FEBS Letters 584 (2010) 124–128

FEBS Letters

journal homepage: www.FEBSLetters.org

mTOR/S6K1 and MAPK/RSK signaling pathways coordinately regulate estrogen receptor α serine 167 phosphorylation

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ARTICLE INFO

Article history:
Received 29 September 2009
Revised 10 November 2009
Accepted 12 November 2009
Available online 16 November 2009

Edited by Lukas Huber

Keywords:
p70 S6 kinase 1
p90 ribosomal S6 kinase
Mammalian target of rapamycin
Rapamycin
Estrogen receptor α
Breast cancer

ABSTRACT

Resistance to anti-estrogen therapy is a major clinical concern in treatment of breast cancer. Estrogen-independent phosphorylation of estrogen receptor α, specifically on Ser167, is one of the contributing causes to development of resistance, and a prognostic marker for the disease. Here, we dissect the signaling pathways responsible for Ser167 phosphorylation. We report that the mTOR/S6K1 and MAPK/RSK contribute non-overlapping inputs into ERα activation via Ser167 phosphorylation. This cooperation may be targeted in breast cancer treatment by a combination of mTOR and MAPK inhibitors.

1. Introduction

The mammalian target of rapamycin (mTOR) is a conserved protein kinase that is a key regulator of cell growth and proliferation in response to nutrient availability and growth stimuli. Rapamycin is a naturally-derived inhibitor of mTOR, and an inhibitor of cell proliferation, as manifested by its potent immunosuppressive properties and activity against solid tumors [1]. Recent work led to the realization that rapamycin does not perturb all mTOR functions because mTOR exists in two complexes in eukaryotic cells, mTOR complexes 1 and 2 (mTORC1 and 2). mTORC1 and mTORC2 consist of distinct sets of proteins and perform non-redundant functions [2]. This work focuses on the rapamycin-sensitive mTORC1 signaling.

In response to a variety of stimuli, including mitogens and hormones, the mitogen-activated protein kinase (MAPK) and mTORC1 pathways regulate important cellular processes such as cell growth, proliferation, and survival [3,4]. There exists an extensive cross-talk between MAPK and mTORC1 signaling in cells. Correspondingly, the effectors of these pathways, the p90 ribosomal S6 kinase (RSK) and the p70 S6 kinase 1 (S6K1) have been shown to converge on a common set of targets, most notably in control of protein translation [5–7]. In this study, we identify estrogen receptor α (ERα) as a recipient of coordinated phosphorylation inputs from the MAPK and mTORC1 pathways.

ERα mediates the proliferative effects of estrogen and represents an important clinical target in treatment of breast cancer. Tamoxifen is an anti-estrogen that has become the standard agent for the treatment of ER-positive breast cancer, where it acts as an antagonist. However, resistance to tamoxifen, and other endocrine or anti-estrogen therapies develops in many cases [8,9]. One mechanism by which resistance develops is through phosphorylation of ERα, allowing it to act in estrogen-independent manner. As illustrated in Fig. 1, the N-terminal estrogen-independent activation AF-1 domain of ERα is responsible for ligand-independent transactivation function of ERα. ERα phosphorylation within the AF-1 domain occurs on residues Ser104/106, Ser118, and Ser167. Ser104/106 phosphorylation is regulated by cdk [10], and Ser118 phosphorylation is regulated by MAPK [11,12], although it has been suggested that MAPK controls this event indirectly [13]. Phosphorylation of Ser167 has been previously attributed to Akt and RSK [14,15], while we have demonstrated that S6K1 is the physiological ERα Ser167 kinase and it phosphorylates this site in

Abbreviations: S6K1, p70 S6 kinase 1; MAPK, mitogen-activated protein kinase; RSK, p90 ribosomal S6 kinase; mTOR, mammalian target of rapamycin; ERα, estrogen receptor α; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C

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0014-5793/$36.00
doi:10.1016/j.febslet.2009.11.041

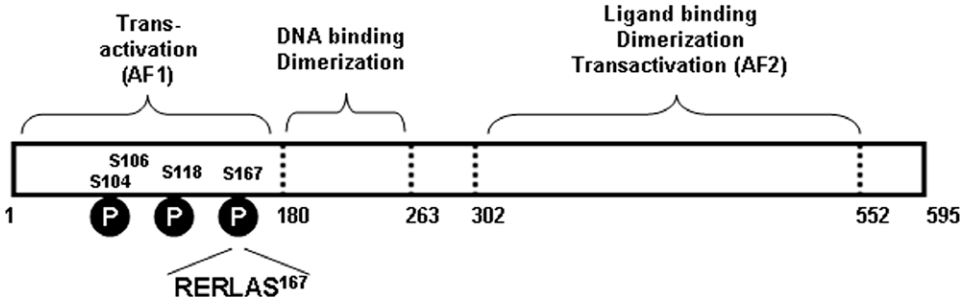

Fig. 1. Domain architecture of estrogen receptor α (ERα), and location of phosphorylation sites within the AF-1 domain.

rapamycin-sensitive fashion [16]. Importantly, Ser167 phosphorylation correlates with resistance to tamoxifen [14] and is a prognostic marker for disease progression and survival [17]. Thus, the identity of the kinase(s) responsible for this phosphorylation event has important clinical consequences.

RSK and S6K1 recognize identical consensus phosphorylation sequence RxRxxS/T, where x is any amino acid, and they share common phosphorylation targets [5,6]. ERα contains a phosphorylation motif RERLAS^{167} (Fig. 1), and both kinases have been shown to directly phosphorylate this site in in vitro kinase assays [15,16]. Because of the different kinetics of mitogen-mediated activation of the mTORC1/S6K1 and MAPK/RSK signaling pathways, it is possible that RSK may play a physiological role in phosphorylation of ERα. Therefore, we set out to determine the relative contributions of the MAPK/RSK and mTORC1/S6K1 signaling pathways to phosphorylation and activation of ERα. In this study, we demonstrate that in response to activating stimuli S6K1 and RSK phosphorylate ERα, allowing for coordinate regulation of ERα activation.

2. Materials and methods

2.1. Reporter and expression vectors

pGL2-3xERE-TATA-luc was kindly provided by Donald P. McDonnell (Duke University, Durham, NC), and pIS2 renilla luciferase reporter was kindly provided by David Bartel (MIT, Cambridge, MA).

2.2. Cell culture

MCF7 cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS).

2.3. RNAi against RSK1/2

For the siRNA studies, double-stranded RNAs for RSK1 and RSK2 were a kind gift from John Blenis (Harvard Medical School, Boston, MA). MCF7 cells were transfected using Lipofectamine2000 (Invitrogen) according to the manufacturer's recommendations. After 24 h post-transfection, cells were deprived of serum overnight, treated with agents as indicated in the figure legend.

2.4. Reporter gene assays

For luciferase reporter assays, cells were transfected using Lipofectamine2000 (Invitrogen) using the manufacturer's protocol with plasmids encoding for firefly luciferase under control of three ERE, and control renilla luciferase. At 24 h post-transfection, rapamycin (20 ng/mL) and/or U0126 (Biomol, 10 μM) were added where indicated. At 48 h post-transfection, cells were harvested using 1× Passive Lysis Buffer (Promega), and relative luciferase activity was measured using the Dual Luciferase Reporter Assay System and Glomax 20/20 luminometer (Promega).

2.5. Immunoblots

Cells were lysed using 1× Passive Lysis Buffer (Promega). Whole-cell lysates (10% of total cell extract) were resolved by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) (10%). Proteins were transferred to a nitrocellulose membrane (Schleicher and Schuell) and blotted with the indicated antibodies. Anti-phospho-ERK1/2 antibodies were from Sigma. Anti-phospho-S6K1 Thr389, anti-phospho-S6 Ser235/236, anti-phospho-S6 Ser240/244, and anti-phospho-ER Ser167 antibodies were purchased from Cell Signaling Technology. Anti-ER antibodies were from Santa Cruz Biotechnology. Anti RSK1 and RSK2 antibodies were from Zymed. For immunoblotting, anti-rabbit, anti-mouse, and anti-goat horseradish peroxidase (HRP)-conjugated antibodies were purchased from Amersham, Chemicon, and Santa Cruz Biotechnology, respectively. Immunoblots were developed using enhanced chemiluminescence reagents (Pierce) and Chemidoc XRS imager with Quantity One software (Bio-Rad).

3. Results and discussion

3.1. Rapamycin-resistant ERα Ser167 phosphorylation in cells growing in serum-supplemented media

To evaluate the relative contribution of the MAPK and S6K1 signaling pathways to phosphorylation of ERα Ser167, MCF7 cells growing in 10% FBS were treated with the mTORC1 inhibitor rapamycin and MEK inhibitor U0126. As shown in Fig. 2A, treatment with rapamycin severely reduced Ser167 phosphorylation, however, some rapamycin-insensitive phosphorylation remained. While treatment with U0126 only slightly reduced phosphorylation levels, treatment with a combination of rapamycin and U0126 resulted in complete inhibition of Ser167 phosphorylation. This indicated that there exists a rapamycin-insensitive MAPK input into Ser167 phosphorylation.

3.2. Acute mitogenic stimulation of ERα Ser167 phosphorylation reveals MAPK and mTORC1/S6K1 inputs

To dissect the signaling inputs into ERα Ser167 phosphorylation, we interrogated the sensitivity of Ser167 phosphorylation to acute mitogenic stimulation. We deprived MCF7 cells of serum, and acutely stimulated them insulin, which preferentially activates the mTORC1/S6K1 pathway, or the phorbol ester phorbol 12-myristate 13-acetate (PMA), which activates both the mTORC1/S6K1 and MAPK pathways. We also investigated the effect of rapamycin and U0126 inhibitors on insulin- and PMA-stimulated ERα Ser167 phosphorylation. As shown in Fig. 2B, 30 min treatment with insu-

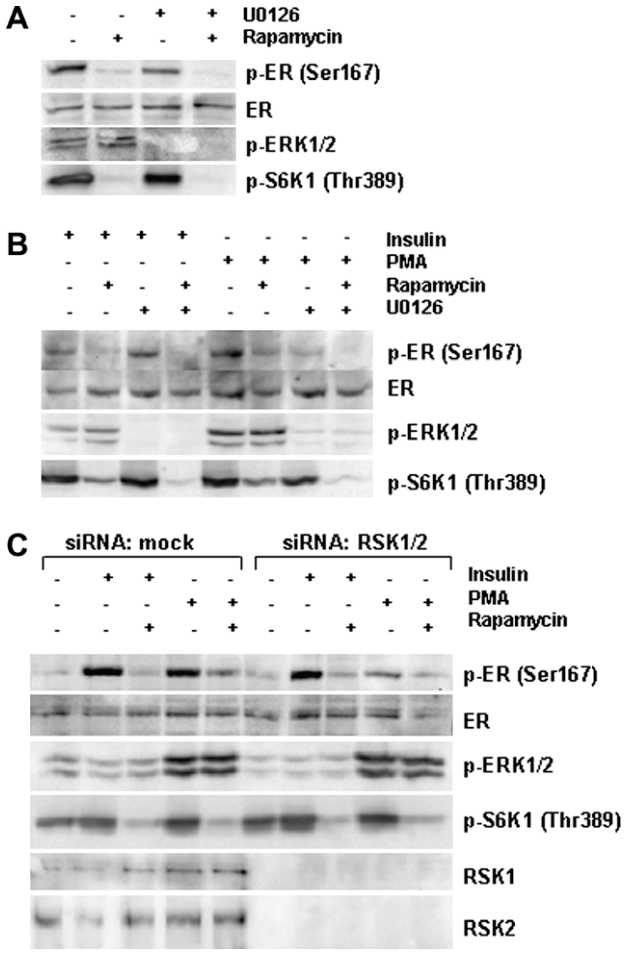

Fig. 2. Rapamycin-resistant ERα Ser167 phosphorylation is mediated by mitogen-activated protein kinase (MAPK)/p90 ribosomal S6 kinase (RSK) signaling. (A) Cells were grown in media containing 10% FBS, supplemented with 10 μM U0126 and/or 20 ng/mL rapamycin overnight, where indicated, and proteins were analyzed by immunoblots. (B) Cells were deprived of serum for 24 h, then pre-treated with 20 ng/mL rapamycin for 30 min and/or 10 μM U0126 for 2 h, and stimulated with 100 nM insulin or 100 ng/mL phorbol 12-myristate 13-acetate (PMA) for 30 min. Proteins were analyzed by immunoblots. (C) RNAi suppressing RSK1/2 expression reduces ERα Ser167 phosphorylation. Cells were transfected with siRNA targeting RSK1/2 or mock oligonucleotides. At 24 h post-transfection, cells were deprived of serum for additional 24 h. Cells were pre-treated with 20 ng/mL rapamycin for 30 min, and then stimulated with 100 nM insulin or 100 ng/mL PMA for 30 min. Proteins were immunoblotted with the indicated antibodies.

lin resulted in rapamycin-sensitive Ser167 phosphorylation. U0126 alone did not appear to inhibit ERα Ser167 phosphorylation, however, we observed a residual rapamycin-insensitive phosphorylation of ERα Ser167 phosphorylation. Accordingly, a combination of rapamycin and U0126 resulted in a greater inhibition of Ser167 phosphorylation than rapamycin alone. These findings indicated that S6K1 is the major contributor to Ser167 phosphorylation following insulin stimulation, while the MAPK pathway has a minor role. We next looked at the effect of PMA stimulation on Ser167 phosphorylation. Similar to insulin treatment, 30 min stimulation with PMA resulted in rapamycin-sensitive Ser167 phosphorylation. However, PMA-induced Ser167 phosphorylation was also partially U0126-sensitive. Treatment with a combination of rapamycin and U0126 abolished ERα Ser167 phosphorylation. Therefore, while the mTORC1/S6K1 pathway is the major signaling pathway phosphorylating Ser167, in response to activating stimuli, the MAPK pathway also contributes to this phosphorylation.

3.3. RNAi of RSK1/2 suppresses ERα Ser167 phosphorylation

Since PMA stimulation leads to activation of RSK, we inquired whether RSK may mediate rapamycin-insensitive Ser167 phosphorylation. To that end, we reduced expression of RSK isoforms RSK1 and RSK2 in MCF7 cells by means of siRNA, and acutely stimulated these cells with insulin or PMA, with or without rapamycin. As shown in Fig. 2C, while RNAi against RSK1/2 did not have an effect on insulin-mediated ERα Ser167 phosphorylation, PMA-induced phosphorylation was reduced in cells with RSK1/2 knockdown. Thus, RSK mediates rapamycin-insensitive ERα Ser167 phosphorylation in response to PMA stimulation.

3.4. Temporal regulation of ERα Ser167 phosphorylation by MAPK/RSK and S6K1

The S6K1 and MAPK pathways exhibit differential patterns of temporal activation. We compared the timing of RSK and S6K1 contributions to Ser167 phosphorylation. As shown in Fig. 3, we performed a time-course of stimulation of MCF7 cells with insulin or PMA. Interestingly, the kinetics of Ser167 phosphorylation were different after insulin and PMA stimulation. Insulin-induced Ser167 stimulation peaked at 30 min, which correlated with activation of S6K1 measured by phosphorylation at Thr389 and phosphorylation of S6 at Ser240/244. In contrast, PMA-stimulated Ser167 phosphorylation was detectable as early as 10 min, preceding full activation of S6K1 and phosphorylation of S6 at Ser240/244. Notably, phosphorylation of S6 at Ser235/236, residues that are also phosphorylated by RSK [6], mirrored the kinetics of Ser167 phosphorylation, indicating that the MAPK/RSK pathway contributes to phosphorylation of Ser167 during the early phase of stimulation with PMA.

3.5. Differential kinetics of S6K1 and RSK activation lead to biphasic pattern of ERα Ser167 phosphorylation

In order to further delineate the contribution of S6K1 and RSK to Ser167 phosphorylation, we compared the kinetics of PMA-stimulated phosphorylation in the presence of rapamycin and/or U0126. As shown in Fig. 4, PMA-induced Ser167 phosphorylation was observed as early as after 5 min of treatment, peaking at 30 min, and preceding full activation of S6K1. Treatment with rapamycin greatly reduced Ser167 phosphorylation, however, the inhibition was more pronounced at the later time points (after 15 min). Treatment with U0126 reduced early phosphorylation of Ser167, without having much effect on later time points. A combination of rapamycin and U0126 abrogated Ser167 phosphorylation. This indicated that two phases of Ser167 phosphorylation exist: an

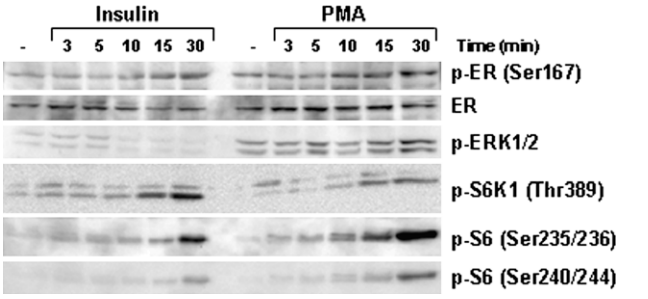

Fig. 3. Temporal regulation of ERα Ser167 phosphorylation by MAPK/RSK and p70 S6 kinase 1 (S6K1). Cells were deprived of serum for 24 h, and stimulated with 100 nM insulin or 100 ng/mL PMA, as indicated. Proteins were analyzed by imunoblots.

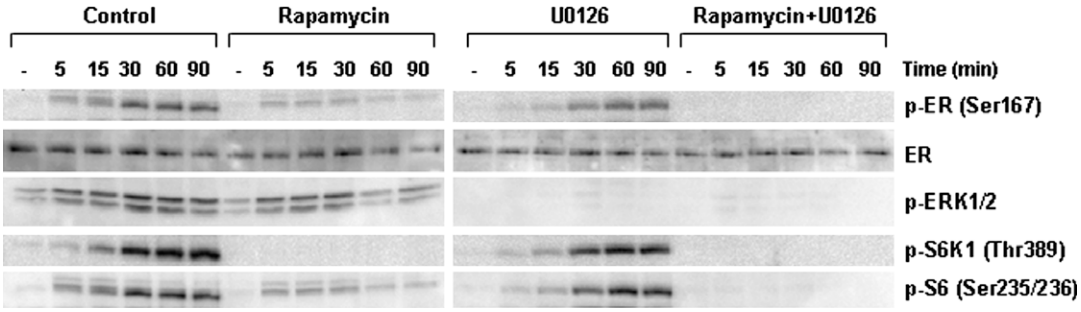

Fig. 4. Biphasic pattern of ERα Ser167 phosphorylation by S6K1 and RSK. Cells were deprived of serum for 24 h. Cells were pre-treated with 20 ng/mL rapamycin for 30 min and/or 10 μM U0126 for 2 h, and then stimulated with 100 ng/mL PMA, as indicated. Immunoblots were analyzed with the indicated antibodies. To accommodate the number of lanes shown in the figure, samples were resolved using two gels, the immunoblots were developed and exposed simultaneously, and shown using two panels in the figure.

early phase that is RSK-mediated and U0126-sensitive, and a late phase that is S6K1-mediated and rapamycin-sensitive.

Thus, S6K1 and RSK perform a non-redundant role with regard to ERα Ser167 phosphorylation and are responsible for a biphasic phosphorylation pattern. The early-activated RSK acts in a priming role, while the late-responding S6K1 is responsible for the maintenance of high-level phosphorylation. This may represent an important mechanism by which the cells insure timely activation of ERα using a combination of phosphorylating kinases.

3.6. Synergistic regulation of ERα transcriptional activity by S6K1 and MAPK/RSK

Ser167 phosphorylation is important for the ability of ERα to bind DNA and affect transcription of estrogen-response genes [16]. Since we observed that both rapamycin and U0126 affected Ser167 phosphorylation, we investigated the effect of a combination of these drugs on transcriptional activity mediated by ERα. We transfected MCF7 cells with a reporter construct that contains the firefly luciferase gene under the control of three estrogen-response elements (ERE), and a control construct expressing renilla luciferase under the control of SV40 promoter for luciferase activity normalization. As shown in Fig. 5, both rapamycin and U0126 significantly reduced ERE-mediated transcriptional activity. Additionally, a combination of the two agents resulted in synergistic inhibition. Thus S6K1 and MAPK pathways cooperate in regulation of ERα activity (Fig. 6).

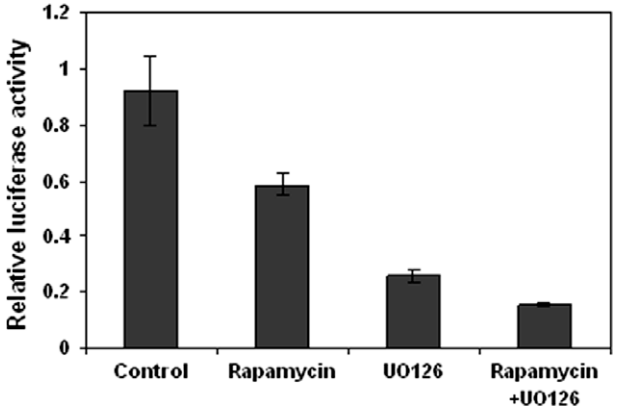

Fig. 5. Synergistic inhibition of ERα transcriptional activity by rapamycin and U0126. Cells were transfected with plasmids encoding for ERE-regulated firefly luciferase, and control renilla luciferase. After 24 h transfection, cells were incubated with full serum media in the presence or absence of 20 ng/mL rapamycin and/or 10 μM U0126 for additional 24 h. ERE-regulated luciferase expression was measured and normalized to control renilla luciferase. The data are presented as means ± S.D. of each experiment performed in triplicate.

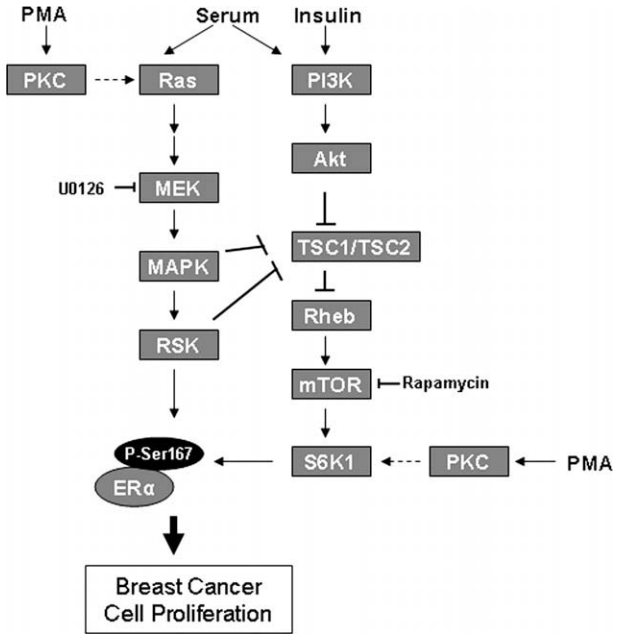

Fig. 6. mTORC1/S6K1 and MAPK/RSK pathways cooperate in promoting ERα Ser167 phosphorylation and activation. Insulin activates mTORC1 and S6K1 by signaling via PI3K and Akt to suppress TSC and thus activate Rheb and mTORC1. Serum signals through the PI3K pathway, as well as the Ras pathway to activate the MEK/MAPK/RSK cascade. MAPK and RSK also inhibit TSC via PI3K/Akt-independent phosphorylation of TSC2. PMA indirectly activates both S6K1 and RSK by signaling via the protein kinase C (PKC) pathway. Thus, when activated, the MAPK and mTORC1/S6K1 pathways signal to coordinately phosphorylate and activate ERα in control of breast cancer cell proliferation.

Estrogen receptor is an important clinical target is treatment of breast cancer. However, resistance to anti-estrogen therapy often develops. One of the mechanisms of resistance is through mitogenic estrogen-independent activation of ERα. Ser167 is an important clinical indicator of response to anti-estrogen therapy, prognosis, and survival. Here we demonstrate that Ser167 phosphorylation is a result of cooperation between two important signaling pathways in the cell, mTORC1 and MAPK. Clinically, MAPK and mTORC1 activation status serve as predictors for endocrine treatment responsiveness [18,19]. These findings may serve as a basis for combating chemotherapy resistance with regard to anti-estrogen or endocrine therapy. Rapamycin and its analogs, as well as MEK inhibitors, are currently being tested alone and in combination in different types of cancer, such as melanoma [20] and lung cancer [21,22]. While rapamycin shown an effect in early clinical trials, the effectiveness of rapamycin in treatment of breast cancer has been variable. We and others have demonstrated that breast

cells overexpressing S6K1, such as MCF7 cells are exquisitely sensitive to rapamycin [16,23], suggesting a possible way to target a population of patients most likely to respond to rapamycin treatment. Thus, a question arises whether dual inhibition of the mTORC1/S6K1 and MAPK/RSK, two major pathways leading to ERα activation and stimulation of breast cancer cell proliferation, may result in a better clinical response. The data presented here provide rationale for combining rapamycin therapy with MEK inhibitors in breast cancer with S6K1 overexpression.

Acknowledgements

This work is supported by a grant from the Atol Charitable Trust (MKH), Wendy Will Case Cancer Fund (MKH) and a Yeshiva University graduate research fellowship (RLY).

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