S6 Kinase 1 Regulates Estrogen Receptor α in Control of Breast Cancer Cell Proliferation*

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The 40 S ribosomal S6 kinase 1 (S6K1) acts downstream of mTOR (mammalian target of rapamycin) and is sensitive to inhibition by rapamycin. The chromosomal region 17q23 containing the RPS6KB1 gene is frequently amplified in breast cancer cells, leading to S6K1 overexpression. The role of S6K1 in disease development and progression is supported by the observation that S6K1 overexpression is associated with poor prognosis in breast cancer patients. However, the identity of mammary cell-specific S6K1 targets is not well understood. In this study, we report that overexpression of S6K1 endows breast cancer cells with a proliferative advantage in low serum conditions and enhanced sensitivity to rapamycin. We investigate the molecular mechanism behind this observation to show that S6K1 regulates estrogen receptor α (ERα) by phosphorylating it on serine 167, leading to transcriptional activation of ERs. By contributing to the activation of ERα, S6K1 promotes ERα-mediated cell proliferation and may be a target of therapeutic intervention in breast cancer.

mTOR (mammalian target of rapamycin) is a conserved protein kinase that is a key regulator of cell growth and proliferation in response to extracellular cues, including nutrient availability and growth stimuli. Rapamycin is a naturally derived inhibitor of mTOR that was revealed to be an inhibitor of cell proliferation, as manifested by its potent immunosuppressive properties and activity against solid tumors (1). The 40 S ribosomal S6 kinase 1 (S6K1) is one of the best characterized downstream targets of mTOR. Rapamycin treatment results in rapid dephosphorylation and inactivation of S6K1 (2). S6K1 is an important regulator of cell size control, protein translation, and cell proliferation (3). The 40 S ribosomal protein S6 is the best characterized target of S6K1 (4). Other targets have been reported, some of which include the apoptotic protein Bad, the eukaryotic elongation factor 2 kinase, the eukaryotic translation initiation factor 4B (eIF4B), the RNA-binding protein SKAR, and the translational inhibitor PDCD4 (5–9). It appears that S6K1 regulates its targets to increase the biosynthetic capacity of the cell that is necessary for cell division (9–11).

Data suggest that S6K1 is implicated in breast cancer. S6K1 is encoded by the RPS6KB1 gene localized to the chromosomal region 17q23. Region 17q23 is amplified in several breast cancer cell lines and in ~30% of primary tumors (12), whereas S6K1 is overexpressed in the majority of cell lines and primary tumors with this amplification (13–17). Furthermore, the role of S6K1 in disease development and progression is supported by the observation that RPS6KB1 amplification and S6K1 overexpression are associated with poor prognosis in breast cancer patients (13, 18).

Interestingly, whereas RPS6KB1 is amplified in several cancer types, high level (multicopy) amplification of RPS6KB1 is limited to breast cancer (16). This suggests that S6K1 may have a specific role in regulating the growth of breast cancer cells. Overexpression of S6K1 has been linked to rapamycin sensitivity of breast cancer cells (19). However, the molecular mechanism explaining this observation was not determined. Therefore, identification of mammary cell-specific downstream effectors of S6K1 in control of proliferation can provide us with insight for the development of new anticancer strategies.

In this study, we focus on estrogen receptor α (ERα) as a target of S6K1 in control of breast cancer cell proliferation. Clinically, up to 60% of breast cancers are ER-positive, making them a target of endocrine therapy. However, resistance to endocrine therapy develops in most cases. Thus, specifically targeting S6K1 in combination with endocrine therapy may be an important strategy to combat resistant breast tumors.

The addition of the ERα ligand, 17β-estradiol, and growth factors leads to ERα hyperphosphorylation, DNA binding, and transcriptional activity. One of the phosphorylation sites, Ser167, is found within the sequence RERLAS167, which conforms to the S6K1 consensus phosphorylation motif. This residue has been previously shown to be regulated by RSK and Akt kinases (20, 21). However, because both p90 ribosomal S6 kinase (RSK) and Akt are upstream activators of S6K1 via inhibition of TSC2, it is possible that S6K1 is the predominant kinase for Ser167 as a result of RSK and/or Akt signaling. There are precedents supporting this hypothesis. mTOR, PDCD4, and GSK3 are phosphorylated by S6K1 under conditions where Akt is not active (9, 22, 23). eIF4B and ribosomal protein S6 are differentially phosphorylated by both S6K1 and RSK (8, 24, 25).
**S6K1 Phosphorylates and Regulates ERα**

**FIGURE 1. Ser<sup>167</sup> phosphorylation of ERα in cells and in vitro is rapamycin-sensitive and correlates with S6K1 expression and activation.** A, T47D and MCF7 cells were grown in full serum with or without 20 ng/ml rapamycin (Rap) for 24 h. Cells were lysed using NE-PER reagent. Ser<sup>167</sup> phosphorylation and ERα levels were determined by immunoblotting in the nuclear fraction, whereas S6K1 and phospho-S6 levels were analyzed in the cytoplasmic fraction. B, MCF7 cells were starved of serum for 24 h and stimulated with 100 mM insulin or 100 ng/ml PMA for 30 min with or without pretreatment with 20 ng/ml rapamycin for 30 min. Ser<sup>167</sup> phosphorylation was determined by immunoblotting and compared with activation of extracellular signal-regulated kinase (ERK), Akt, and S6. C, endogenous ER was immunoprecipitated from serum-starved T47D cells. S6K1 was immunoprecipitated separately from MCF7 cells that were serum-starved for 24 h and treated with PMA (100 ng/ml) for 30 min with or without pretreatment of cells with rapamycin (20 ng/ml) for 30 min. An in vitro kinase assay for ER was performed by addition of S6K1 or a control immunoprecipitate to the ER immunoprecipitate and incubation in a kinase reaction containing ATP for 60 min as described under “Experimental Procedures.” Levels of phospho-Ser<sup>167</sup>, ERα, and S6K1 in the reactions were determined by immunoblotting. D, HEK293E cells were transfected with WT, KD, or activated rapamycin-resistant alleles of HA-S6K1; serum-starved for 24 h; treated with PMA (100 ng/ml) for 30 min with or without pretreatment of cells with rapamycin (20 ng/ml) for 30 min; and immunoprecipitated with anti-HA antibodies. ER-expressing HEK293E cells were serum-starved and separately lysed, and ER was precipitated using anti-ER antibodies. An in vitro kinase assay for ER was performed by addition of WT, KD, or RR alleles of S6K1 or a control immunoprecipitate and incubation in a kinase reaction containing ATP for 60 min as described under “Experimental Procedures.” Levels of phospho-Ser<sup>167</sup>, ERα, and HA-S6K1 in the reactions were determined by immunoblotting.

Thus, we sought to investigate the role of S6K1 in ERα phosphorylation and breast cancer cell proliferation.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—Generation of the hemagglutinin (HA)-S6K1 constructs in the pRK7 expression vector has been described previously (26–28). VP16-ERα and pGL2–3xERE-TATA-luc were kindly provided by Donald P. McDonnell (Duke University, Durham, NC), pIS2 Renilla luciferase reporter was kindly provided by David Bartel (Michigan Institute of Technology, Cambridge, MA).

**Antibodies**—Anti-HA and anti-phospho-ERK1/2 antibodies were purchased from Sigma. Anti-phospho-S6 Ser<sup>240/244</sup> and anti-phospho-ER Ser<sup>167</sup> antibodies were provided by David Bartel (Michigan Institute of Technology, New Jersey). Anti-ER and anti-actin antibodies were purchased from Santa Cruz Biotechnology. For immunoblotting, horseradish peroxidase-conjugated anti-rabbit, anti-mouse, and anti-goat antibodies were purchased from Amersham Biosciences, Chemicon, and Santa Cruz Biotechnology, respectively.

**Cell Culture, Transfections, and Generation of Stable Cell Lines**—ZR-75-1 cells were a gift from William Hahn (Dana Farber Cancer Institute, Boston, MA). MDA-MB-231 cells were from Joan Massague (Memorial Sloan-Kettering Cancer Center, New York, NY). MDA-MB-435 and T47D cells were generously provided by Jeffrey Segall (Albert Einstein College of Medicine, Bronx, NY). MCF10a, MCF7, MDA-MB-468, HEK293E, and BT-474 cells were a gift from John Blenis (Harvard Medical School, Boston). MDA-MB-361 and MDA-MB-436 were from ATCC. All cell lines were maintained according to the suppliers’ instructions.

For transfection studies, HEK293E cells were transfected using the calcium phosphate method as described previously (22). T47D cells were transfected using Lipofectamine 2000 (Invitrogen) following the manufacturer’s protocol. Lysates were prepared at 48 h post-transfection. To stably suppress S6K1, we used pLKO.1 lentiviral small hairpin RNA constructs generated by William Hahn. Lentiviral infections of the indicated cell lines were performed, and stable cell lines were selected in puromycin.

**Cell Proliferation Measurements**—For proliferation assays, cells were seeded in quadruplicate at a density of 5000 cells/well in 96-well plates and grown overnight. Media were changed to assay media with or without the agents (as indicated in the figure legends). Cell proliferation was assayed after 96 h using the supravalent dye neutral red (NR) incorporation (29). The medium was removed, 0.2 ml of medium containing 0.04 mg/ml NR was added per well, and incubation was continued for 0.5 h at 37 °C. Cells were then rapidly washed and fixed with a 0.2-ml solution of 0.5% formalin, 1% CaCl<sub>2</sub> (v/v), and the NR incorporated into the viable cells was released into the supernatant with a 0.2-ml solution of 1% acetic acid, 50% ethanol. Absorbance was recorded at 540 nm with a microtiter plate spectrophotometer. Cell density was averaged and plotted using Excel.

**Cell Extract Preparation**—Cells were grown in media and stimulated (as indicated in the figure legends), and cells were lysed in radioimmune precipitation assay buffer with 1 mM sodium orthovanadate, 40 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 5 μg/ml pepstatin at 4 °C. Lysates were cleared of insoluble material by centrifugation at 15,000 × g for 10 min. Where indicated, NE-PER nuclear cytoplasmic
extraction reagent (Pierce) or 1× passive lysis buffer (Promega) was used.

Immunoblots—Whole-cell lysates (10% of total cell extract) were resolved by SDS-PAGE (10% or as indicated). Proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell) and blotted with the indicated antibodies. Immunoblots were developed using enhanced chemiluminescence reagents (Pierce) and a ChemiDoc XRS imager with Quantity One software (Bio-Rad).

Immunoprecipitations and Immune Complex Kinase Assays—ERα or S6K1 was separately immunoprecipitated with anti-ERα, anti-HA, or anti-S6K1 antibodies (as indicated in the figure legends) and protein A/G-Sepharose beads (Sigma). Immunoprecipitates were stringently washed once in 1 ml each of buffers A (10 mM Tris, 1% Nonidet P-40, 0.5% sodium deoxycholate, 100 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 2 mM dithiothreitol, 40 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 5 μg/ml pepstatin, pH 7.2), B (same as buffer A, except for 0.1% Nonidet P-40 and 1 mM NaCl), and ST (50 mM Tris-HCl, 5 mM Tris base, 150 mM NaCl), and resuspended in 20 mM HEPES, pH 7.2, 10 mM MgCl₂, 0.1 mg/ml bovine serum albumin, 3 mM 2-mercaptoethanol.

Kinase assays were performed with ER-containing immunoprecipitates as substrate by the addition of endogenous S6K1 immunoprecipitated separately from PMA-stimulated MCF7 cells or rapamycin-pre-treated T47D cells. The reaction products were resolved using SDS-PAGE, and the amount of phospho-ERα was assessed by immunoblotting with anti-phospho-ERα Ser167 antibody.

Luciferase Reporter Assays—HEK293E cells were transfected with plasmids encoding ERα, firefly luciferase under the control of three estrogen response elements (EREs), control Renilla luciferase, and the alleles of S6K1 or vector pRK7. After 24 h, rapamycin and/or 4-hydroxytamoxifen was added as indicated. At 48 h post-transfection, cells were harvested using 1× passive lysis buffer, and relative luciferase activity was measured using the Dual-Luciferase reporter assay system and the GloMax 20/20 luminometer (Promega).

RESULTS

We sought to determine whether S6K1 directly phosphorylates ERα in cells, leading to its activation. ERα contains a putative S6K1 phosphorylation motif, RERLASx[S/T], conforming to the S6K1 consensus phosphorylation motif RxRx[S/T], where x is any amino acid. First, we compared phosphorylation of Ser167 in two ERα-positive cell lines, T47D and MCF7, the former with low S6K1 expression and the latter with high S6K1 levels. As shown in Fig. 2A, we found that phospho-Ser167 levels were considerably higher in MCF7 than in T47D cells. Treatment with rapamycin inhibited Ser167 phosphorylation in MCF7 cells. Thus, Ser167 phosphorylation correlates with S6K1 levels and is rapamycin-sensitive.

Because signaling by Akt (21) and RSK (20) has been linked to Ser167 phosphorylation, we attempted to dissect the signaling pathways leading to Ser167 phosphorylation (Fig. 1B). First, we stimulated MCF7 cells with insulin, which activates the phosphatidylinositol 3-kinase/Akt and mTOR/S6K1 but not MAPK/RSK pathways. We observed that under these conditions, Ser167 was phosphorylated in concert with activation of S6K1 and Akt. The MAPK/RSK pathway was not activated. However, insulin-stimulated Ser167 phosphorylation was rapamycin-sensitive, eliminating the possibility that Akt was the kinase directly responsible for this event. We next stimulated MCF7 with the phorbol ester phorbol 12-myristate 13-acetate (PMA), which activates the mTOR/S6K1 and MAPK/RSK pathways but not the phosphatidylinositol 3-kinase/Akt pathway. We observed PMA-stimulated Ser167 phosphorylation, which correlated with activated S6K1 and MAPK/RSK pathways but not with Akt. Yet again, Ser167 phosphorylation was rapamycin-sensitive, indicating that the MAPK/RSK pathway was not directly responsible for the phosphorylation of Ser167 under these conditions. Thus, S6K1 phosphorylates Ser167 in insulin and PMA-stimulated cells.

We also investigated whether S6K1 could directly phosphorylate Ser167 in vitro. We performed an in vitro kinase assay with ERα as a substrate. We immunoprecipitated endogenous ERα from serum-deprived T47D cells and performed a kinase assay by the addition of endogenous S6K1 immunoprecipitated separately from PMA-stimulated MCF7 cells or rapamycin-pre-
We observed rapamycin-sensitive phosphorylation of Ser\textsuperscript{167} that was seen only after the addition of PMA-stimulated S6K1 and not rapamycin-pretreated S6K1 or the control immunoprecipitate. We confirmed this observation by repeating the kinase assay with transfected ER\textsubscript{X} and the wild type (WT), kinase-dead (KD; containing a K100R mutation), and activated rapamycin-resistant (RR; containing an F5A-T389E-R3A mutation) alleles of HA-S6K1 (Fig. 1D). Kinase reaction with WT-S6K1 resulted in a rapamycin-sensitive increase in Ser\textsuperscript{167} phosphorylation compared with the KD-S6K1 control, whereas RR-S6K1-mediated phosphorylation was rapamycin-insensitive. Thus, S6K1 appears to directly phosphorylate ER\textsubscript{X}.

If S6K1 is the physiological kinase for Ser\textsuperscript{167}, then modulation of its levels should affect phospho-Ser\textsuperscript{167} levels. First, we investigated the effect of S6K1 knockdown in MCF7 cells on Ser\textsuperscript{167} phosphorylation (Fig. 2A). Suppression of S6K1 expression resulted in a great decrease in insulin-stimulated, rapamycin-sensitive Ser\textsuperscript{167} phosphorylation. Additionally, overexpression of S6K1 in T47D cells increased rapamycin-sensitive phospho-Ser\textsuperscript{167} levels (Fig. 2B). In both instances, changes in Ser\textsuperscript{167} phosphorylation were mirrored by phospho-pS6 levels, indicating that S6K1 is responsible for the phosphorylation of both substrates in cells.

The transcriptional activity of ER\textsubscript{X} has been previously shown to be sensitive to rapamycin (30). To determine whether S6K1 regulates the transcriptional activity of ER\textsubscript{X}, we transfected HEK293E cells with ER\textsubscript{X}, a reporter construct that contains the firefly luciferase gene under the control of three EREs, a control construct expressing Renilla luciferase under the control of the SV40 promoter for luciferase activity normalization, and WT, KD, and RR alleles of S6K1. Overexpression of WT and RR alleles of S6K1 resulted in a 3-fold increase in
ERE-mediated transcriptional activity, compared with KD-S6K1 or vector expression (Fig. 3A). Moreover, rapamycin and 4-HT in combination on the transcriptional activity (19). Indeed, we found that although the growth rates of all cell lines were sensitive to rapamycin, those with S6K1 overexpression were growing at 30–40% capacity compared with untreated cells, whereas cells with low S6K1 expression were growing at 60–70% capacity compared with untreated cells (Fig. 4B). Thus, mammary epithelial cells with S6K1 overexpression exhibit enhanced rapamycin sensitivity. We were unable to include MDA-MB-361 cells in the study because the very long doubling time of these cells precluded meaningful comparison with the other cell lines.

To characterize the relationship between S6K1 and the proliferative capacity of mammary epithelial cells, we examined the levels of S6K1 in various mammary epithelial cell lines. As shown in Fig. 4A, we determined that S6K1 was overexpressed in four of five cell lines with 17q23 amplification, specifically MCF7, BT-474, MDA-MB-361, and ZR-75-1. T47D cells, despite having the amplification, express S6K1 to levels similar to cells with no 17q23 amplification. Next, we determined the relationship between S6K1 overexpression and sensitivity to rapamycin in our panel of cell lines. It was previously suggested that S6K1 overexpression may correlate with rapamycin sensitivity (19). Indeed, we found that although the growth rates of all cell lines were sensitive to rapamycin, those with S6K1 overexpression were growing at 30–40% capacity compared with untreated cells, whereas cells with low S6K1 expression were growing at 60–70% capacity compared with untreated cells (Fig. 4B). Thus, mammary epithelial cells with S6K1 overexpression exhibit enhanced rapamycin sensitivity. We were unable to include MDA-MB-361 cells in the study because the very long doubling time of these cells precluded meaningful comparison with the other cell lines.

As an important regulator of cell growth and cell cycle progression (31), S6K1 overexpression may play a role in cancer pathogenesis and progression. It has been proposed that overexpression of S6K1 may allow cells to proliferate under conditions of low serum, a hallmark of neoplastic transformation (31). We examined the correlation between S6K1 levels in mammary cell lines and their ability to proliferate in low (1%) serum (Fig. 4B). We found that cells with low S6K1 expression were growing at 20–30% capacity compared with cells in full serum, in contrast to S6K1-overexpressing cells that were able to grow at 60–70% capacity compared with cells in full serum. Thus, S6K1 overexpression confers a proliferative advantage to mammary epithelial cells in low serum conditions, thus contributing to their neoplastic transformation.

Subsequently, we sought to determine the effect of suppression of S6K1 expression on growth in low serum. We generated cell lines in which the expression of S6K1 was reduced by small hairpin RNA (Fig. 5A). As expected, because of its role as a cell cycle regulator, S6K1 suppression resulted in decrease in cell proliferation of all cell lines growing in full serum (Fig. 5B).
However, low S6K1-expressing cells experienced only an ∼20% decrease, whereas S6K1-overexpressing cell lines lost ∼50% proliferative capacity. However, the proliferation defect was even more pronounced in low serum conditions. Whereas low S6K1-expressing cells did not experience a further reduction in proliferation following S6K1 knockdown, the proliferative

FIGURE 5. Alteration of S6K1 expression in breast cancer cells modulates their ability to proliferate in low serum and rapamycin. A, protein expression was suppressed by small hairpin RNA (shRNA) against S6K1 or the green fluorescent protein (GFP) control as described under “Experimental Procedures.” Proteins from the indicated cell extracts were resolved on 10% gel. S6K1 protein levels were determined by immunoblotting and compared with actin expression. B, proliferation of cells in full serum with S6K1 or green fluorescent protein knockdown was determined by NR assay as described under “Experimental Procedures.” The data are presented as mean ± S.D. of each experiment performed in quadruplicate. Numbers above the bars indicate percent proliferative capacity of cells with S6K1 knockdown compared with each cell line’s matched control. C, proliferation of cells in low serum with S6K1 or green fluorescent protein knockdown was determined by NR assay as described under “Experimental Procedures.” The data are presented as mean ± S.D. of each experiment performed in quadruplicate. Numbers above the bars indicate percent proliferative capacity of cells with S6K1 knockdown compared with each cell line’s matched control. D, overexpression of S6K1 was achieved by stable expression of HA-tagged S6K1 alleles in MCF10a cells. Levels of HA-S6K1 were determined by immunoblotting. E, proliferation of cells in the serum-deprived, 1% or 5% serum with 20 ng/ml rapamycin medium was determined by NR assay as described under “Experimental Procedures” and expressed as percent proliferation in 5% serum. The data are presented as mean ± S.D. of each experiment performed in quadruplicate. *, p < 0.05 by two-tailed paired Student’s t test.
capacity of MCF7 cells was reduced by ~55% compared with the cells in full serum and that of BT-474 cells by ~65% (Fig. 5C). This may indicate that S6K1 overexpression renders cells dependent on the continued high level expression and function of S6K1 to sustain proliferative capacity under conditions of low serum.

We next investigated whether overexpression of S6K1 in cells with low endogenous S6K1 expression would alter their sensitivity to rapamycin and serum levels. As shown Fig. 5D, we stably overexpressed S6K1 in MCF10a cells using low expressing pBABE retroviral vector, followed by selection in puromycin. Cells were passaged for 2 months (~20 passages) to allow them to adapt to elevated levels of S6K1. We used vector control and the WT, KD, and activated RR alleles of S6K1. We examined proliferation of these cell lines in media with low (1%) serum (Fig. 5E). All cell lines had a similar proliferation rate in serum-deprived media. However, in media with 1% serum, WT- and RR-S6K1-expressing cells grew ~10% more than the vector or KD-S6K1-expressing controls. Additionally, when grown in the presence of rapamycin (Fig. 5E), WT-S6K1-overexpressing cells demonstrated a small but statistically significant decrease in proliferation compared with vector and KD-S6K1 controls. Interestingly, RR-S6K1-overexpressing cells not only were not inhibited by rapamycin compared with controls but acquired a proliferative advantage greater than overexpression of WT-S6K1. Thus, even a modest increase in S6K1 activity renders cells more rapamycin-sensitive.

Moreover, S6K1 overexpression also allows cells to better proliferate in low serum.

It has been previously shown that some ERα-positive cells are sensitive to rapamycin; however, that observation attributed rapamycin sensitivity to other components of the mTOR pathway, such as Akt activation or phosphorylation of eIF4E (30, 32). We hypothesized that rapamycin sensitivity of ERα-positive cells may be specifically due to inhibition of S6K1 and reduction in ERα Ser167 phosphorylation. We cultured MDA-MB-231, T47D, MCF7, and ZR-75-1 cell lines in the presence of 20 ng/ml rapamycin, $10^{-7}$ M 4-HT, or a combination of the two agents and compared this with proliferation in 10% serum. As shown in Fig. 6A, ERα-negative and low S6K1-expressing MDA-MB-231 cells were not inhibited by a combination of rapamycin and 4-HT any more than rapamycin alone. ERα-positive and low S6K1-expressing T47D cells were not inhibited by a combination of rapamycin and 4-HT more than 4-HT alone. Finally, ERα-positive and S6K1-overexpressing MCF7 and ZR-75-1 cells showed a greater inhibition by a combination of rapamycin and 4-HT than each drug alone. This indicated that the mTOR/S6K1 pathway converges onto ERα to promote cell proliferation.
S6K1 Phosphorylates and Regulates ERα

DISCUSSION

The efficacy of endocrine therapy for ER-positive breast cancer increased following the introduction of aromatase inhibitors; however, de novo or acquired resistance remains a major clinical issue (33). Recent research into the mechanisms of resistance has revealed that estrogen receptor activation can occur through estrogen-independent growth factor signaling pathways, and oncogenes involved in the signal transduction cascade become activated and used by breast cancer cells to bypass normal endocrine responsiveness (34).

In this study, we conclusively demonstrate that S6K1 is a physiological kinase for Ser167 of ERα. Importantly, S6K1 phosphorylates ERs transcriptional activity, thus contributing to proliferation of ER-positive breast cancer cells (Fig. 6d). Moreover, we determine that S6K1 overexpression in breast cancer cells renders them dependent on the continuous activity of this kinase for proliferation. S6K1 expression levels strongly correlate with the ability of cells to proliferate in low serum conditions, whereas suppression of S6K1 expression results in a decrease in proliferation that is very pronounced in S6K1-overexpressing cells. This apparent dependence on S6K1 is similar to oncogene addiction of cancer cells expressing Bcr-Abl, Her-2/neu receptor, or mutant c-Myc or K-Ras.

This conclusion has important clinical implications because many clinical trials are underway that evaluate the efficacy of inhibition of the mTOR pathway in breast cancer. Because of the role of the mTOR pathway in cell growth and proliferation, rapamycin and its analogues CCI-779, AP23573, and RAD-001 are considered among the most promising drugs in the anticaner pipeline. Although mTOR inhibitors have shown an effect in early clinical trials, the response has been variable among patients. Consequently, there exists an urgent clinical need to identify those patients who stand to benefit most from mTOR inhibitor treatment. Because a significant number of patients carry S6K1 amplification correlating with a poor prognosis, identifying and treating these patients represents a very significant opportunity to make a clinical impact. Therefore, clinical use of rapamycin and its analogues can augment the responsiveness of S6K1-overexpressing and ER-positive breast cancer to endocrine therapy.

Importantly, while having an overall low adverse reaction profile, such concerns as immunosuppressive activity, cutaneous toxicity, and nephrotoxicity are a consideration with rapamycin and its analogues (35). The toxicity may arise due to nondiscriminatory inhibition of the entire rapamycin-sensitive mTOR pathway. Therefore, we hope that by focusing on the S6K1 arm of the mTOR pathway, we have established S6K1 as a specific target of therapeutic intervention, whose inhibition may result in fewer side effects. The long term objective would be to develop and test new inhibitors of S6K1 in the treatment of tumors with S6K1 amplification.

Finally, the role of S6K1 in the phosphorylation and activation of ERα may have implications in pathogenesis and treatment of other diseases. One example is lymphangiolyomatois (LAM) (reviewed in Refs. 36 and 37). LAM is a rare disease, arising either spontaneously or in conjunction with a mutation of the tuberous sclerosis complex. The pathology of LAM is represented by the proliferation of immature smooth muscle cells in the walls of airways, venules, and lymphatic vessels in the lung. Curiously, LAM affects women of childbearing age almost exclusively and shows a dependence on estrogen. Supporting this notion, estrogen receptor expression has been detected in the smooth muscle cells of LAM patients. It has also been demonstrated that increased LAM cell growth is associated with deregulated S6K1 activation, leading to the aberrant cell proliferation seen in LAM disease (38). Thus, it is important to investigate whether S6K1 contributes to LAM disease through phosphorylation and activation of ERα.

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