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The role of S6K1 in ER-positive breast cancer

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The 40S ribosomal S6 kinase 1 (S6K1) is a conserved serine/threonine protein kinase that belongs to the AGC family of protein kinases, which also includes Akt and many others. S6K1 is the principal kinase effector downstream of the mammalian target of rapamycin complex 1 (mTORC1). S6K1 is sensitive to a wide range of signaling inputs, including growth factors, amino acids, energy levels and hypoxia. S6K1 relays these signals to regulate a growing list of substrates and interacting proteins in control of oncogenic processes, such as cell growth and proliferation, cell survival and apoptosis and cell migration and invasion. Several lines of evidence suggest an important role for S6K1 in estrogen receptor (ER)-positive breast cancer. S6K1 directly phosphorylates and activates ERα. Furthermore, S6K1 expression is estrogenically regulated. Therefore, hyperactivation of mTORC1/S6K1 signaling may be closely related to ER-positive status in breast cancer and may be utilized as a marker for prognosis and a therapeutic target.

Keywords: mTORC1, S6K1, ERα, estrogen, rapamycin, 17q23 amplicon

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Introduction

The mTORC1 signaling pathway. Since its cloning and biochemical purification almost 20 y ago, mTOR, a conserved protein kinase, emerged as a central node in the plexus coordinating cellular growth and proliferation in response to numerous extracellular cues, including nutrient availability and growth stimuli. In eukaryotic cells, mTOR exists in two complexes. mTORC1 and mTORC2 consist of distinct sets of proteins and perform non-redundant functions.[1] Rapamycin is a naturally derived inhibitor of mTORC1 and an inhibitor of cell proliferation, as manifested by its potent immunosuppressive properties and activity against solid tumors.[2] Growth factor signaling to mTORC1 is primarily mediated by the phosphatidylinositol-3 kinase (PI3K) pathway and inactivation of the tuberous sclerosis complex protein TSC2 (tuberin). As illustrated in **Figure 1**, in response to extracellular activating stimuli, PI3K mediates cell membrane recruitment and activation of the serine/threonine protein kinases phosphatidylinositol-dependent kinase-1 (PDK1) and Akt.[3,4] The lipid phosphatase PTEN opposes the action of PI3K. TSC2 functions as a heterodimer with TSC1 (hamartin) to negatively regulate mTORC1 signaling by acting as a GTPase-activating protein (GAP) for the small GTPase Rheb.[5] Rheb directly binds to mTORC1 and regulates its activity in a GTP-dependent manner.[6] Thus, TSC2 inhibits Rheb-dependent activation of mTORC1. Phosphorylation and inactivation of TSC2 has first been shown to occur via PI3K/Akt.[7] Subsequently, several groups have shown that the Ras/ERK pathway also converges on the TSC1/2 complex. Ras-activated ERK1/2 directly phosphorylates TSC2 at sites different than Akt, resulting in TSC2 inactivation.[8] The downstream effector of ERK, RSK, also phosphorylates TSC2 at a unique site, thus inactivating it.[9] Therefore, TSC2 serves as a convergence point for multiple signaling inputs to mTORC1.

Activation of S6K1. S6K1 is one of the best-characterized downstream targets of mTORC1, and rapamycin treatment results in rapid dephosphorylation and inactivation of S6K1.[10] Regulation

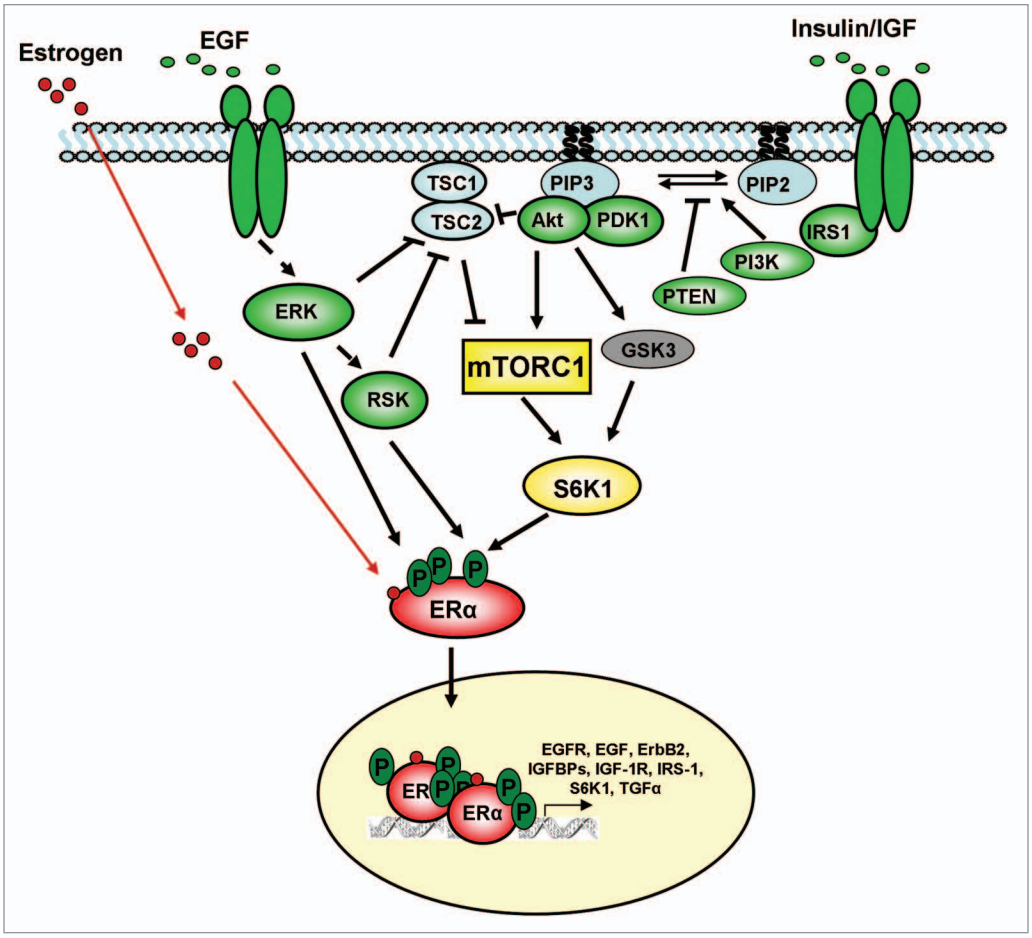

Figure 1. Reciprocal crosstalk between estrogen receptor (ER) α and growth factor receptor signaling pathways. Growth factor receptors activate phosphatidylinositol 3-kinase (PI3K) and MAPK signaling pathways. The effectors of these pathways, including ERK, RSK and S6K1 can then phosphorylate and activate ERα. ERα can also become activated by binding of its ligand, estrogen. When activated, ERα dimerizes, translocates to the nucleus, binds DNA and activates transcription of genes encoding components of growth factor signaling pathways, promoting a positive feedforward pathway activation loop.

of S6K1 is complex, involving several mTORC1- and PI3K-mediated phosphorylation events (reviewed in ref. 11). Phosphorylation of the proline-directed sites in the C terminus (Ser411, Ser418, Ser421 and Thr424) is hypothesized to prime S6K1 for subsequent phosphorylation at critical regulatory sites. Although the kinase(s) responsible for phosphorylation of these four C-terminal sites is not known, ERK- and p38-MAPK have been implicated in their regulation. The turn motif is phosphorylated at Ser371, a site essential for S6K1 activity by a mechanism involving glycogen synthase kinase-3 (GSK-3).[12] The hydrophobic motif Thr389 is also an essential and rapamycin-sensitive phosphorylation site. mTORC1 directly phosphorylates this site in vitro. Moreover, autophosphorylation has also been shown to contribute to the regulation of this site.[13] Phosphorylated Thr389 creates a docking site for PDK1, which phosphorylates Thr229 at the activation loop of S6K1. Other PI3K-dependent inputs linked to the activation of S6K1 are Akt, PKCζ and λ and the small G proteins Rac1 and Cdc42.[11]

Signaling by S6K1. S6K1 is an important regulator of cell size control, protein translation and cell proliferation.[14] Results from *S6k1* mouse knockouts uncovered a specific role for S6K1 in regulation of cell growth.[15] S6K1 phosphorylates proteins that function in RNA processing and protein biogenesis to increase cellular size, which is the limiting factor for cell division.[16-20] The 40S ribosomal protein S6 is the best-characterized target of S6K1.[21] S6K1 has also been shown to control the proliferative aspect of cell division. Specifically, S6K1 has been reported to drive the G_1/S cell cycle progression, and overexpression of S6K1 provides a significant proliferative advantage in low serum conditions,[18,22,23] a hallmark of neoplastic transformation. Significantly, the upstream regulators of the mTORC1/S6K1 pathway, such as PIK3CA, PTEN, AKT, PDK1 and TSC1/2, are frequently mutated in cancer, leading to inappropriate hyperactivation of S6K1.[24,25]

S6K1 in ER-Positive Breast Cancer

ER-positive breast cancer and endocrine resistance. Clinically, up to 60% of breast cancers are ER-positive, indicative of estrogen dependence for cancer

cell growth.[26] ER-positive breast cancers can be targeted therapeutically by antiestrogens (such as tamoxifen) or aromatase inhibitors (AIs). However, only about half of ER-positive breast cancers respond to endocrine treatments,[27] and resistance frequently develops.[28] Third-generation aromatase inhibitors (e.g., letrozole and anastrozole) are now considered to be the first-line treatment strategy for breast cancer.[29] However, response rates range between 35% and 70% in neoadjuvant studies and tend to be less effective in advanced disease.[30] Development of primary or de novo resistance frequently occurs, and even patients who have a response eventually relapse (acquired resistance). Although great strides have been made in understanding the mechanisms of resistance, for instance, implicating increased signaling via the MAPK, PI3K and mTORC1 pathways, the precise details are not fully understood.[31]

Regulation of ERα activity. The binding of 17β-estradiol (estrogen), the physiological ligand of ERα, allows ERα to dissociate from the inhibitory heat shock proteins, undergo phosphorylation, dimerization and translocation to the nucleus, where ERα activates transcription of responsive genes. ERα can also become activated via phosphorylation by effectors of growth factor signaling pathways, which can either potentiate estrogen signaling or mediate estrogen-independent activation.

Several phosphorylation events occur within ERα that are essential for transcriptional activity and responsive to either estrogen, growth factors or both activating stimuli. The phosphorylation of Ser104/106 is sensitive to estrogen only and not growth factors. The kinases that have been implicated in this event are GSK3, cdk2 and MAPK.[32-34] Ser118 phosphorylation is sensitive to both estrogen and growth factor signaling. While the identity of the kinase(s) that mediates estrogenic phosphorylation of Ser118 is unknown, cdk7, IKKα, MAPK and GSK3 have been shown to have direct and indirect growth factor-induced phosphorylation.[32,35-37] The phosphorylation of Ser167 is mainly sensitive to growth factor stimuli and is mediated primarily by S6K1,[18,38] while p90Rsk and Akt may play a secondary or indirect role.[19,39] Importantly, this site is associated with tamoxifen resistance.[40] PKA phosphorylates Ser236, which appears to promote the stability of ERα,[41,42] as well as Ser305. Ser305 appears to be important for dimerization and transactivation activity of ERα and is phosphorylated by PKA in the presence of estrogen[43] and by Akt through the IGF-I signaling pathway.[44]

Crosstalk between estrogen receptor and growth factor signaling pathways. Growing evidence indicates there exists a close interaction between the mTORC1/S6K1 pathway and ER signaling (**Fig. 1**). Notably, endocrine resistance is often associated with ligand-independent activation of ERα signaling due to hyperactivation of the mTORC1 signaling pathway[45] and can be reversed by the mTORC1 inhibitor everolimus in vitro.[40,46,47] S6K1 directly phosphorylates ERα, leading to ligand-independent activation.[18,19] S6K1 is also one of the kinases required for hormone-independent breast cancer cell proliferation.[48] In addition, mTORC1 serves as an important signaling node for several growth factor signaling pathways that are implicated in endocrine resistance, such as MAPK and PI3K. Thus, mTORC1 hyperactivation by converging stimuli would be targeted by the addition of mTOR inhibitors to endocrine therapy.

S6K1 activates ERα and promotes proliferation of ER-positive breast cancer cells. A large body of evidence reveals that S6K1 signaling may be particularly important for proliferation of ER-positive breast cancer cells. One molecular mechanism by which S6K1 controls proliferation is direct phosphorylation of ERα on Ser167, leading to increased ERα transcriptional activity and ER-dependent breast cancer cell proliferation. P-S6K1, a marker of S6K1 activation, has been determined to correlate with a poorer prognosis in patients with ER-positive (but not in patients with ER-negative) tumors.[49] S6K1 expression levels strongly correlate with the ability of cells to proliferate in low serum conditions, a hallmark of neoplastic transformation, while suppression of S6K1 expression results in a decrease in proliferation that is very pronounced in S6K1-overexpressing cells.[19] Moreover, overexpression of S6K1 increases rapamycin sensitivity of breast cancer cells.[19,50] Finally, it is evident that S6K1 overexpression in breast cancer cells renders them dependent on the continuous activity of this kinase for proliferation. The apparent dependence on S6K1 is similar to oncogene addiction of cancer cells expressing Bcr-Abl, Her-2/neu receptor or a mutant c-Myc or K-ras.

17q23 amplicon in breast cancer. Genomic amplification of genes critical for tumor initiation and progression is an important mechanism to augment the expression and activity of oncogenes. Several well-characterized examples of oncogenes amplified in breast cancer include *ERRB2* (at 17q12), *MYCN* (at 8q24) and *CCND1* (at 11q13). Owing to its biological role, S6K1 is a kinase whose activation by gene amplification would contribute to its oncogenicity. S6K1 is encoded by the *RPS6KB1* gene localized to the chromosomal region 17q23, which is amplified in several types of cancer.[51] Interestingly, high-level (multi-copy) amplification of *RPS6KB1* is limited to breast cancer.[51] This suggests that S6K1 may have a specific role in regulating the growth of breast cancer cells. The role of S6K1 in breast cancer development and progression is further supported by the observation that *RPS6KB1* amplification and S6K1 overexpression and activation are associated with poor prognosis in breast cancer patients.[52-55] Region 17q23 is amplified in several breast cancer cell lines and in up to 30% of primary tumors,[56] while S6K1 is overexpressed and hyperactivated in the majority of cell lines and primary tumors with this amplification, more predominantly in breast cancer with *BRCA1/2* mutations.[19,51,52,57-59] In fact, *BRCA1/2* mutations may be directly responsible for chromosomal breakage and amplifications in the 17q23 region.[60]

Estrogenic regulation of S6K1 expression within the 17q23 amplicon. Our group recently determined that S6K1 expression is regulated in human breast cancer cell lines and murine mammary epithelia by estrogen/ERα in a mechanism that may involve the transcription factor GATA-3.[61] S6K1 can directly phosphorylate ERα on Ser167; conversely, ERα activation leads to increased S6K1 expression, resulting in a positive co-regulatory loop.[18,19,61] Thus, it appears

that S6K1 expression is maintained in two modes: a basal level of expression, typical of normal cells, and an estrogen/ERα-dependent specific upregulation.[61] Others have observed estrogen-mediated regulation of *RPS6KB1* transcription in ZR-75–1 and MCF7 ER-positive breast cancer cell lines.[62,63] The estrogenic mode of S6K1 upregulation does not seem to be limited to breast epithelia. *Rps6kb1* was also observed to be estrogenically induced in uteri of ovariectomized mice.[64]

The existence of the feedforward regulatory loop whereby S6K1 activates ERα transcriptional activity, leading to increased expression of S6K1, is of great interest, since it not only sheds light on the mode of transcriptional regulation of S6K1 expression, but may also help in understanding the role of S6K1 overexpression in the development and progression of breast cancer. Significantly, a feedforward mechanism of transcriptional activation by ERα exists for many other genes involved in growth factor signaling pathways upstream of PI3K/mTORC1/S6K1, including IGF1-R, ErbB2, IRS-1, IGFBPs, EGFR, EGF and others,[30] which underscores the importance of sustained pathway activation in breast cancer cells. Moreover, chronic activation of the PI3K/mTORC1 pathway has been associated with development of resistance to endocrine therapy.

Remarkably, the 17q23 amplicon contains the coding sequence for mir-21, a microRNA gene with oncogenic properties, located about 20kb upstream of the *RPS6KB1* transcriptional unit. Mir-21 is upregulated in breast cancer and is regulated by estrogen.[65,66] Mir-21 target genes include PTEN, PDCD4 and bcl-2. *RPS6KB1* expression was observed to be induced by estrogen in a study focusing on estradiol-regulated microRNAs.[65] This may indicate a secondary role of miRNAs in estrogen-induced regulation of *RPS6KB1*. Coincidently, PTEN and PDCD4 function biochemically upstream and downstream of S6K1, respectively. The coordinate estrogenic regulation of expression of these genes together with *RPS6KB1* may confer a selective proliferative advantage to breast cancer cells.

The instability of the 17q23 genomic region also results in a tandem duplication of *RPS6KB1*, producing a gene fusion of *RPS6KB1* with *VMP1* (also known as *TMEM49*).[67] Curiously, *MIR21* is included in the tandem duplication structure. The consequences of this instability include deregulated expression of cancer-associated elements such as *MIR21* and *RPS6KB1*.

S6K as a Prognostic and a Therapeutic Target in Breast Cancer

mTOR inhibitors and markers of sensitivity. mTOR kinase is allosterically inhibited by rapamycin and its analogs (rapalogs), such as temsirolimus (CCI-779), everolimus (RAD-001) as well as a newer class of catalytic mTOR inhibitors. While early studies demonstrated an effect for rapalogs, especially for breast cancer,[50,68] the clinical effect was variable among patients. Thus, there exists an urgent need to identify signaling proteins whose inappropriate expression or activation is predictive of the patients' response to mTORC1 inhibition. Various upstream and downstream players in the mTORC1 pathway have been considered as markers for sensitivity. For instance, deficiency of PTEN, a gene frequently mutated in breast cancer, has been observed to correlate with rapamycin sensitivity in cell lines.[50,68] Disappointingly, clinical trials proved to be inconclusive, probably owing to a relatively low incidence of *PTEN* mutation in sporadic breast cancer.[69] A similar trial in the context of activating *PIK3CA* mutations didn't produce any major objective responses.[70] Akt is frequently activated by upstream *PTEN* or *PIK3CA* mutations, leading to hyperactivation of mTOR and S6K1. These mutations may make cells more sensitive to Akt or mTOR inhibitors, as the growth of the cells becomes dependent on elevated mTOR signaling. Preclinical studies of the role of Akt in sensitizing cells to mTOR inhibitors alone or in combination with endocrine agents have determined that rapamycin reverses endocrine resistance or synergizes with anti-estrogens in cells expressing activated Akt.[46,71,72]

Results from clinical trials with mTORC1 inhibitors in ER-positive breast cancer have been recently reported. For example, a pre-operative study of letrozole with or without everolimus reported greater tumor shrinkage for the combination.[73] A very recent breast cancer trial of oral everolimus-(BOLERO-2), a phase 3 study in patients with advanced disease, showed that the addition of everolimus to endocrine therapy results in an improved clinical outcome.[29] However, these studies were performed in an unselected group of patients with ER-positive disease, resulting in a variable response among patients. Work attempting to identify predictors of response following single-agent everolimus treatment noted gene expression changes in responding vs. resistant tumors, but no clear-cut pattern was observed.[74]

S6K1 as a marker of sensitivity to mTORC1 inhibitors. mTOR inhibition was observed to result in the reversal of resistance in a breast cancer cell line rendered cross-resistant to tamoxifen and fulvestrant, accompanied by reduction in ERα phosphorylated on Ser167.[75] Considering this is an S6K1-dependent phosphorylation site, these data imply that reversal of endocrine resistance involves S6K1 inactivation. P-S6K1 and P-S6 positively correlated with high proliferative index in letrozole-treated patients, indicative of endocrine resistance and poor patient outcome.[48] Since a significant number of patients carry S6K1 amplification and exhibit S6K1 overexpression or overexpression, S6K1 could be utilized as a prognostic marker. Detection of S6K1 expression in breast cancer could be an effective way to target and treat patients most likely to respond to the combination of mTORC1 and endocrine inhibitors.

S6K1 as a therapeutic target in ER-positive breast cancer. Targeting S6K1 may be an advantageous strategy based on the following data. First, 17q23 amplification strongly correlates with ER-positive status[76] and is one of the most frequent aberrations in ER-positive invasive ductal carcinoma.[77] Similarly, many other common mutations in breast cancer leading to aberrant expression and activation in IGF-1R, ErbB2, FGFR, PIK3CA, PTEN and Akt1/2 result in S6K1 hyperactivation.[78] Second, *RPS6KB1* was determined to be a gene whose gain in ER-positive tumors is prognostic of the metastatic capacity of human breast

cancer.[55] Moreover, expression of activated S6K1 in ER-positive, but not ER-negative, tumors was prognostic of a poorer prognosis and development of endocrine resistance. Third, *RPS6KB1* amplification and S6K1 overexpression in ER-driven breast cancers may stem from the genomic and non-genomic co-stimulatory relationship between ERα and S6K1. Indeed, our group and others have shown that S6K1 regulates ERα transactivational activity in control of breast cancer cell proliferation by directly phosphorylating ERα on Ser167.[18,19,38] Reversal of endocrine resistance by addition of everolimus resulted in decreased Ser167 phosphorylation, suggesting that this effect may be mediated through inhibition of S6K1.[40] Moreover, we have shown that S6K1 expression is estrogenically regulated via ERα.[61] Inhibition of the estrogenic activation of S6K1 may also be the target of action of letrozole, which has been shown to reduce phosphorylation of S6K1 and its targets, p-mTOR Ser2448 and p-S6.[48,73,79] Therefore, maintaining high co-overexpression of both S6K1 and ERα may provide a selective advantage for breast cancer cells and contribute to disregulated proliferation of cells during the progression to carcinogenesis and breast cancer. Finally, S6K1 was revealed as a significant hit in a genome-wide screen for kinases important for growth of MCF7/LTED cells (ER-positive cells selected after long-term estrogen deprivation.[48]

Future Directions

The current challenge in the treatment of ER-positive breast cancer with regard to utilization of mTORC1 pathway inhibitors remains two-fold. The first task is to obtain insights into the mechanistic effects of combination of endocrine and mTORC1 inhibitors. In addition, there exists a need to identify the downstream effectors of mTORC1 signaling that distinguish resistant and responsive cancers, which can subsequently be used as molecular predictors of response to mTORC1-targeted therapy. The goal is to identify and treat those patients most likely to benefit from the combination.

The identification of *RPS6KB1* as an ERα-regulated gene may help further elucidate the mechanisms of breast cancer pathogenesis and may lead to the development of new targeted therapies. The investigation of the relationship between mTORC1/S6K1 and ERα would identify new mediators and determinants of endocrine escape and help develop novel cancer treatments to inhibit downstream pathways of estrogen receptor action.

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