2-ERG fusion and activation of mTOR/S6K pathway (Faraj *et al.*, 2013; King *et al.*, 2009). The third studied transcription factor was T-box transcription activator Eomesodermin (or TBR2) (Conlon *et al.*, 2001). Earlier it was regarded as a target for anticancer therapy. It was detected that siRNA knockdown of Eomesodermin in human hepatocellular carcinoma significantly affected anchorage-independent cell growth (Gao *et al.*, 2014). Besides, it is involved in lymphocyte differentiation. It should be noted that TOR signaling is involved in modulation of TBR2 activity; it was demonstrated that TOR signaling was the central regulator of transcriptional programs by regulation of expression of transcription factors T-bet and Eomesodermin, that determined effector or memory cell fates in CD8+ T cells (Cui *et al.*, 2016).

Immunofluorescence analysis of subcellular distribution of S6K1 and mentioned transcription factors in MCF-7 cells revealed that ERG transcription factor either was present in scant quantities or not determined at all in MCF-7 cells (Dataset 4; (Kosach *et al.*, 2018d)). CDX2 was determined as positive dots predominantly in the nuclei, CDX-2 and S6K1 colocalization was not detectable by confocal microscopy (Dataset 4; (Kosach *et al.*, 2018d)). TBR2/Eomesodermin positive granules were

observed in the cytoplasm as well as in nuclei (Figure 6A, B). In both cases, partial but bright colocalization of TBR2 and S6K1 was revealed. Moreover, in low-density monolayer, when S6K1 localized mainly in the nuclei, TBR2 was observed predominantly in the nuclei as well. At high-density monolayer, S6K1 was redistributed in the cytoplasm, and TBR2 repeated the pattern of immunofluorescent reaction (Figure 6A, B).

Dataset 4. Unedited images of S6K1 colocalization with transcription factors TBR2 (Figure 6), ERG, and CDX2

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For quantitative characterization of S6K1 and TBR2 colocalization, Pearson coefficient (Rr) and Manders coefficient (M1 and M2) analysis was performed on background-subtracted images using JACoP ImageJ plugin (Bolte & Cordelières, 2006). M1 shown the colocalization of S6K1 with TBR2, whereas M2 expressed the pool of TBR2 colocalizing with S6K1. Colocalization analysis of S6K1 and TBR2 in low density monolayer revealed Pearson coefficient $Rr = 0.55 \pm 0.113$, $M1 = 0.999 \pm 0.01$, $M2 = 0.84 \pm 0.087$. To validate and describe the obtained degree of colocalization pre-defined image sets from Colocalization Benchmark Source were used. The closest benchmark was CBS007RGM that corresponded to 60% colocalization, thus indicating the medium level of colocalization between TBR2 and S6K1. A slightly lower but reliable colocalization of S6K1 and TBR2 was observed in a monolayer with a high density. Namely Pearson coefficient was $Rr = 0.47 \pm 0.064$, Manders

coefficients were $M1 = 0.995 \pm 0.004$ and $M2 = 0.62 \pm 0.187$. So, a slightly higher level of S6K1 and TBR2 colocalization was revealed in MCF-7 cells grown in low density monolayer, when S6K1 and TBR2 localized mainly in the nuclei.

The application of immunoprecipitation confirmed the interaction of S6K1 and TBR2 (Figure 7). Protein complexes containing S6K1 were extracted from cultured MCF-7 cell lysate using anti-S6K1 antibodies and then blotted with antibodies to TBR2. Obtained results revealed protein complex formation of S6K1 and TBR2 suggesting the possibility of TBR2 regulation via S6K1 mediated phosphorylation.

Dataset 5. Unedited western blot images of co-immunoprecipitation of S6K1 and TBR2 used in Figure 7

<http://dx.doi.org/10.5256/f1000research.15447.d214434>

Further computational prediction of phosphorylation sites in TBR2 (GPS 2.1) indeed revealed several sites, and three of them (Tyr421, Tyr423, Ser646) can be phosphorylated by S6K1 with a high score (Figure 8). Interestingly, both Tyr421 and Tyr423 are located in the DNA binding domain and one can assume that their phosphorylation could be related to the binding ability of the transcription factor to the targeted DNA. Another phosphorylation site (Ser646) is located within transcription activation domain at C-terminus of TBR2, which is involved in transcription activation. So, S6K1 can be involved in the regulation of TBR2 transcription activity. However, further research is needed to find if S6K1 phosphorylates TBR2 *in vitro* and *in vivo*.

Figure 6. S6K1 partially colocalizes with transcription factor TBR2 in MCF-7 cells. (A) Immunofluorescence image of low density monolayer culture of the MCF-7 cells double stained with anti-S6K1 (green) and anti-TBR2 (magenta). DNA was counterstained with DAPI (blue). Scale bars correspond to 20 μ m. Arrows point to the regions of S6K1 and TBR2 colocalization. (B) Immunofluorescence image of high density monolayer culture of the MCF-7 cells double stained with anti-S6K1 (green) and anti-TBR2 (magenta). DNA was counterstained with DAPI (blue). Scale bars correspond to 20 μ m. Arrows point out to the regions of S6K1 and TBR2 colocalization. The images are representative of two independent experiments.

Figure 7. Co-Immunoprecipitation of S6K1 and TBR2 protein complex in the MCF-7 cells. Endogenous S6K1 was precipitated with anti-S6K1 mouse monoclonal antibodies immobilized on protein A/G PLUS Agarose beads (Santa Cruz Biotechnology). As a control, protein agarose beads were incubated with monoclonal antibodies or cell lysates alone. Immune complexes were analyzed by immunoblotting with anti-TBR2 rabbit antibodies (Abcam, ab23345) or anti-S6K1 C-terminal rabbit polyclonal antibodies. The data are representative of two independent experiments.

Figure 8. S6K1 possibly phosphorylates TBR2 at several residues. Free software Group-based Prediction System v2.1 was used for bioinformatics analysis. It revealed that TBR2 contained three sites that could be phosphorylated by S6K1 with a high probability (**A**). Two of them, T421 and T423, situate in the DNA binding domain of the TBR2. Third site S646 is within transcription activation domain at C-terminus of TBR2 (**B**). Possible phosphorylation of these residues by S6K1 could significantly influence the TBR2 activity. However, this finding warrants further research.

Concerning TBR2 targets, in the course of organism development, Eomesodermin can induce virtually the entire spectrum of mesodermal genes in all types of mesodermal cells, which could appear in malignant cells of non-mesodermal origin (Reim *et al.*, 2017; Russ *et al.*, 2000).

Considering the multiplicity of S6K1 substrates, phosphorylation of the TBR2 transcription factor is not the only reason for the movement of the kinase from the cytoplasm into the nucleus of migrating cells. However, the proposed interaction can partially explain the accumulation of kinase in the nucleus of moving cells. The existence of several kinase isoforms may explain the partial colocalization of the kinase and the transcription factor (Amaral *et al.*, 2016). In addition to the previously known classical nuclear substrates of S6K1, in case of breast cancer, it is necessary to note that this kinase can activate estrogen receptor- α , which is a nuclear transcription factor by its phosphorylation at Ser167 in a ligand-independent manner (Yamnik & Holz, 2010). Besides, recent data indicate that S6K1 is targeted by histone acetyltransferases p300 and p300/CBP-associated factor (PCAF). The significance of this acetylation is not fully clear, but by analogy with S6K2, it is assumed that S6K1 is involved in the regulation of the transcription process (Fenton *et al.*, 2010). Summing up, there are a number of data confirming the nuclear localization of S6K1, but the role that S6K1 performs in the nucleus of migrating malignant cells require further investigation.

Conclusions

For the first time, this study revealed S6K1 relocation from the cytoplasm to the nuclei in migrating cells using the model of spheroids MCF-7 cells transformation into monolayer culture. Such relocation could be linked to the S6K1 driven activation of transcription factors responsible for cell locomotion. Particularly, colocalization and interaction of S6K1 and transcription factor TBR2 were revealed using confocal microscopy and co-immunoprecipitation. In addition, bioinformatics analysis of phosphorylation sites in TBR2 supports a prediction about S6K1 mediated phosphorylation and regulation of TBR2.

Data availability

F1000Research: Dataset 1. Unedited images that were used in Figure 1 and Figure 2, showing S6K1 subcellular localization in breast normal tissue, cancer tissue, and in MCF-7 cells monolayer., 10.5256/f1000research.15447.d214430 (Kosach *et al.*, 2018a).

F1000Research: Dataset 2. Unedited images from Figure 3., 10.5256/f1000research.15447.d214431 (Kosach *et al.*, 2018b).

F1000Research: Dataset 3. Unedited images that were used in Figure 4 and Figure 5, showing S6K1 and phospho-S6K1 (T389) subcellular localization during MCF-7 cell migration., 10.5256/f1000research.15447.d214432 (Kosach *et al.*, 2018c).

F1000Research: Dataset 4. Unedited images of S6K1 colocalization with transcription factors TBR2 (Figure 6), ERG, and CDX2., 10.5256/f1000research.15447.d214433 (Kosach *et al.*, 2018d).

F1000Research: Dataset 5. Unedited western blot images of co-immunoprecipitation of S6K1 and TBR2 used in Figure 7., 10.5256/f1000research.15447.d214434 (Kosach *et al.*, 2018e).

Grant information

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgments

We would like to thank Prof. Valeriy Filonenko for support, expertise, and discussions that greatly assisted the research. We also thank Dr. Volodymyr Shablui for antibodies against transcription factors (TBR2, CDX2, ERG), and Dr. Serhiy Karakhim for help in obtaining the confocal images at Light Microscopy Facility of the O.V. Palladin Institute of Biochemistry of NAS of Ukraine.

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Svetlana Dokudovskaya

French National Centre for Scientific Research(CNRS), UMR 8126, Institut Gustave Roussy (IGR), University of Paris-Sud (University of Paris XI), Villejuif, France

The MS by Kosach et al. describes an interesting phenomenon, nucleocytoplasmic shuttling of S6K1 in MCF-7 cells. The authors established a relationship between MCF-7 monolayer density and subcellular localization of the kinase. The work is worthy of publication, but the MS should be significantly shortened as it contains some redundant information and speculations. Proofreading by a native English-speaker is also recommended.

Major point:

The putative phosphorylation of TBR2 by S6K1 is too speculative and not convincing to use it as one of the major conclusions of the papers. It should be either confirmed experimentally or removed from the abstract and the main results of the paper.

Minor points:

Material and Methods:

p4 Please remove "if you are interested in obtaining this antibody, please contact the corresponding author";

p4 The anti-ERG and anti-CDX antibodies are not used in the main Figures. Please move information about these antibodies from the Materials and Methods section to legends of the supplementary figures;

Results and discussion:

P5 Please remove the first paragraph or move it to the introduction section.

P5 "For the first step of the present study" is redundant

Figure 1,5 legend. Please replace "positive reaction" with "staining"

P6 The following phrase is unclear: "Since cell spreading can be considered as a stage of

migration, the previous data led to the hypothesis..."

Figure 6. It is unclear whether the images represent confocal sections or reconstituted 3D images. Colocalization of S6K1 and TBR2 should be analyzed quantitatively, eg using ImageJ tools for colocalization analysis. It is also unclear whether TBR2 shuttles between the nucleus and the cytoplasm similarly to S6K1.

P9. S6K1 is a serine threonine kinase and the putative phosphorylation of TBR2 by S6K1 should concern threonines rather than tyrosines. I also propose to remove the bioinformatic part and the Figure 8 as they are too speculative. Indeed, many other kinases may potentially phosphorylate TBR2 basing on the bioinformatic analysis, and the authors did not even show whether TBR2 is indeed phosphorylated.

Figure 7. TBR2 has a molecular weight of 84 kDa of TBR2, but the major band has a lower molecular weight. This should be explained in the Results section.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Not applicable

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

We confirm that we have read this submission and believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.

Author Response 03 Dec 2018

Viktoriia Kosach, National Academy of Sciences of Ukraine, Kyiv, Ukraine

We thank the reviewers for deep and knowledgeable revision of this manuscript. Please, find below our response to the reviewers' comments point by point.

1. *Major point: The putative phosphorylation of TBR2 by S6K1 is too speculative and not convincing to use it as one of the major conclusions of the papers. It should be either confirmed experimentally or removed from the abstract and the main results of the paper.*

We have withdrawn the mentioned statements from the conclusions and the abstract, and

left as a hypothesis in the text of our article.

2. Minor points

p4 Please remove "if you are interested in obtaining this antibody, please contact the corresponding author";

We could not remove it, as this sentence is a recommendation of the F1000Research Editorial board, and it informs the other researchers, where they could obtain these antibodies to replicate the study.

p4 The anti-ERG and anti-CDX antibodies are not used in the main Figures. Please move information about these antibodies from the Materials and Methods section to legends of the supplementary figures;

We agree. We have moved mentioned information to the legend of Dataset 4.

Results and discussion:

P5 Please remove the first paragraph or move it to the introduction section.

P5 "For the first step of the present study" is redundant

Figure 1,5 legend. Please replace "positive reaction" with "staining"

P6 The following phrase is unclear: "Since cell spreading can be considered as a stage of migration, the previous data led to the hypothesis..."

We agree with all comments. We have corrected them in version 2 according to reviewers' queries.

Figure 6. It is unclear whether the images represent confocal sections or reconstituted 3D images. Colocalization of S6K1 and TBR2 should be analyzed quantitatively, eg using ImageJ tools for colocalization analysis. It is also unclear whether TBR2 shuttles between the nucleus and the cytoplasm similarly to S6K1.

The images represent confocal sections. Colocalization of S6K1 and TBR2 was analysed using ImageJ JaCoP plugin, as it is indicated in article. As well as S6K1, we observed TBR2 nucleo/cytoplasmic relocalization that depended on cell density. Moreover, TBR2 shuttled between cytoplasm and nuclei similarly to S6K1 during spheroid to monolayer reversion. But quantitatively the level of colocalization of S6K1 and TBR2 we detected on MCF-7 monolayer at different density, because the cells are in more similar condition reliable for image analysis than in course of 3D spheroid transformation into 2D monolayer colony.

P9. S6K1 is a serine threonine kinase and the putative phosphorylation of TBR2 by S6K1 should concern threonines rather than tyrosines. I also propose to remove the bioinformatic part and the Figure 8 as they are too speculative. Indeed, many other kinases may potentially phosphorylate TBR2 basing on the bioinformatic analysis, and the authors did not even show whether TBR2 is indeed phosphorylated.

We agree that there was erratum concerning tyrosin and threonine. We have fixed it.

As co-immunoprecipitation revealed the existence of S6K1-TBR2 protein complex in MCF-7 cells, we considered the theoretical probability of TBR2 phosphorylation by S6K1 kinase. Of course, this does not exclude the presence of other effectors of this transcription factor, but gives us the prerequisites for hypothesis. We removed this statement from the conclusions of the article.

Figure 7. TBR2 has a molecular weight of 84 kDa of TBR2, but the major band has a lower molecular weight. This should be explained in the Results section.

The antibody supplier also state that anti-TBR2 antibodies detects two bands in immunoblot analysis. We have found information that 4 splice isoforms of TBR2 with lower molecular weight are known today (<https://www.uniprot.org/uniprot/O95936>).

Competing Interests: No competing interests were disclosed.

Reviewer Report 28 September 2018

<https://doi.org/10.5256/f1000research.16834.r37832>

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Olivier E. Pardo

Division of Cancer, Department of Surgery and Cancer, Imperial College London, London, UK

The study by Kosach et al. clearly shows for the first time differential nucleo-cytoplasmic localisation of S6K1 in response to cell density and migration. It also provides some information about the type of role that S6K1 may play in the nucleus and the biological processes that may be associated.

The results shown are convincing and the quality of the data is sufficient to support the conclusions of the authors. Therefore, it is the opinion of this reviewer that the present manuscript should be accepted. However, the authors may want to address the comments below so as to improve the quality/impact of the manuscript:

1. At the end of the fourth paragraph of the Introduction, the authors refer to EGF as "endothelial growth factor". This should be corrected to "epithelial growth factor".
2. The value of the first paragraph of the Results section (There are several... of different function.") is not clear and should be deleted.
3. On Page 13 of the manuscript, the authors refer to 2 residues on TRB2, Tyr421 and Tyr423, as potential sites phosphorylated by S6K1. S6K2 is a Ser/Thr kinase and therefore unable to phosphorylate Tyr residues. Are the authors referring to Thr sites instead? Please clarify.
4. The authors argue that interaction of S6K1 with TRB2 may be involved in the migration of cells out of microspheres onto 2D layers. This is an interesting proposition and the

manuscript would benefit from an experiment being performed using siRNAs to TRB2 and showing that this impacts either S6K1 distribution or the movement of cells out of the microspheres.

5. The style of the paper is poor in places and could be improved prior to indexing.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Not applicable

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 03 Dec 2018

Viktoriia Kosach, National Academy of Sciences of Ukraine, Kyiv, Ukraine

We thank the reviewer for expert opinion and insightful comments on our study. We appreciate these comments, and we have tried to answer step by step. Please, find below our response to the reviewers' comments.

1. *At the end of the fourth paragraph of the Introduction, the authors refer to EGF as "endothelial growth factor". This should be corrected to "epithelial growth factor".*

We agree. It was erratum concerning EGF, and we have fixed it.

1. *The value of the first paragraph of the Results section (There are several... of different function.) is not clear and should be deleted.*

We have deleted mentioned paragraph.

1. *On Page 13 of the manuscript, the authors refer to 2 residues on TRB2, Tyr421 and Tyr423, as potential sites phosphorylated by S6K1. S6K1 is a Ser/Thr kinase and therefore unable to phosphorylate Tyr residues. Are the authors referring to Thr sites instead? Please clarify.*

Yes, of course, S6K1 is Ser/Thr protein kinase, and we wrote about Threonine 421 and Threonine 423 residues on TRB2, but, unfortunately, there was erratum in the text. We have fixed it in version 2.

1. *The authors argue that interaction of S6K1 with TRB2 may be involved in the migration of cells out of microspheres onto 2D layers. This is an interesting proposition and the manuscript would benefit from an experiment being performed using siRNAs to TRB2 and showing that this impacts either S6K1 distribution or the movement of cells out of the microspheres.*

This is a very interesting suggestion. Also, to our mind, interaction of S6K1 and TBR2 could be just one from the possible reasons of S6K1 subcellular redistribution in course of MCF-7 cell migration. The level of their colocalization is statistically significant, but not very high, so, under TBR2 down-regulation we can obtain partial alteration of S6K1 relocation that will be difficult to assay. Therefore, in the present work, we so far only point to the revealed colocalization. And we hope to develop this area of research in future.

1. *The style of the paper is poor in places and could be improved prior to indexing.*

The manuscript was proofread by a person with advanced level of scientific English. We believe that this greatly improved the style of the text.

Competing Interests: No competing interests were disclosed.

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