Sridharan S., Involvement of S6 kinase in breast cancer. Doctor of Philosophy (Cancer Biology), November, 2013, 129 pp, 19 illustrations, 215 references.

The 40S ribosomal protein S6 kinase (S6K) is activated downstream of the mammalian target of rapamycin (mTOR) and is believed to play an important role in protein translation. In mammalian cells S6K is represented by two highly homologous proteins, S6K1 and S6K2. Both homologs have been shown to be amplified and overexpressed in breast cancer cells and tissues. While the regulation and functions of S6K1 have been addressed, little is known about those of S6K2. Hence we sought to examine the causes and consequences of elevated S6K2 levels in breast cancer cells. While the depletion of S6K1 decreased breast cancer cell death, silencing of S6K2 substantially increased it in response to apoptotic and chemotherapeutic agents. We then explored the mechanism by which S6K2 mediates survival and observed that in contrast to S6K1, S6K2 depletion decreased the activation of the prosurvival protein Akt and increased the level of proapoptotic proteins p53 and bid. Following this observation, we sought to determine the pathway(s) contributing to the overexpression of S6K2 in breast cancer cells. Due to its role as a prognostic indicator in estrogen receptor (ER) – positive tumors, we studied the role of the estrogen signaling pathway in regulating S6K2 levels. Estradiol and estrogen receptor alpha (ERα) positively regulated S6K2 protein but did not affect its mRNA levels, suggesting post-transcriptional regulation. We further observed that

S6K2 regulated cell survival downstream of estrogen in ER-positive breast cancer cells. These findings strongly suggest that S6K2 is critical for the survival of breast cancer cells and that targeting S6K2 in combination with chemotherapeutic agents is a novel strategy to promote breast cancer cell death.

INVOLVEMENT OF S6 KINASE IN BREAST CANCER

DISSERTATION

Presented to the Graduate Council of the

University of North Texas

Health Science Center at Fort Worth

in Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

Savitha Sridharan

Fort Worth, Texas

November 2013

Acknowledgements

Any quest, big or small, is made possible and successful by a guide (or a couple of them as in this case). I realize that I would not have been able to embark on and see the end to this quest without the constant support and encouragement provided by the people around me. It is now my turn to acknowledge and thank these folk who made it possible.

First, I would like to express my gratitude towards my mentor Dr. Alakananda Basu for believing in and accepting me into her lab. I have greatly benefitted from her example and gained immensely by observing her approach work and life. I would like to thank my committee members Drs. Robert Mallet, Jamboor Vishwanatha, Hriday Das and Ignacy and Karol Gryczynski for their words of encouragement, insightful comments and constructive criticisms which have helped me shape and improve my work and myself as a scientist in numerous ways.

I strongly believe that any achievement, big or small is made in the presence of a nurturing environment. The group around me made me grow as a researcher and a person every day. I would like to thank the present and past members of the Basu lab – Ms. Deepanwita Pal, Ms. Kirti Jain, Drs. Shalini Outram, Eswar Shakar, Qiang Zeng, Soumya Krishnamurthy and Archana Sehrawat – for their help. A special shout out to Deepanwita and Kirti, who have remained a constant source of support and amusement without which my sanity might have come into question! I would also like to mention Ms. Smrithi Rajendiran, Dr. Mukesh Sahu and all my friends who have directly and indirectly helped me during my PhD.

This section would be amiss if I did not mention the help and assistance that the staff of the department of Molecular Biology and Immunology have provided throughout the course of my PhD. I would like to thank Ms. Lydia St.John, Ms. Melissa Henson, Ms. Jacklyn Crisp, Ms. Sandra Clapp and Ms. Georgia Quintero for everything they have done for me.

Every journey starts at home. I am deeply grateful to my parents, Mr. Sridharan Iyer and Mrs. Uma Sridharan and my sister Ms. Madhu Sridharan for their blessings, faith and support in all my endeavors. I would also like to acknowledge the understanding and encouragement provided by my extended family, Mr. Krishnan Swaminathan and Mrs. Yogam Krishnan.

Last but not the least, I would like to thank my better half, Mr. Sudarsan Krishnan. Words fail me when I think of the unconditional love, encouragement and patience he has shown me these past five years so that I see this through. It would have been impossible for me to achieve this without him to share the ride.

I would like to dedicate this work in its entirety to my family who are the reason for me being here today.

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List of abbreviations

4EBP1…….. eIF4E-binding protein

E2…….. 17-β-estradiol

eEF2K …….eukaryotic elongation factor 2K

eIF4B …… eukaryotic initiation factor 4B

ERα…….. estrogen receptor alpha

FoxO3A…… forkhead box O3 A

GOBO…….. Gene expression based Outcome for Breast cancer Online

HDM2…….. human homolog of mouse double minute 2

IRS1 ……. insulin receptor substrate 1

MAPK …….. mitogen activated protein kinase

Mcl-1 …….. myeloid cell leukemia 1

MDM2 ……. mouse double minute 2

MEK…….. mitogen activated protein kinase kinase

mTOR…….. mammalian target of rapamycin

NLS ……..nuclear localization signal

PDCD4…….. programmed cell death 4

PDK1…….. phosphoinositide-dependent kinase 1

PDZ……..postsynaptic density 95, PSD-85; discs large, Dlg; zonula occludens-1, ZO-1

PHLPP…….. PH domain and Leucine rich repeat Protein Phosphatase

PI3K……. phosphotidylinositol-3kinase

PIP2…….. phosphatidylinositol-4,5-bisphosphate

PIP3…….. phosphatidylinositol-3,4,5-triphosphate

PKC…….. protein kinase C

PP2A……. protein phosphatase PP2A

PTEN …….. phosphatase and tensin homolog

RHEB …….. ras homolog enriched in brain

rpS6 ……. ribosomal protein S6

RSK…….. ribosomal S6 kinase

S6K …….. S6 kinase

SGK1 …….. serum- and glucocorticoid-induced protein kinase 1

SH ……. Src homology

TNF ……. tumor necrosis factor alpha

TRAIL …….. TNF-related apoptosis inducing ligand

TSC ……. tuberous sclerosis complex

XIAP …….. X-linked inhibitor of apoptosis

CHAPTER I

Introduction

Breast cancer

Breast cancer is the most common cancer among women in the United States and the leading cause of cancer-related deaths in women besides lung cancer (1). It is estimated that about 1 in 8 women will develop invasive breast cancer over the course of her lifetime (Breastcancer.org). Conventional methods of treating breast cancer include surgical resection of the tumor along with chemo- and radiation therapy and targeted therapies such as Her-2/neu inhibitors and anti-estrogens. However, undesirable side effects, the development of resistance to such targeted therapy and metastatic spread resulting in secondary tumors pose major challenges in the treatment of breast cancer. At the cellular level, the existence of redundancy and compensatory mechanisms in signaling pathways leads to the failure of targeted therapies. Hence current research is aimed at identifying and characterizing more suitable molecular targets for designing combinatorial therapies to achieve effective eradication of breast cancer cells.

The PI3K/Akt/mTOR pathway in breast cancer

Cancer is characterized by a deregulation in signaling pathways that control cell survival and proliferation in response to environmental cues. Over 70% of breast cancers exhibit mutations in genes that constitute the phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway implying its importance in mediating breast cancer pathogenesis (2). Akt represents an important cell survival-promoting node along this pathway. It mediates its effects on cell survival by decreasing or inhibiting several apoptosis-inducing proteins such as p53 (3) and FoxO3A (4) and promoting the activation or increase in the levels of various anti-apoptotic proteins such as HDM2 (5) and Mcl-1 (6). Thus targeted therapies that promote the inhibition of Akt and result in subsequent tumor cell death represent suitable approaches for cancer therapy.

Growth factors bind to their cognate receptor tyrosine kinases and lead to the activation of PI3K subsequently resulting in Akt and mTOR activation (7, 8). mTOR exists in either of the two distinct complexes, mTOR complex 1 and 2 (mTORC1 and mTORC2) (9). mTORC2 functions in the phosphorylation and activation of Akt, serum- and glucocorticoid-induced protein kinase 1 (SGK1) and protein kinase C (PKC) (10, 11). However, the regulation of mTORC2 is poorly understood. mTORC1 is activated in response to growth factors via the PI3K pathway and promotes translation of a subset of mRNAs that harbor a 5' terminal oligopyrimidine tract (5' TOP) which code for components of ribosomes and hence cell growth and proliferation (12). Owing to these functions, there is great interest in better understanding the pathways that mediate mTOR activation.

Growth factor-mediated activation of receptor tyrosine kinases promote PI3K activation which then phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) to produce phosphatidylinositol-3,4,5-triphosphate (PIP3) which is reversed by the tumor suppressor phosphatase and tensin homolog (PTEN). This leads to the membrane recruitment and activation of pleckstrin homology domain-containing proteins such as phosphoinositide-dependent kinase 1 (PDK1). Activation of PDK1 leads to the phosphorylation and activation of several drivers of cell survival and proliferation such as Akt which then promotes mTORC1 activation by negatively regulating tuberous sclerosis complex (TSC), a tumor suppressor complex (13) mutated in hamartomas (14). TSC inhibition allows activation of the small GTPase ras homolog enriched in brain (RHEB) (15, 16), and subsequent mTORC1 activation resulting in downstream signaling and cap-dependent translation by phosphorylating and inhibiting the eIF4E-binding protein (4EBP) and activating S6 kinase (S6K) (Fig. 1). Thus due to its oncogenic roles, mTOR and its downstream target S6K are attractive targets for cancer therapy. The immunosuppressant drug rapamycin and its analogs that inhibit mTOR are currently being evaluated for their potential as anti-cancer agents (17).

Figure 1

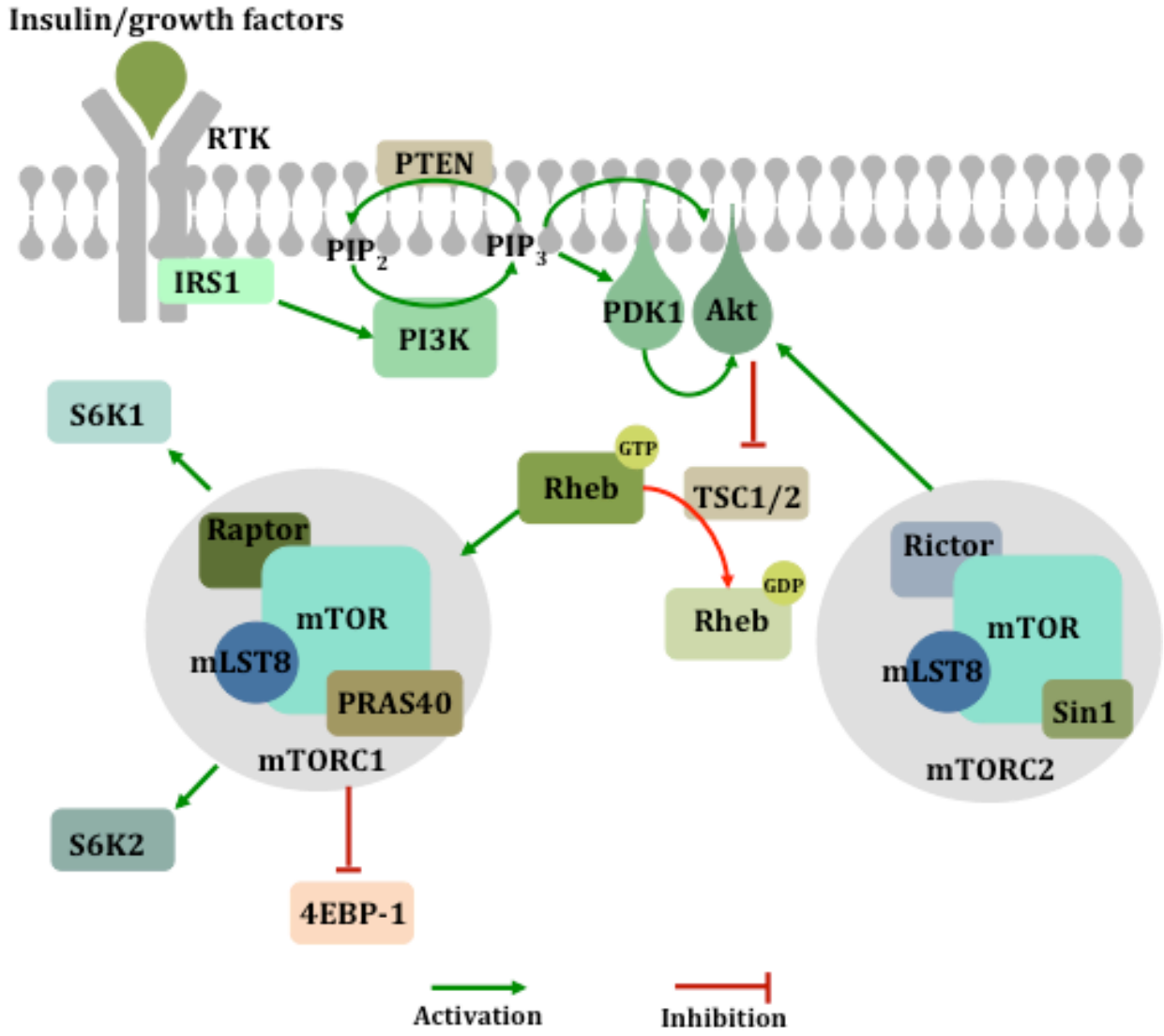

Fig. 1. The activation of mTOR. Growth factor-mediated activation of the PI3K pathway leads to the membrane recruitment and activation of PDK1 and Akt, which then phosphorylates and inactivates the tuberous sclerosis complex (TSC1/2), a negative regulator of Rheb ultimately resulting in the activation of mTOR within complex 1. mTORC1 mediates its downstream effects chiefly via the inhibition of 4EBP-1 and the activation of S6 kinase (S6K).

S6 kinase

Studies addressing the highly conserved inducible phosphorylation of ribosomal protein S6 in somatic cells led to the discovery of S6K1 (S6Kα) or p70S6 kinase (18, 19). It was originally identified as the serine/threonine kinase that mediated the mitogen-inducible phosphorylation of ribosomal protein S6 (rpS6). However, the observation that rpS6 phosphorylation was not affected in $S6K^{-/-}$ knockout mice led to the identification of a close homolog of S6K1, S6K2 (S6Kβ) which was later shown to be the major kinase mediating rpS6 phosphorylation (20-24). S6K1 and S6K2 are encoded by *RPS6KB1* on chromosome 17 and *RPS6KB2* on chromosome 11 respectively. Both genes code for two isoforms each with the use of alternative translation start sites: p70 S6K (S6KαII) and p85 S6K (S6KαI) in the case of S6K1 and p54 S6K (S6KβII) and p56 S6K (S6KβI) for S6K2 (21, 25). The N-terminal extensions of the longer forms of both S6K1 and S6K2 harbor a functional nuclear localization signal (NLS) making them constitutively nuclear. However, the shorter isoforms represent the predominant forms for both homologs and will be referred to as S6K1 and S6K2 henceforth.

Both S6K1 and S6K2 exhibit a modular structure consisting of an N-terminal regulatory region, the kinase domain followed by the kinase extension domain and a C-terminal regulatory region harboring the autoinhibitory/pseudosubstrate domain (Fig. 2). S6K1 and S6K2 share over 80% homology in the amino acids sequence of their kinase domain and a high degree of similarity in the adjacent kinase extension and pseudosubstrate or autoinhibitory domains with conserved sites critical for their activation (21, 22). However, important differences exist in the extreme N- and C-terminal regions. S6K1 possesses a C-terminal PDZ-binding domain which promotes association with actin cytoskeleton (26)

whereas S6K2 but not S6K1 harbors a functional nuclear localization signal (NLS) and a proline-rich domain which may promote interaction with SH3-domain containing proteins at its C-terminus (21) (Fig. 2). It is believed that these key differences between S6K1 and S6K2 will result in differential localization, binding partners and hence distinct functions for the two proteins (27).

Figure 2

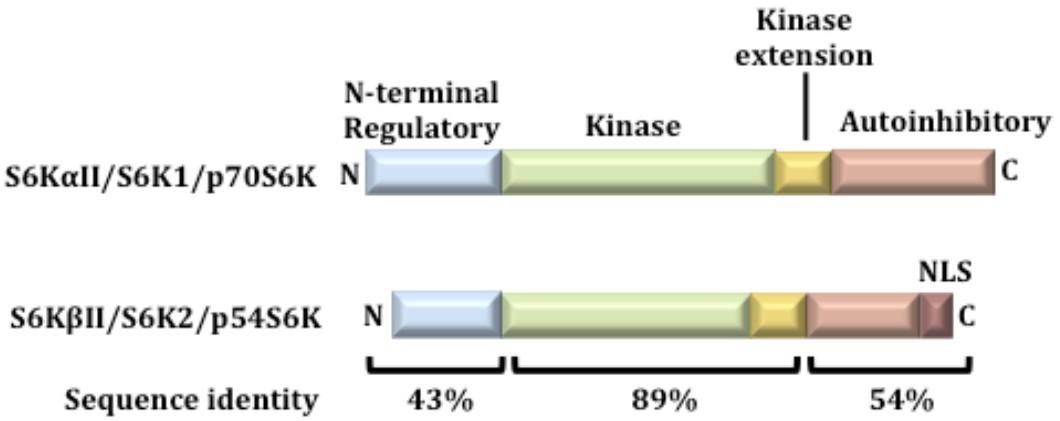

Fig. 2. Modular structure of S6K. While sharing significant homology in their kinase domains, the shorter isoforms of both S6K1 and S6K2 which are the predominant forms, exhibit substantial divergence in the extreme N- and C-terminal regions.

Regulation of S6 kinase:

Activation of S6K

Sensitivity of S6K to the immunosuppressant drug rapamycin implied its regulation by mTOR (28-30). Further studies suggested that the phospho-mimetic mutation of a conserved phosphorylation site in the kinase-extension domain (T389 in S6K1 and T388 in S6K2) known as the hydrophobic motif, by mTOR leads to resistance to rapamycin (31). Raptor within the mTORC1 complex (Fig. 1) binds to the tor signaling motif (TOS), a conserved amino acid sequence, found in S6K (32) and promotes its interaction with mTOR which mediates the hydrophobic motif phosphorylation (33). Following this, the activation of S6K is achieved by PDK-1-mediated phosphorylation at a threonine residue in the activation loop (T229 in S6K1 and T228 in S6K2) within the kinase domain (34).

However, in order for mTORC1 and PDK1 to be able to access their target sites on S6K, a series of phosphorylation events at C-terminal serine residues first must occur so as to render the pseudosubstrate/autoinhibitory domain inactive and expose the activation loop making it accessible by PDK1 (Fig. 3). The C-terminal autoinhibitory domain phosphorylations are believed to be carried by members of the mitogen activated protein kinase (MAPK) family (35).

Thus the current model of S6K activation follows that the initial phosphorylation events in the C-terminal pseudosubstrate domain expose the kinase extension and kinase domain and promote mTORC1-mediated phosphorylation followed by the activating phosphorylation by PDK1 (27, 36) (Fig. 3).

While S6K1 and S6K2 share the majority of conserved phosphorylation sites, they are believed to exhibit differences in their sensitivities to rapamycin and inputs from the MAPK signaling pathway. For example, in H510 lung cancer cells which are characterized by highly active mitogen activated protein kinase kinase (MEK) signaling, S6K2 was less responsive to rapamycin but highly sensitive to MEK inhibition (37). These and other observations suggest that the MAPK-mediated C-terminal phosphorylations are more critical for the activation of S6K2 than that of S6K1 (37, 38).

Growth factor-mediated activation of S6K1 is followed by its rapid dephosphorylation and desensitization via the serine/threonine protein phosphatase PP2A (39-42). In addition to the phosphorylation and activation of S6K, mTOR leads to increase in level of the serine/threonine protein phosphatase PHLPP (43) which aids in switching off S6K activity by dephosphorylating the hydrophobic motif (44). Thus functional mTOR not only promotes the activation of S6K but also ensures tight regulation of its activity via dephosphorylation.

Additional modes of regulation – subcellular localization and protein turnover

The presence of a nuclear localization sequence at its C-terminus suggests S6K2 but not S6K1 is nuclear where its activation can be regulated by a pool of nuclear mTOR (45). Several phosphorylation events have been linked to the regulation of S6K localization. For example, S6K2 has been shown to shuttle between the nucleus and cytoplasm. This shuttling is believed to be regulated by growth factor-induced phosphorylation by PKC in C-terminus of S6K2 close to its nuclear localization signal thus inhibiting its nuclear translocation. This mechanism is believed to maintain a pool of active S6K2 in the

cytoplasm (46). Similarly, the phosphorylation of S6K1 by casein kinase 2 prevents its nuclear translocation (47) which has been shown to occur upon growth factor stimulation despite the lack of a nuclear localization signal (47, 48). While these phosphorylation events do not directly correlate with S6K activity, they are believed to recruit them to specific cellular compartments or serve to bring them in the proximity of their targets thus determining their functions.

S6Ks have been shown to be regulated via protein degradation and stabilization. For example, acetylation of S6Ks causes stabilization and increase in their levels (49). Similarly both S6K1 and S6K2 have been reported to be ubiquitinated and degraded in the proteasome. Indeed, the Roc1 ubiquitin ligase was shown to ubiquitinate S6K1 although the identity of the ubiquitin ligase mediating the proteasomal degradation of S6K2 remains unknown (50, 51).

Thus while the core activation mechanisms are conserved between S6K1 and S6K2, there may exist important differences in their sensitivities to upstream signaling pathways, localization and modes of protein turnover.

Figure 3

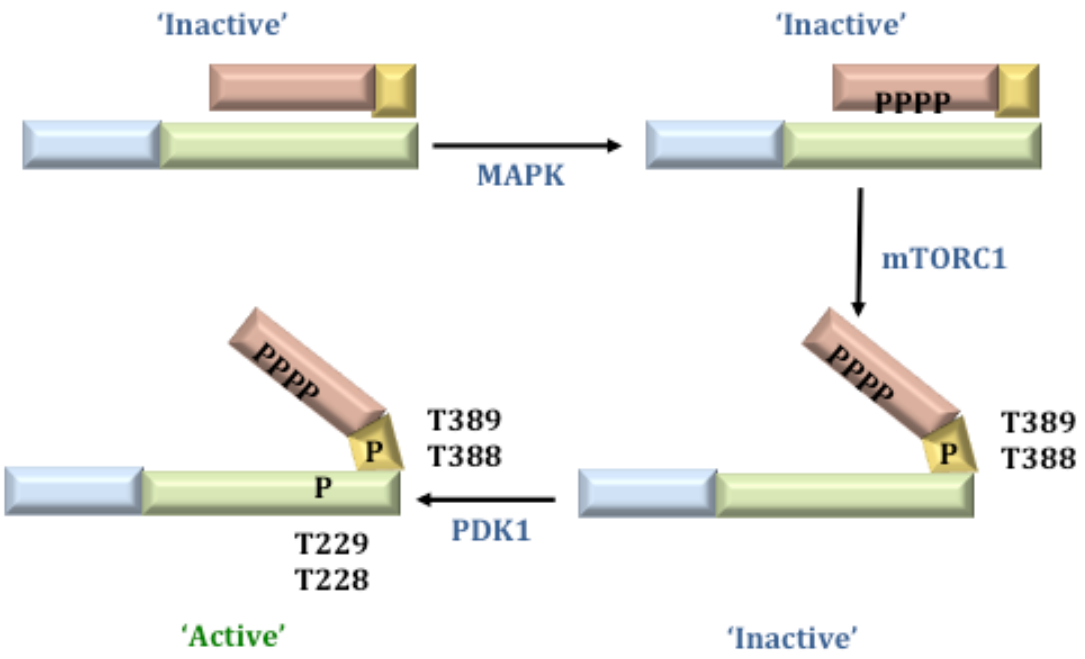

Fig. 3. Activation of S6K. The activation of S6Ks involves a series of sequential phosphorylation events. Components of the MAPK signaling pathway mediate the phosphorylation of C-terminal residues which displaces the pseudosubstrate domain and exposing phosphorylation sites in the kinase-extension and kinase domains which are then phosphorylated by mTORC1 and PDK1 respectively, rendering the protein fully active (reviewed in (36)).

Cellular functions of S6 kinase

Initial studies on the physiological role of S6K came from Drosophila which possess a single S6K (dS6K) gene. The disruption of this gene decreases the probability of survival to adulthood with a marked decrease in body size which was associated with a decrease in cell size rather than total cell numbers. This suggests a role for dS6K in regulating cell growth in those that reach adulthood (52).

Similar to Drosophila, S6K1$^{-/-}$ mice exhibit defects such as small size, hypoinsulinemia and glucose intolerance associated with decreased pancreatic beta cell size (53). It has been observed that there is an upregulation of S6K2 expression in tissues from S6K1 knockout mice, which compensate for the decrease in rpS6 phosphorylation in these mice (54). Conversely, rpS6 phosphorylation is abrogated in S6K2$^{-/-}$ mice suggesting that physiologically S6K2 is the principal kinase for rpS6. While S6K1/S6K2 double knockout mice exhibit perinatal lethality, S6K2$^{-/-}$ mice survive to adulthood with no apparent phenotype (54).

What then is the physiological role of the highly conserved rpS6 phosphorylation? The mitogen-inducible phosphorylation of rpS6 occurs at five C-terminal serine residues (55) and is mediated by several distinct kinases (56). In vivo knock-in mouse models which harbor a non-phosphorylatable mutant of rpS6 showed small size, hypoinsulinemia and decreased beta cell size and muscle weakness – phenotypes similar to those of S6K1 knockout mice (57, 58) which is counterintuitive since S6K2 seems to be the primary kinase mediating rpS6 phosphorylation (57). Several explanations have been put forth to resolve this discrepancy. For example, given their distinct localization patterns, a pool of

rpS6 that is accessible only by S6K1 may be required for mediating cell growth. Similarly, S6K1 but not S6K2 may be activated during a particular developmental stage when rpS6 phosphorylation is required (27). Further studies in mice deficient for rpS6 phosphorylation revealed that this highly conserved phosphorylation event was dispensable for the translation of 5' TOP mRNAs, an event long considered to depend on it (57).

Although S6K was originally identified as the kinase mediating rpS6 phosphorylation, several other cellular S6K substrates, specifically of S6K1, have since been reported which play important roles in gene transcription, protein translation, insulin resistance and cell survival. For example, S6K1 has been reported to phosphorylate and regulate components of the translation apparatus such as eIF4B (59) and eEF2K (60) and regulators of protein synthesis such as programmed cell death 4 (PDCD4) which inhibits the translation machinery (61). It is believed to regulate cell survival via the phosphorylation and regulation of MDM2 (62), a negative regulator of p53 and BAD (63), a cell death promoting protein.

While glucose intolerance has been observed in $S6K1^{-/-}$ mice, it has also been shown to promote the phosphorylation and feedback inhibition of insulin receptor substrate 1 (IRS1) (64-67). Insulin- and amino acids-mediated activation of mTOR/S6K via the PI3K pathway leads to a negative feedback loop resulting in the phosphorylation and downregulation of IRS1 by S6K1 and eventually insulin resistance and type 2 diabetes. In addition to diabetes, mTOR/S6K1-mediated feedback inhibition of IRS1 and by extension the oncogenic PI3K/Akt pathway poses drawbacks for cancer therapy and limits the cytotoxic effects of rapamycin-based therapeutic approaches (68). In addition to its role in the inhibition of

IRS1, S6K1 has also been implicated in the phosphorylation and negative regulation of Rictor, a component of the mTORC2 complex which mediates Akt activation, although this conclusion remains controversial (69-72).

While several studies have documented the cellular functions and substrates of S6K1, little is known about those of S6K2. Given the lack of a phenotype in S6K2$^{-/-}$ mice, the physiological role of S6K2 is poorly understood. Some reports suggest that it plays roles in cell proliferation via interaction with heterogeneous nuclear ribonucleoproteins (hnRNPs) (73) and histone H3 (74). S6K2 has also been shown to participate in fibroblast growth factor-2 (FGF-2)–mediated chemoresistance in lung cancer cells (75) and translational regulation via the inhibition of PDCD4 (76). However, the functions of S6K2 in other disease scenarios and cancers remain largely unknown.

S6 kinase and breast cancer

Immunohistochemical analysis demonstrated that both S6K1 and S6K2 are overexpressed in breast cancer with S6K1 primarily cytosolic and S6K2 predominantly nuclear in localization (77, 78). Furthermore, nuclear S6K2 correlated with nuclear staining of proliferation markers such as Ki-67 and proliferating cell nuclear antigen (PCNA) suggesting a role for S6K2 in breast cancer pathogenesis (77).

S6K1 and S6K2 are encoded by the genes *RPS6KB1* and *RPS6KB2* located on chromosomes 17q23 and 11q13 respectively, which harbor several key mediators of breast cancer. Gene amplification of both *RPS6KB1* and *RPS6KB2* are often observed in breast cancer tissues (79, 80). Furthermore, a co-amplification of *RPS6KB2* and *4EBP1* has been reported

suggesting a synergy between these mTOR targets in breast cancer development and progression (81).

RPS6KB1 amplification (≥ 4 copies) has been reported in 10.7% and gene gains (≥3 copies) have been reported in 21.4% breast cancers (80). Furthermore, this has been associated with loco-regional recurrence (82). While amplification of *RPS6KB2* is only associated with 4.3% of breast cancers, a large number of sample (21.3%) exhibit gains, suggesting that *RPS6KB2* gain rather than amplification is a major event in breast cancer (27).

Estrogen receptor (ER)-positive breast cancers account for over half of all breast cancers and hence constitute the major subtype (83). The canonical or genomic ER signaling is characterized by the binding of estrogen to and subsequent activation of ERα which then translocates to the nucleus and regulates its target genes by either promoting or repressing their transcription (84). Activation of ERα is associated with its phosphorylation by several different kinases including S6K1 (85-87). Further studies showed that S6K1 and ERα constitute a positive feed forward loop where the phosphorylation of ERα by S6K1 promotes is activity which in turn promotes transcription of *RPS6KB1* to mediate breast cancer cell proliferation (88, 89). While the role and regulation of S6K1 in breast cancer has been addressed, little is known the causes and consequence of S6K2 overexpression.

The availability of anti-estrogen therapies suggests a favorable prognosis for patients with ER-positive breast cancers. However a substantial number of patients fail to respond to therapy (90) and the development of resistance further complicates treatment (83). Studies in breast cancer tissues reported *RPS6KB2* gains/amplifications correlate with ER-positivity (80). Also, distant-recurrence free survival was substantially reduced in patients

with ER-positive tumors associated with *RPS6KB2* gains/amplifications (80). Furthermore, 11q13 amplifications have been intimately linked to ER-positive breast cancers (81, 91-94) and constitute a high-risk subgroup of ER-positive cancers (91) suggesting that this region may play an important role in response and resistance of breast cancer cells to death induced by anti-estrogen therapy.

Apoptosis

Apoptosis or programmed cell death is fundamental for organismal development and homeostasis (95). It involves a series of controlled events characterized by the activation of proteases called caspases that results in the destruction of cellular components and cell shrinkage facilitating its phagocytosis by the immune system. The ability of transformed cells to evade apoptosis results in cancer.

Apoptosis can be triggered either by a receptor-mediated (extrinsic) pathway or mitochondrial (intrinsic) pathway. Binding of apoptosis inducing ligands such as tumor necrosis factor-alpha (TNF) or TRAIL (TNF-related apoptosis inducing ligand) to their receptors recruits initiator caspase-8/10 resulting in the formation of a death-inducing signaling complex (96). The mitochondrial pathway is activated following the perturbation of the mitochondrial membrane resulting in cytochrome-c release and initiator caspase-9 activation via the apoptosome (97). Both initiator caspases then cleave and activate effector caspase such as caspase-3 and -7 to execute cell death via the cleavage of downstream molecules involved in processes such as cell cycle progression and DNA repair (Fig. 4). While death domain containing receptors play an important role in the extrinsic pathway, the mitochondrial pathway is primarily regulated by the Bcl-2 family of proteins

(98). The Bcl-2 family comprises both anti-apoptotic proteins such as Bcl-2, Mcl-1 and Bcl-xl and pro-apoptotic proteins such as Bax, Bim and Bid. The balance between the pro- and anti-apoptotic Bcl-2 family members at the mitochondrial membrane dictates cell fate. The status of the tumor suppressor protein p53 plays an important role in determining this balance. At the transcriptional level, p53 promotes the expression of pro-apoptotic Bcl-2 family members such as Bid and inhibits the expression and function of anti-apoptotic members such as Bcl-2 and Mcl-1 (99).

Based on their response to apoptosis, cells have been characterized as either type I or type II. While the activation of the extrinsic pathway seems sufficient to induce effective killing of type I cells, the activation of the intrinsic pathway seems essential for effective execution of apoptosis in type II cells. Cancer cells are often categorized as type II cells due to the overexpression of the X-linked inhibitor of apoptosis (XIAP) protein (100) and in order for the receptor-initiated pathway to be effective in these cells, it is essential for the extrinsic pathway to amplify cell death via the intrinsic cell death pathway (101). It has been established that the extrinsic cell death pathway communicates with the mitochondrial pathway via the pro-apoptotic Bcl-2 family member Bid. Following caspase-8-mediated cleavage, truncated Bid translocates to the mitochondria where it amplifies cell death via the intrinsic pathway (102, 103). The regulation of the levels, activation status, and localization of the Bcl-2 family proteins plays an essential role in determining death of cancer cells in response to therapy.

Figure 4

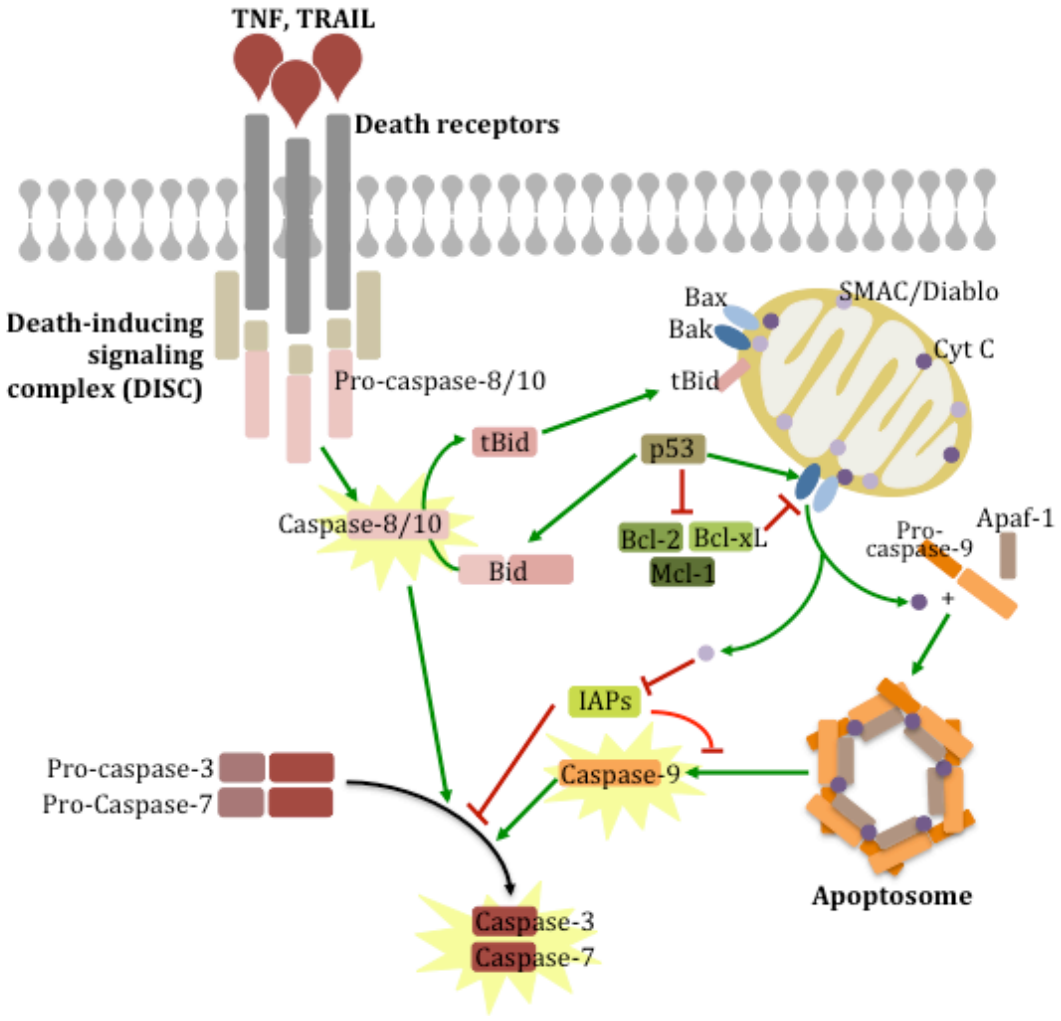

Fig. 4. The apoptotic signaling pathway. Death-inducing ligands bind to cell surface death receptors and result in the recruitment and activation of initiator caspase-8/10 while intracellular death signals promote the release of cytochrome C from the mitochondria via the Bcl-2 family members and result in the activation of initiator caspase-9. Following their activation, the initiator caspases cleave and activate effector caspase-3/7. The extrinsic apoptotic pathway communicates with the mitochondrial pathway via the pro-apoptotic Bcl-2 family member Bid.

mTOR – S6K signaling as a target for cancer therapy

Given the role of the mTOR-S6K1 pathway in promoting protein translation, cell growth and proliferation, it is an attractive target for cancer therapy (17). Rapamycin and its analogs that inhibit mTOR are being tested for their clinical potential in several cancers (104). Indeed, rapamycin analogs have already been approved for the treatment of advanced renal cell carcinoma (105). Furthermore, due to the association between *RPS6KB1* amplifications and cancer, there is considerable interest in the development of inhibitors of S6K1. LYS6K2 (106) and PF-4708671 (107) are believed to be highly specific for S6K1 with no observable activity against other related kinases such as S6K2 and ribosomal S6 kinases (RSKs). Specific inhibitors of S6K2 are yet to be developed.

The therapeutic efficacy of mTOR and S6K1 inhibitors, however, is reduced by the existence of a negative feedback loop between PI3K/Akt and mTOR/S6K1 signaling (68). While S6K1 activation promotes phosphorylation and degradation of IRS1 resulting in PI3K inactivation, mTOR also mediates feedback inhibition on insulin signaling independent of S6K1 via Grb10 (108, 109). These observations dampen the enthusiasm for rapamycin- and S6K1-inhibitor based approaches for cancer therapy. Currently combinatorial approaches using dual specificity inhibitors of PI3K/Akt and mTOR are being evaluated (110). Furthermore, the observation that $S6K1^{-/-}$ mice are characterized by small size and exhibit hypoinsulinemia suggests that targeting S6K1 for cancer therapy may be associated with significant side effects (53).

The normal development and the lack of an apparent phenotype in $S6K2^{-/-}$ mice suggests that it is a potential target in the treatment of endometrial (111), gastric (112) and breast

cancers (77, 78, 80) which have been shown to have *RPS6KB2* amplification or elevated S6K2 expression. However, little is known about the role and regulation of S6K2 in breast cancers. Hence there is a need to identify its cellular functions, targets and the molecular pathways that mediate its overexpression. This study should help in determining the suitability of S6K2 as a target for cancer therapy and if validated, aid in the designing and development of novel S6K2-based therapeutic strategies for cancer.

Hypothesis and specific aims

The mammalian target of rapamycin (mTOR) plays important roles in mediating protein synthesis, cell proliferation and cell growth and hence is an attractive target for cancer therapy. mTOR mediates its downstream effects primarily via eIF4E-binding protein (4E-BP) and 40S ribosomal S6 kinases (S6K) 1 and 2. Rapamycin and its analogs that inhibit mTOR are being tested for their potential as anti-cancer agents albeit with limited success primarily due to feedback activation of the growth- and survival-promoting phosphoinositide-3-kinase (PI3K)/Akt pathway following mTOR/S6K1 inhibition via the insulin receptor substrate (IRS). While both S6K1 and S6K2 are overexpressed in breast cancer, little is known about the pathways that promote S6K2 overexpression and how it contributes to breast cancer. A recent study utilizing breast cancer tissue specimens established the role of S6K2 as a prognostic marker in estrogen receptor (ER)-positive breast cancer suggesting a functional relationship at the cellular level between S6K and ER signaling. Furthermore, the identification of IRS as a substrate for S6K1 but not S6K2 suggests that it may not participate in the feedback regulation of the PI3K/Akt pathway. Based on these observations, we hypothesize that upregulation of S6K1 and S6K2 exert distinct effects on Akt activation and breast cancer cell survival. We further propose that targeting S6K2, in combination with chemotherapeutic agents is a novel strategy to promote breast cancer cell death.

We propose the following specific aims to test our hypothesis (as outlined in Fig. 5):

Aim 1: To determine the role of S6K in breast cancer cell survival. The working hypothesis is that the depletion/inhibition of S6K2 but not S6K1 promotes breast cancer cell death.

Aim 2: To determine the role of the estrogen signaling pathway in regulating S6K2 expression. The working hypothesis is that the estrogen signaling pathway contributes to elevated S6K2 levels in breast cancer cells.

Aim 3: To determine the mechanism by which S6K regulates cell survival. The working hypothesis is that S6K1 and S6K2 have distinct effects on Akt, and S6K2 promotes cell survival by regulating the Bcl-2 family proteins.

Figure 5

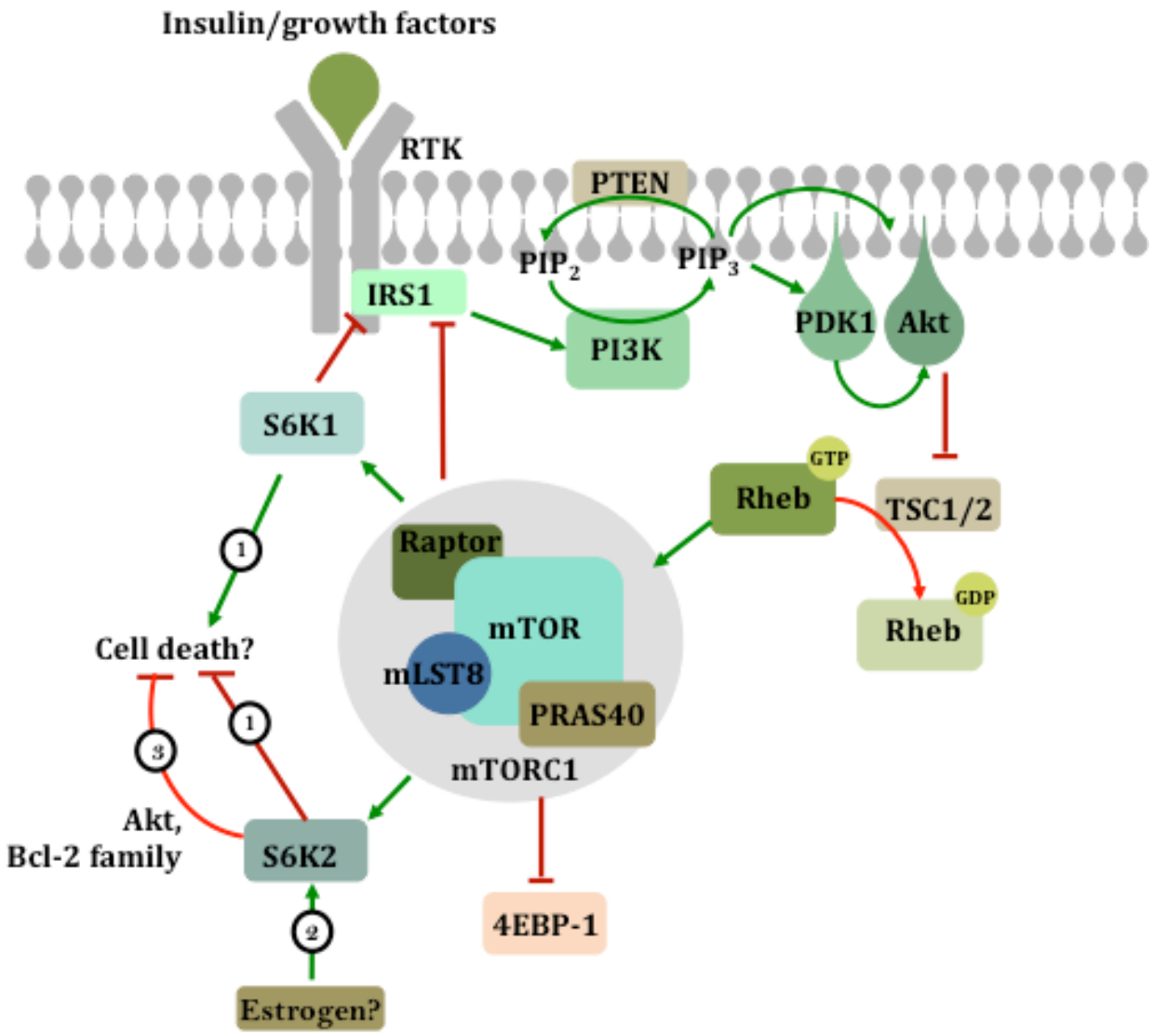

***Fig. 5.* Akt/mTOR/S6K signaling and the questions addressed in this study.** The role and regulation of S6K2 in breast cancer in comparison to S6K1 are addressed in this study.

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CHAPTER II

S6 Kinase 2 Promotes Breast Cancer Cell Survival via Akt

Savitha Sridharan and Alakananda Basu

Key Words: S6K, Akt, Bid, TNF, p53, apoptosis, breast cancer

Abstract

The 40S ribosomal protein S6 kinase (S6K) acts downstream of the mammalian target of rapamycin (mTOR), which plays important roles in cell proliferation, protein translation and cell survival and is a target for cancer therapy. mTOR inhibitors are, however, of limited success. Although Akt is believed to act upstream of mTOR, persistent inhibition of p70 S6 kinase or S6K1 can activate Akt via a negative feedback loop. S6K exists as two homologs, S6K1 and S6K2 but little is known about the function of S6K2. In the present study, we have examined the effects of S6K2 on Akt activation and cell survival. Silencing of S6K1 caused a modest decrease whereas knockdown of S6K2 caused a substantial increase in tumor necrosis factor-a (TNF)- and TNF-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis. In contrast to S6K1, depletion of S6K2 by siRNA decreased basal and TNF-induced Akt phosphorylation. Ectopic expression of constitutively-active Akt in MCF-7 cells restored cell survival in S6K2-depleted cells. We have previously shown that activation of Akt induces downregulation of Bid via p53. Knockdown of S6K2 caused an increase in p53 and downregulation of p53 by siRNA decreased Bid level. Silencing of Bid blunted the ability of S6K2 deficiency to enhance TNF-induced apoptosis. Taken together, our study demonstrates that the two homologs of S6K have distinct effects on Akt activation and cell survival. Thus, targeting S6K2 may be an effective therapeutic strategy to treat cancers.

Introduction

Akt or protein kinase B (PKB), a serine/threonine kinase, is the cellular homolog of the oncogene product v-Akt (1). It is activated downstream of phosphatidyl inositol-3-kinase (PI3K) in response to growth factors or cytokines. Akt performs diverse cellular functions, including cell growth, proliferation and survival (2). It is deregulated in many cancers, including breast cancer and confers resistance to chemotherapeutic drugs (3). Phosphorylation of Akt at Thr308 and Ser473 sites results in its activation (4).

Tumor necrosis factor-a (TNF) was originally identified as a cytokine that induces necrosis in tumors and regression of cancer in animals (5). It causes selective destruction of tumor tissues but has no effect on normal tissues (6). The presence of antiapoptotic proteins, however, can counteract cell death mediated by TNF. It has been reported that TNF causes activation of Akt through phosphorylation at Ser473 (7). Binding of TNF to its cell surface receptors causes activation of initiator caspase-8 followed by activation of effector caspases, such as caspase-3 and -7, resulting in the cleavage of critical cellular proteins and cell death (8, 9). Although caspase-8 is the apical caspase in the death receptor pathway, there is cross-talk between the receptor-initiated and mitochondrial pathway (10-12). The members of the Bcl-2 family proteins play important roles in regulating the intrinsic or mitochondrial cell death pathway (13, 14). Capase-8 catalyzes the cleavage of the Bcl-2 family protein Bid (10-12). The truncated Bid (tBid) translocates to mitochondria causing release of cytochrome c and activation of caspase-9 (10-12). It has been reported that Akt can exert its antiapoptotic function by inhibiting the function of proapoptotic Bcl-2 family proteins (15-20).

Several cellular functions of Akt are mediated by the mammalian target of rapamycin (mTOR), which is considered the master controller of protein synthesis and cell proliferation (21). Activated Akt can phosphorylate and inactivate tuberous sclerosis complex 2 (TSC2), which negatively regulates mTOR (22). mTOR interacts with either raptor or rictor to form mTOR complex I (mTORC1) or mTOR complex 2 (mTORC2), respectively (22). While phosphoinositide-dependent kinase 1 (PDK1), which acts downstream of PI3K, phosphorylates Akt at Thr308 site, rictor complexed with mTORC2 can phosphorylate Akt at Ser473 (22). mTORC1 is inhibited by rapamycin, which is currently being tested for use in cancer therapy albeit with limited success (23).

The 40S ribosomal protein S6 kinase (S6K) is a downstream target of mTORC1 (24). S6K is represented by two homologous cellular proteins, S6K1 and S6K2, both of which act downstream of mTOR and phosphorylate S6 (25). Persistent inhibition of S6K1 has been shown to activate Akt via feedback inhibition of the PI3K pathway where S6K1 phosphorylates several sites on insulin receptor substrate-1 (IRS-1) and inhibits it (26-30). The limited therapeutic efficacy of rapamycin and its analogs has been attributed to the activation of Akt via this negative feedback loop due to inhibition of S6K1 (26, 29) and the inability of rapamycin to completely activate 4E-BP, another downstream target of mTORC1 (31-33).

Although there are two homologs of S6K (25, 34), most of the studies have been focused on S6K1 and little is known about the function of S6K2. S6K1-deficient mice phosphorylated S6 but had a small body phenotype (35). S6K1/2 double knockout mice also exhibit normal proliferation and growth reduction (36). Similarly, S6K1/2 double knockout mouse embryo fibroblasts and myoblasts show defects in size but not proliferation (31, 36). These results

suggest that these two homologs have redundant as well as non-overlapping functions. It has been reported that S6K2 but not S6K1 was important for FGF2-induced chemoresistance of small cell lung cancer cells (37). A recent study demonstrated that S6K2 but not S6K1 was important for cell proliferation in response to mTOR activation (38).

Since the Akt/mTOR/S6K axis plays a critical role in cell survival yet targeting mTOR has been of limited success due to feedback activation of Akt, we have examined if the two homologs of S6 kinase perform distinct functions in mediating breast cancer cell survival. We report for the first time that S6K2 regulates cell survival via the Akt pathway. We have shown that in contrast to S6K1, silencing of S6K2 inhibits Akt and induces cell death via the proapoptotic Bcl-2 family protein Bid. Thus, selective targeting of S6K2 rather than mTOR or S6K1 may be a more effective therapeutic strategy to treat cancers.

Materials and Methods

Materials. Human recombinant TNF and TRAIL were purchased from R&D Systems (Minneapolis, MN). Monoclonal antibodies to PARP and p53, and polyclonal antibody to caspase-9 were obtained from Pharmingen (San Diego, CA). Polyclonal antibody to Akt, phospho-Akt (Ser473), S6K1 and phospho-FOXO3a were obtained from Cell Signaling Technology (Beverly, MA). Polyclonal antibody to S6K2 was from Santa Cruz Biotechnology (Santa Cruz, CA) and Bethyl Laboratories (Montgomery, TX). Polyclonal antibody to Bid and monoclonal antibody to caspase-8 were purchased from BioSource (Camarillo, CA). Actin was purchased from Sigma-Aldrich (St Louis, MO). Yo-Pro, annexin V conjugated to Alexa Fluor 488 and propidium iodide (25) were purchased from Molecular Probes/Invitrogen (Carlsbad, CA). Caspase-3 fluorometric assay kit was obtained from BioVision (Palo Alto, CA, USA). Horseradish peroxidase conjugated goat anti-mouse and donkey anti-rabbit antibodies were obtained from JacksonImmuno Research Lab. Inc. (West Grove, PA). Control non-targeting siRNA and siRNA specific for S6K1, S6K2, Bid, Bax and p53 were obtained from Dharmacon (Lafayette, CO). Polyvinylidene difluoride membrane was from Millipore (Bedford, MA) and enhanced chemiluminescence detection kit was from Amersham (Arlington Heights, IL).

Cell Culture and Transfection. MCF-7 and ZR-75-1 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 2 mM glutamine. MCF-7 cells were obtained from Dr. Olivera J. Finn. ZR-75-1 and cells were obtained from the UT Southwestern Medical Center. Cells were kept in a humidified incubator at 370C with 95% air and 5% CO2. All these cells were authenticated by DNA fingerprinting at the UT Southwestern Medical Center and the Department of Forensic Genetics at UNT Health

Science Center. siRNA was transfected using Lipofectamine 2000 transfection reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). Cells were infected with adenovirus vector containing GFP or constitutively-active (myrisotylated) Akt (MOI 10).

Immunoblot Analysis. Equivalent amounts of total cellular extracts were electrophoresed by SDS-PAGE and transferred electrophoretically to polyvinylidene difluoride membrane. Immunoblot analyses were performed as described before (39).

Cell Death Analysis. Cells were labeled with 0.5 μM YO-PRO-1 and 2 μM PI by incubating at 37°C for 15 min and visualized using a Zeiss Axiovert 40 inverted microscope with the AxioVision Rel 4.6 software (Zeiss, Göttingen, Germany).

Annexin V/Propidium Iodide Binding Assay. Cells were treated with or without TNF as indicated in the text. At the end of the incubation, both detached cells and attached cells were collected and washed with PBS. Cells were then stained with Annexin V-Alexa 488 conjugate and PI according to the manufacturer's protocol and analyzed using a flow cytometer (Coulter Epics) (40).

Caspase assay. DEVDase activity was determined at 37C using Ac-DEVD-AFC as the substrate and manufacturer's protocol. The fluorescence liberated from DEVD-AFC was measured using a SpectraMax GeminiXS fluorometer and SOFTmax PRO 3.1.1 software (Molecular Devices, Sunnyvale, CA, USA) with an excitation wavelength of 400-nm and emission wavelength of 505 nm.

Statistical analysis. Data are presented as the mean ± S.D. and n = 4. Statistical significance was determined by paired Student's t-test using PASW Statistics (SPSS, Inc., Chicago, IL). $P < 0.05$ was considered statistically significant.

Results

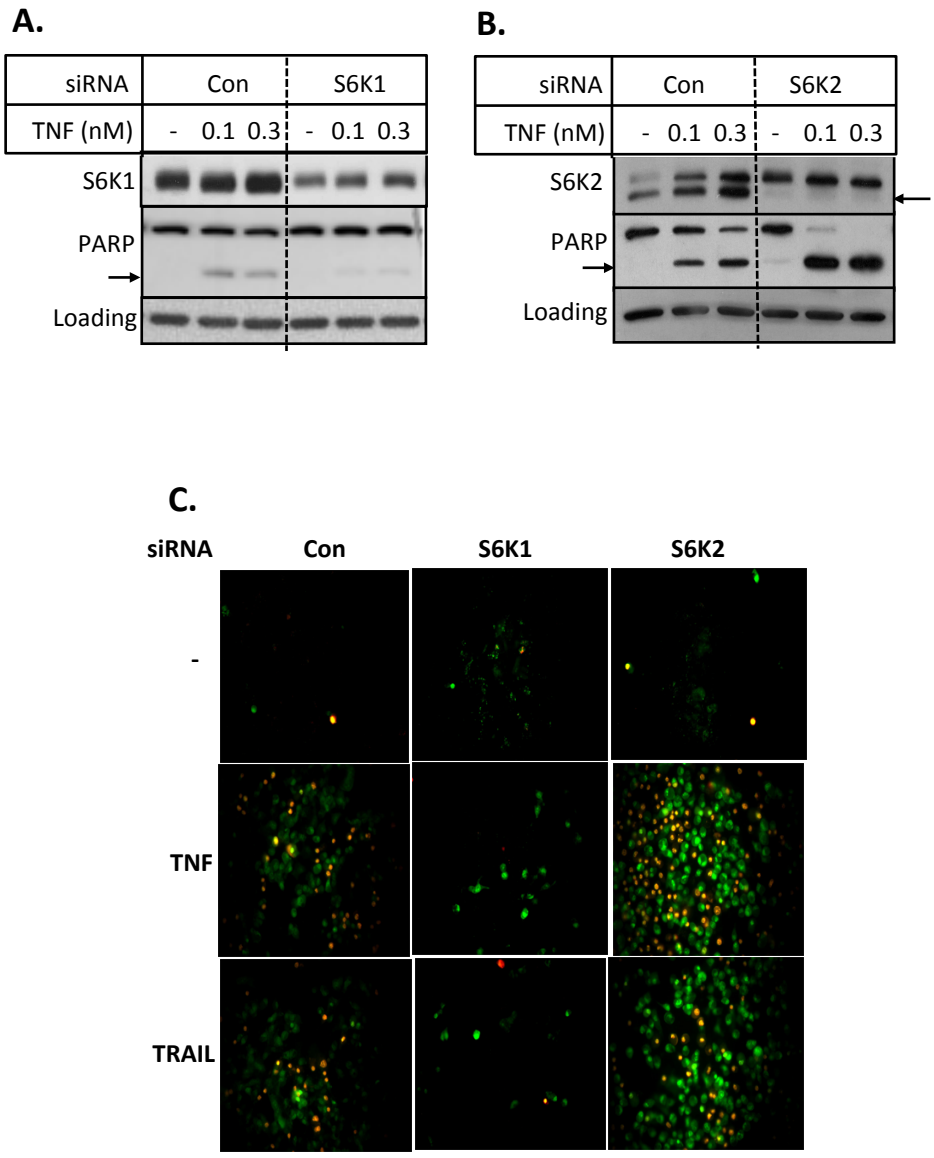
S6K Homologs Exhibit Distinct Effects on TNF-Induced Apoptosis in Breast Cancer MCF-7 Cells.

Since S6K1 is overexpressed in MCF-7 breast cancer cells (41) and has been associated with chemoresistance (42, 43), we examined if S6K1 confers resistance to TNF in MCF-7 breast cancer cells. Figure 1A shows that silencing of S6K1 by siRNA caused a modest decrease rather than an increase in the cleavage of PARP in response to TNF. Since there are two S6K homologs, we examined the effect of S6K2 knockdown on TNF-induced cell death. As shown in Figure 1B, depletion of S6K2 caused a substantial increase in TNF-induced cleavage of the 116-kDa full-length PARP to the 85-kDa form. We also monitored the effect of S6K1 and S6K2 knockdown on cell death by staining cells with YO-PRO-1 and PI (Fig. 1C). Apoptotic cells are permeable to the green fluorescent dye YO-PRO-1 whereas PI (red) is taken up only by necrotic and late-apoptotic cells. S6K2 depletion increased the number of YO-PRO-1/PI-stained cells in response to TNF and TRAIL while S6K1 depletion appears to decrease it. Thus, the two S6K homologs had distinct effects on TNF- and TRAIL-induced cell death.

Figure 1

S6K1 and S6K2 have distinct effects on breast cancer cell survival. MCF-7 cells were transfected with control non-targeting siRNA and either S6K1 (A) or S6K2 (B) siRNA and then treated with indicated concentrations of TNF. Western blot analysis was performed with indicated antibodies. Actin was used to control for loading differences. The arrow indicates 85-kDa cleaved PARP. C, MCF-7 cells transfected with control, S6K1 or S6K2 siRNA were treated without or with TNF or TRAIL. Cells were then stained with YO-PRO-1 (Green) to detect apoptotic cells and propidium iodide (red) to detect necrotic cells using a fluorescence microscope. Merged figures are shown. Results are representative of 3 independent experiments.

Figure 1


A.
siRNA
Con
S6K1
TNF (nM)
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S6K1
S6K2
-
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TRAIL

S6K Homologs Exert Opposite Effects on TNF-Induced Akt Phosphorylation.

Since silencing of S6K1 caused a modest inhibition of TNF- and TRAIL-induced apoptosis (Figs. 1A and 1C), and S6K1 was shown to negatively regulate Akt via a feedback loop (26, 28-30), we examined if knockdown of S6K1 enhances TNF-induced activation of Akt in MCF-7 cells. Figure 2 shows that depletion of S6K1 in MCF-7 breast cancer cells enhanced phosphorylation of Akt. In contrast to S6K1, knockdown of S6K2 decreased both basal and TNF-induced Akt phosphorylation (Fig. 3A). Based on densitometric scanning of four independent experiments, knockdown of S6K2 decreased basal and TNF-induced Akt phosphorylation at Ser473 by 40% and 60%, respectively (Fig. 3B).

We also examined the consequence of S6K2 knockdown on Akt phosphorylation in ZR-75-1 breast cancer cells (Fig. 3C). Knockdown of S6K2 decreased Akt phosphorylation, and enhanced PARP cleavage and caspase activation in ZR-75-1 cells (Fig. 3C).

Figure 2

Knockdown of S6K1 increased Akt phosphorylation. MCF-7 cells transfected with control non-targeting or S6K1 siRNA were treated with indicated concentrations of TNF. Western blot analysis was performed with indicated antibodies.

Figure 2

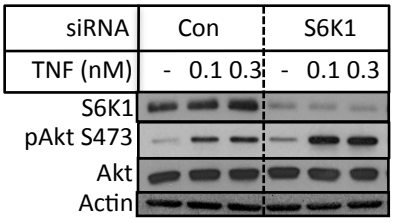
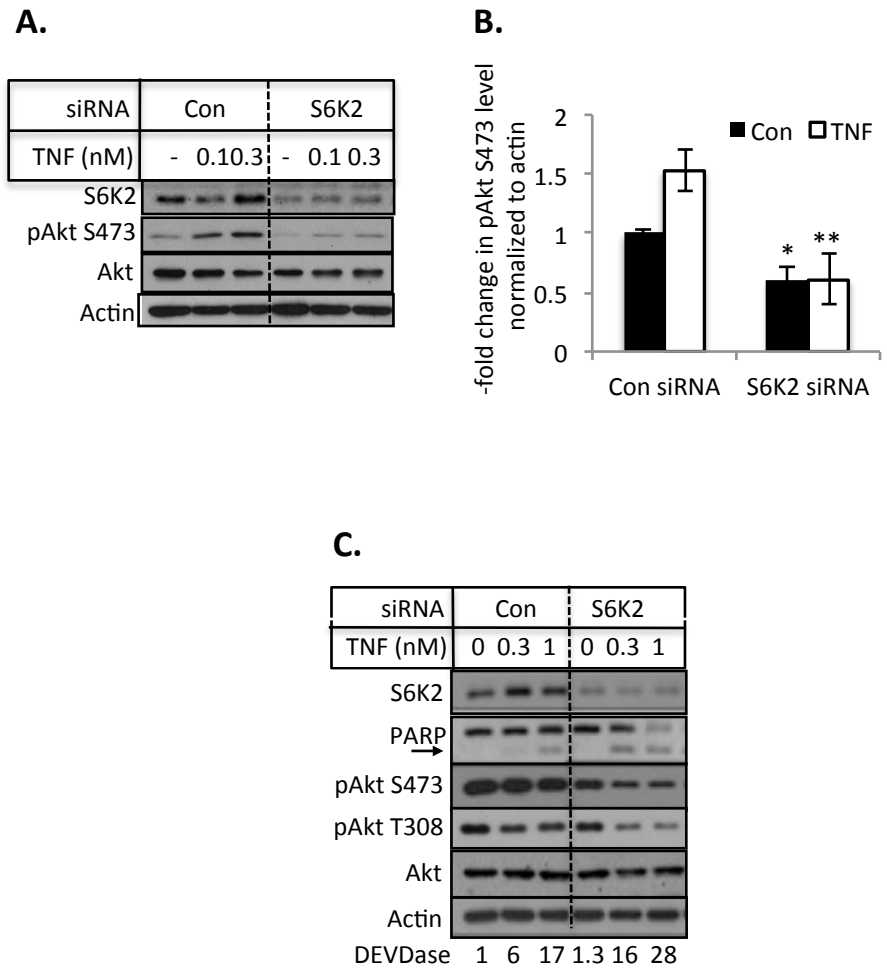


Figure 3

Knockdown of S6K2 decreased Akt phosphorylation. MCF-7 (A) or ZR-75-1 (C) cells were transfected with control non-targeting or S6K2 siRNA. Cells were treated with or without indicated concentrations of TNF and Western blot analysis was performed with indicated antibodies. B, Intensity of phospho-Akt was determined by densitometry and standardized by loading. The data represent the -fold decrease in Akt phosphorylation in S6K2-depleted cells compared to control siRNA-transfected cells. Each bar represents the mean ± S.D. of four independent experiments. The solid bar represents untreated cells and the open bar represents TNF treatment. *, p value < 0.05; **, p value < 0.01 using paired Student's t test.

Figure 3


A.
siRNA
Con
S6K2
TNF (nM)
- 0.1 0.3 - 0.1 0.3
S6K2
pAkt S473
Akt
Actin
B.
-fold change in pAkt S473 level normalized to actin
2
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S6K2
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S6K2
PARP
pAkt S473
pAkt T308
Akt
Actin
DEVDase 1 6 17 1.3 16 28

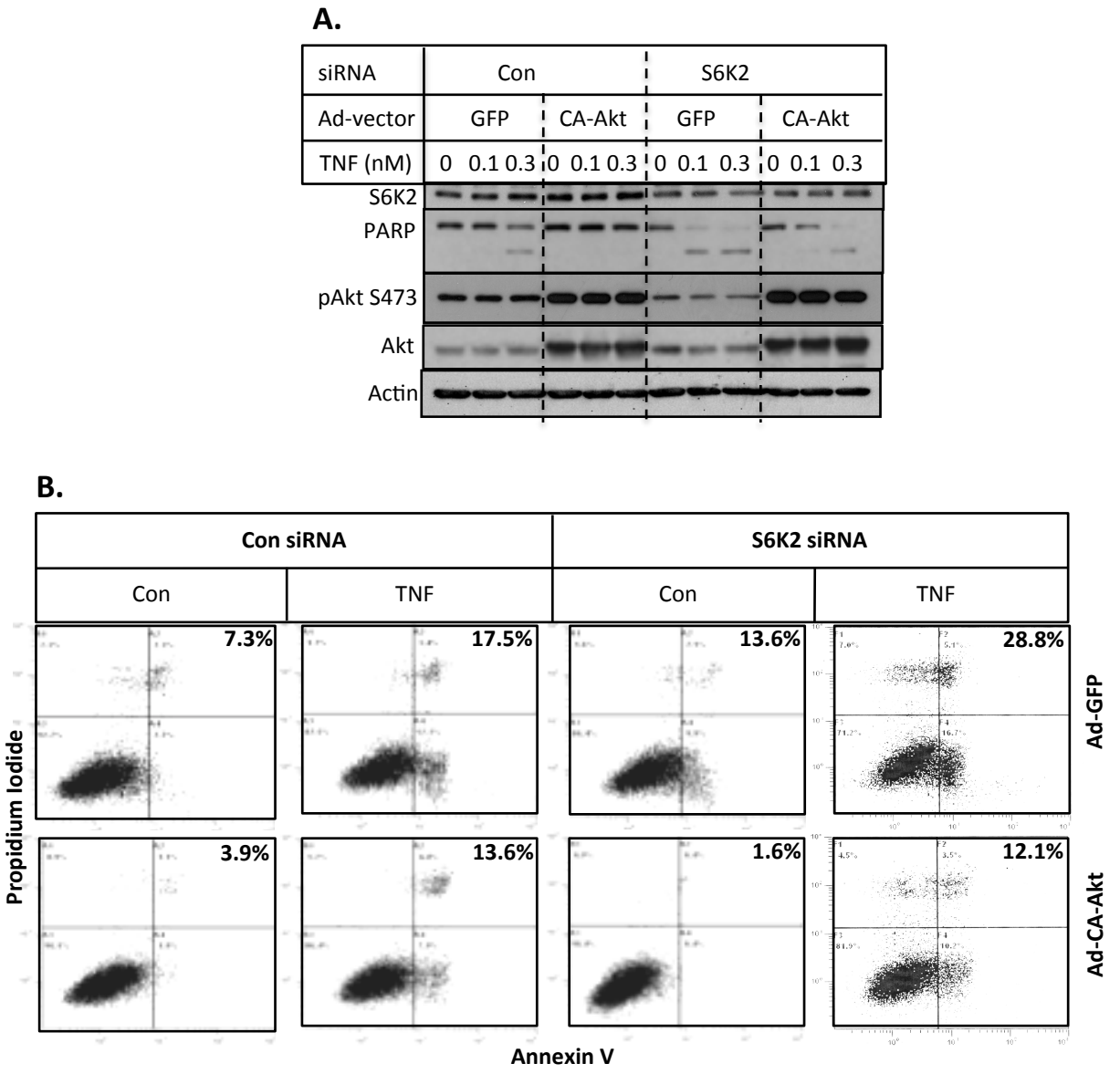
S6K2 Promotes MCF-7 Cell Survival via Akt

Since knockdown of S6K2 inhibits Akt phosphorylation, we examined if S6K2 promotes cell survival via Akt. We examined the ability of constitutively-active (CA) Akt to reverse the potentiation of cell death caused by S6K2 depletion. Figure 4A shows that the adenoviral vector-mediated delivery of CA-Akt in MCF-7 cells decreased TNF-induced PARP cleavage compared to cells transfected with adeno-GFP. While knockdown of S6K2 caused a substantial increase in TNF-induced PARP cleavage, overexpression of CA-Akt inhibited TNF-induced PARP cleavage in S6K2-depleted cells. Similar results were obtained when we monitored cell death by staining cells with Annexin V and PI (Fig. 4B). These results suggest that S6K2 mediates its prosurvival effect via Akt.

Figure 4

Overexpression of Akt reverses the effects of S6K2 knockdown on TNF-induced apoptosis. MCF-7 cells transfected with control or S6K2 siRNA were infected with adenovirus vector containing GFP or CA-Akt construct. Cells were then treated with or without indicated concentrations of TNF. A, Western blot analysis was performed with indicated antibodies. B, Cells were stained with Annexin V-Alexa 488 conjugate and PI, and analyzed using a flow cytometer. Results are representative of 3 independent experiments.

Figure 4


A.
siRNA
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CA-Akt
TNF (nM)
0 0.1 0.3 0 0.1 0.3 0 0.1 0.3 0 0.1 0.3
S6K2
PARP
pAkt S473
Akt
Actin
B.
Con siRNA
S6K2 siRNA
Con
TNF
Con
TNF
7.3%
17.5%
13.6%
28.8%
Ad-GFP
3.9%
13.6%
1.6%
12.1%
Ad-CA-Akt
Propidium Iodide
Annexin V

Knockdown of S6K2 Enhanced Cell Death via Bid

Although TNF and TRAIL trigger cell death via the receptor-initiated pathway, they can also amplify cell death via the mitochondrial pathway (10-12). To determine the mechanism(s) by which depletion of S6K2 potentiates TNF-induced cell death, we monitored TNF-induced caspase activation and processing of Bid. Figure 5A shows that TNF caused an increase in phospho-Akt which was attenuated by S6K2 knockdown. Depletion of S6K2 was associated with enhanced processing of PARP and procaspase-8 in response to TNF. This was accompanied by an increase in the cleavage of Bid, a substrate for caspase-8 (10) and increased processing of procaspase-9, the apical caspase of the mitochondrial cell death pathway. We also compared the effects of S6K1 and S6K2 knockdown on cellular responses to TRAIL (Fig. 5B). Knockdown of S6K2 had little effect on caspase-8 inhibitor c-FLIP but it enhanced processing of procaspase-8, -9 and Bid (Fig. 5B).

To further validate our observation that S6K2 depletion decreases Akt phosphorylation and increases cell death via the mitochondrial pathway, we used four different siRNA constructs against S6K2. Figure 5C shows that siRNAs 1, 2 and 4 against S6K2 decreased Akt phosphorylation, enhanced PARP cleavage and increased processing of procaspase-8 and -9 similar to S6K2 SMARTpool siRNA. In contrast, siRNA 2 was less effective in attenuating Akt phosphorylation and cleavage of PARP, caspase-8 and -9. Thus, a decrease in Akt phosphorylation by S6K2 depletion was associated with an increase in PARP cleavage.

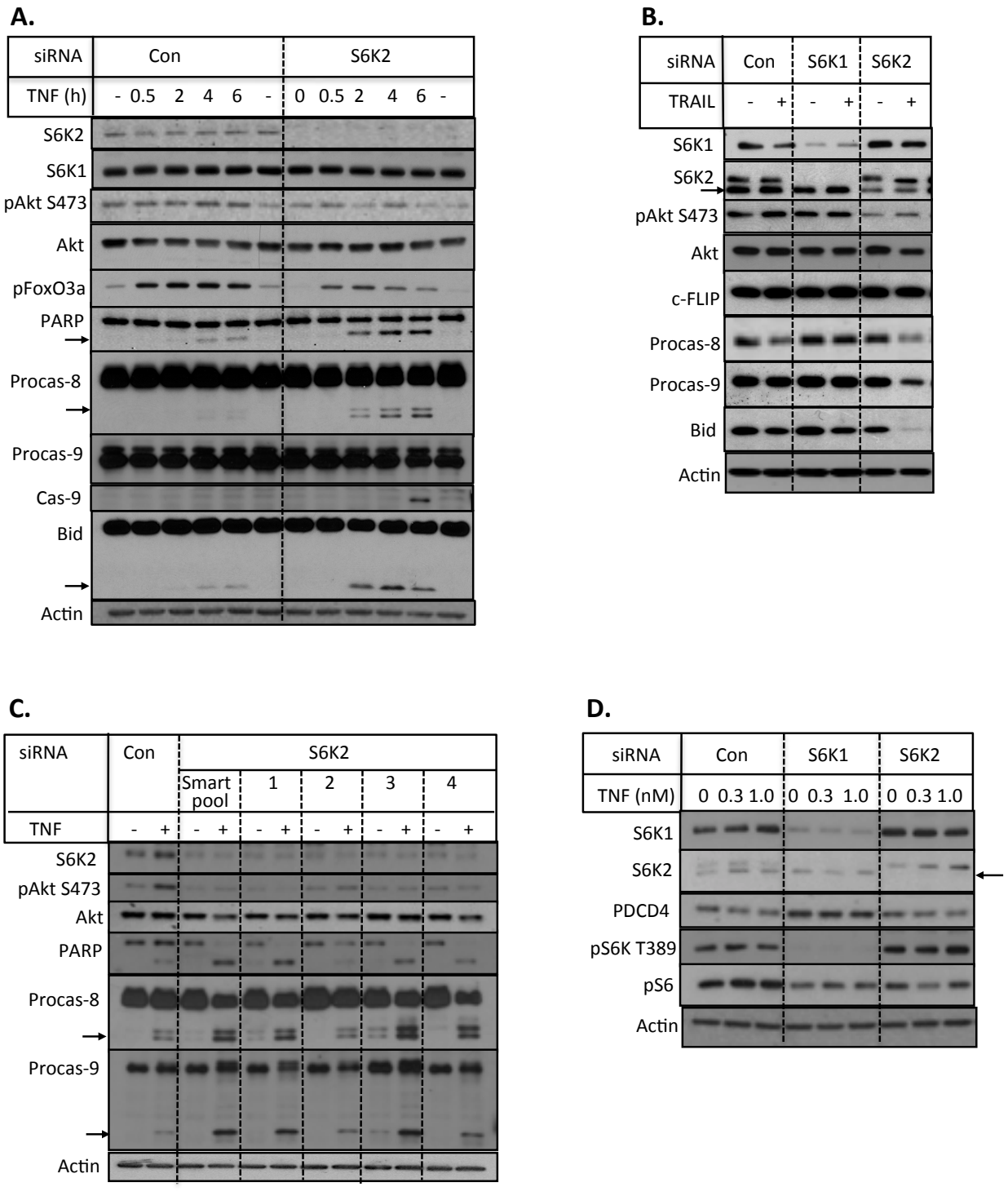
Since PDCD4 has been implicated in TNF-induced apoptosis and acts as a tumor suppressor (44, 45), we have also examined the effects of S6K1 and -2 knockdown on the level of

PDCD4. Silencing of S6K1 or S6K2 effectively depleted the homolog and attenuated phosphorylation of the substrate S6. However, while knockdown of S6K1 consistently increased PDCD4 level, depletion of S6K2 had either no effect or decreased the level of PDCD4 modestly (Fig. 5D and data not shown). Thus, it is unlikely that a decrease in PDCD4 was responsible for the potentiation of cell death caused by S6K2 knockdown.

Figure 5

Knockdown of S6K2 induced apoptosis via the mitochondrial pathway. MCF-7 cells were transfected with control or S6K2 siRNA. A, Cells were treated with 1 nM TNF for indicated periods of time. B, Cells were treated with or without TRAIL. C, Cells were transfected with control, siRNA SMARTpool or four different S6K2 siRNAs and then treated with 0.3 nM TNF. D, Cells were transfected with control, S6K1 or S6K2 siRNA. Western blot analysis was performed with indicated antibodies. Western blot analyses were performed with indicated antibodies. The upper band in the S6K2 blot is likely to be S6K1. Results are representative of 3 independent experiments. The arrows indicate the processed forms of PARP, caspase-8, caspase-9 and Bid.

Figure 5


A.
siRNA
Con
S6K2
TNF (h)
- 0.5 2 4 6 - 0 0.5 2 4 6 -
S6K2
S6K1
pAkt S473
Akt
pFoxO3a
PARP
Procas-8
Procas-9
Cas-9
Bid
Actin
B.
siRNA
Con
S6K1
S6K2
TRAIL
- + - + - +
S6K1
S6K2
pAkt S473
Akt
c-FLIP
Procas-8
Procas-9
Bid
Actin
C.
siRNA
Con
S6K2
Smart pool
1
2
3
4
TNF
- + - + - + - + - + - +
S6K2
pAkt S473
Akt
PARP
Procas-8
Procas-9
Actin
D.
siRNA
Con
S6K1
S6K2
TNF (nM)
0 0.3 1.0 0 0.3 1.0 0 0.3 1.0
S6K1
S6K2
PDCD4
pS6K T389
pS6
Actin

We have previously shown that activation of Akt promotes cell survival by downregulating Bid via p53 (17). We therefore examined if S6K2 knockdown affects p53 level. Figure 6 shows that knockdown of S6K2 enhanced basal and TNF-induced p53 level and silencing of p53 decreased Bid level, suggesting that S6K2 may regulate Bid via p53. Finally, to determine if Bid is indeed involved in the potentiation of cell death caused by S6K2 knockdown, we examined if S6K2 depletion affected Bid levels and sensitizes cells to TNF when Bid is depleted. We compared the effect of Bid with another proapoptotic Bcl-2 family member Bax. Figure 7 shows that knockdown of Bid abolished TNF-induced PARP cleavage. Additionally, knockdown of Bid but not Bax attenuated the ability of S6K2 to enhance TNF-induced PARP cleavage. These results suggest that the mechanism by which S6K2 potentiates receptor-mediated apoptosis involves the proapoptotic protein Bid.

Figure 6

S6K2 depletion increases p53 levels and knockdown of p53 attenuates Bid level. MCF-7 cells were transfected with indicated siRNAs and then treated with or without TNF. Cell lysates were analyzed by Western blotting using the indicated antibodies. Actin was used to control for loading differences. Results are representative of two independent experiments.

Figure 6

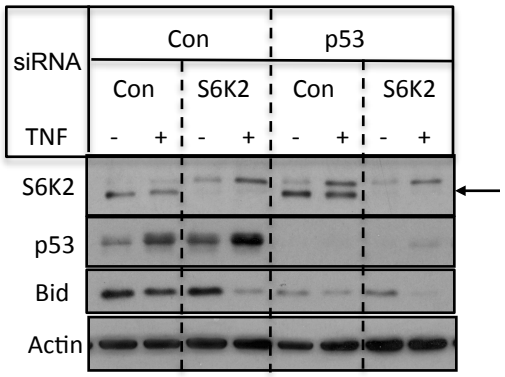
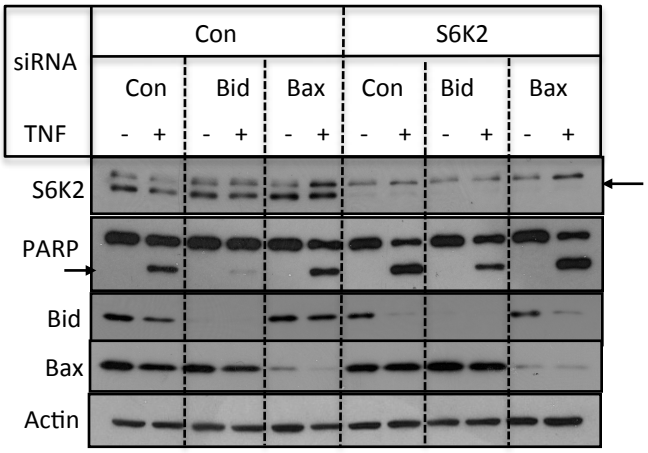


Figure 7

S6K2 depletion enhances TNF-induced apoptosis via Bid. MCF-7 cells transfected with indicated siRNAs were treated with or without TNF and Western blot analysis was performed with indicated antibodies. Results are representative of two independent experiments.

Figure 7


siRNA
Con
S6K2
Con
Bid
Bax
Con
Bid
Bax
TNF
- + - + - + - + - + - +
S6K2
PARP
Bid
Bax
Actin

Discussion

The results of our present study demonstrate that the two S6K homologs, S6K1 and S6K2 exhibit distinct functions on breast cancer cell survival. While it has been reported that S6K1 can negatively regulate Akt via a negative feedback loop, we report for the first time that depletion of S6K2 inhibits Akt activity and promotes breast cancer cell death via the mitochondrial cell death pathway that involves the Bcl-2 family protein Bid.

It is generally believed that activation of PI3K/Akt stimulates the mTOR pathway by phosphorylating and inactivating the tumor suppressor protein tuberous sclerosis complex 2 (TSC2), which negatively regulates mTOR activity. mTOR is required for estrogen-induced breast tumor cell proliferation (46) and constitutive signaling through the mTOR pathway is a cause of treatment failure in breast cancer patients (47). S6K1, a downstream target of mTOR, is an important mediator of mTOR function (48). An elevation/activation of S6K has been associated with several cancers and resistance to chemotherapeutic drugs (41, 43, 49, 50). The S6K1 gene is amplified in approximately 9% of primary breast cancers (51), and S6K1 mRNA is elevated in almost 40% of the tumors (41). The status of the activated S6K1 was shown to be a predictor of patient's survival and treatment response (41, 49, 52). Recently, it has been reported that S6K1 promotes breast cancer cell proliferation by phosphorylating ERa, leading to its transcriptional activation (53). Thus, we anticipated that knockdown of S6K1 would enhance cell death in breast cancer cells. To our surprise, depletion of S6K1 caused a modest decrease in cell death in response to TNF. Our results are, however, consistent with the recent reports that S6K1 deficiency protects against death receptor-mediated apoptosis in hepatocytes (54) and mTOR-S6K1 activates p53-dependent cell death in response to DNA damage (55). As has been reported earlier

that persistent inhibition of mTOR/S6K1 can activate Akt via a negative feedback loop (26, 28-30), we also found that depletion of S6K1 resulted in an increase in TNF-induced Akt phosphorylation and this may explain why S6K1 knockdown inhibits rather than potentiates TNF-induced cell death.

Although most of the published reports have focused on S6K1, there are two homologs of S6K, S6K1 and S6K2 that act downstream of mTOR (25, 34). While the two homologs share overall similarity in structure and exhibit redundant functions, there are also important differences. S6K2 has been shown to potentiate IL3-mediated mitogenic response (56). A recent study demonstrated that S6K2 but not S6K1 interacts with heterogeneous ribonucleoproteins (hnRNPs) F/H to drive cell proliferation (57). We have consistently found that in contrast to S6K1, depletion of S6K2 caused a dramatic increase in TNF- and TRAIL-induced apoptosis, suggesting that S6K2 functions as a prosurvival protein. TNF has been shown to activate mTOR signaling (58) and we have found that TNF preferentially activates S6K1 (data not shown), presumably because the abundance of S6K1 is much greater compared to S6K2 in MCF-7 cells. We made a novel observation that in contrast to S6K1, S6K2 positively regulates Akt. Knockdown of S6K2 caused a decrease in both basal and TNF-induced Akt phosphorylation, which is indicative of its activation status, suggesting that S6K2 promotes cell survival via activation of Akt. In fact, overexpression of CA-Akt blocked increase in cell death caused by S6K2 depletion, suggesting that S6K2 acts upstream of Akt although we cannot rule out the possibility that Akt and S6K2 act in parallel pathways where Akt has a dominant role over S6K2.

There are several potential mechanisms by which S6K2 affects phosphorylation/activity of Akt. Since mTORC2 activates Akt by phosphorylating at the hydrophobic site, it is

conceivable that knockdown of S6K2 decreases Akt phosphorylation by inhibiting mTORC2. Others and we have also shown that Ser473 phosphorylation of Akt is also regulated by DNA-dependent protein kinase (40). Since PTEN inhibits PI3K/Akt, another possibility is that S6K2 knockdown increases PTEN level resulting in inhibition of Akt. It has been reported that a major kinase downstream of mTORC2 is SGK1 (59). Thus, it is also important to determine if S6K2 regulates cell survival via SGK1. Moreover, since activation of Akt would lead to the activation of mTORC1, there may be a positive feedback loop between S6K2 and Akt. Thus, mTORC1 and its downstream targets may mediate some of the effects of the potential functional interaction between S6K2 and Akt. Future studies should discern the mechanisms by which S6K2 regulate Akt and the functional interaction between S6K2 and Akt.

Our results suggest that the mechanism by which S6K2 promotes cell survival via Akt involves the proapoptotic Bcl-2 family protein Bid. We have previously shown that activation of Akt can cause a decrease in p53 levels in MCF-7 cells by phosphorylating and stabilizing Hdm2, which degrades p53 via the ubiquitin proteasome-mediated pathway (17). We have also shown that Bid is a transcriptional target of p53 and Akt can decrease Bid expression by inducing downregulation of p53 (17). The results of our present study demonstrate that knockdown of S6K2 increased p53 and silencing of p53 was associated with a decrease in Bid. However, depletion of Bid was not associated with upregulation of Bid. We have previously shown that overexpression of Bid is sufficient to cause cell death (19). Since Bid is a proapoptotic protein, an increase in Bid may also lead to its cleavage. Therefore, it may be difficult to demonstrate an increase in Bid level. Nevertheless, knockdown of S6K2 had little effect on enhancing TNF-induced cell death when Bid was

depleted by siRNA silencing. Thus, one of the mechanism by which S6K2 promotes cell survival via Akt may involve downregulation of Bid.

S6K2 has also been implicated in fibroblast growth factor-mediated chemoresistance of small cell lung cancer H69 cells (37). It has been reported that PKCe interacts with S6K2 and mediates the prosurvival effects of S6K2 via Raf/MAPK signaling pathway by increasing the levels of antiapoptotic proteins XIAP and Bcl-xL (37). We were unable to detect a decrease in XIAP and Bcl-xL in S6K-2-depleted MCF-7 cells (data not shown) although we cannot rule out the possibility of other Bcl-2 family members. Interestingly, we have previously shown that PKCe also acts upstream of Akt during TNF-induced apoptosis in MCF-7 breast cancer cells (40), and inhibits TNF- and TRAIL-mediated apoptosis by increasing antiapoptotic Bcl-2 and decreasing proapoptotic Bid levels (19). Moreover PKCe caused a decrease in Bid via Akt (17). Thus, depending on the cellular context and apoptotic stimulus, PKCe may promote cell survival either via the Raf/MEK/ERK pathway or via the Akt signaling pathway.

Aberrations in Akt/mTOR/S6K pathway have been associated with many cancers. Consequently, this pathway is an important target for cancer therapy. Rapamycin and its analogues that inhibit mTOR, however, were of limited success (26-30). Since S6K1 and S6K2 appear to have opposite effects on cell death, targeting mTOR which acts upstream of both S6K1 and S6K2 may not be effective. Our observation that S6K2 rather than S6K1 is needed for the survival of breast cancer cells has significant implications in the treatment of the disease. Inhibition of S6K2 rather than of S6K1 should sensitize cancer cells to chemotherapeutic agents, providing a basis for rational combination chemotherapy. Since Akt signaling pathway is often deregulated in cancer, the observation that knockdown of

S6K2 results in inhibition of Akt demonstrates positive feedback regulation of Akt by S6K2, and has significant impact in cancer therapy.

Acknowledgements

We thank Dr. Timothy Break and Dr. Eswar Shankar for help with flow cytometry and YO-PRO-1/PI assay, respectively. We also gratefully acknowledge Ms. Soumya Krishnamurthy and Deepanwita Pal for help with several experiments, and Deepanwita Pal for critical reading of the manuscript. Adenovirus containing constitutively active Akt was a kind gift from Dr. Santosh DeMello (University of Texas, Dallas).

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CHAPTER III

Upregulation of S6 kinase 2 by estrogen signaling contributes to breast cancer cell survival

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Key words: breast cancer, S6K2, estradiol, estrogen receptor alpha, cell death.

Abstract

Estrogen receptor (ER)-positive breast cancer accounts for approximately 70% of all breast cancers. The treatment of ER-positive breast cancers is often complicated by unresponsiveness and the development of resistance to anti-estrogen therapies. Hence, there is a need to identify suitable molecular targets that mediate ER-induced effects. The 40S ribosomal S6 kinases (S6K), S6K1 and S6K2, are often overexpressed in breast cancer cells and tissues. While the role and regulation of S6K1 in breast cancer is understood, little is known about those of S6K2. Here we report that S6K2 is regulated by the estrogen signaling pathway post-transcriptionally. We further demonstrate that it functions downstream of estrogen to mediate survival of breast cancer cells and determine the prognostic value of the relationship between ER-positive status and S6K2 expression on patient survival using the Gene expression based Outcome for Breast cancer Online database. Our results suggest that S6K2 may serve as a novel target for the treatment of ER-positive breast cancers.

Introduction

Breast cancer is a highly heterogeneous disease characterized by molecular differences that dictate treatment and outcome. While triple-negative breast cancers which lack estrogen receptor (ER), progesterone receptor (PR) and HER2/neu pose a challenge for therapy, ER-positive tumors account for approximately 70% of all breast cancers [1]. The availability of anti-estrogen therapies suggests that ER-positive breast cancers are clinically manageable. However, the lack of response [2] and development of endocrine resistance has been a major obstacle in the treatment of this breast cancer subtype [3].

Several studies have reported a relationship between 11q13 amplification and estrogen receptor (ER) positivity in breast cancer [4-7]. Furthermore, among ER-positive tumors, those that harbor 11q13 amplifications have been reported to constitute a high-risk subgroup [4]. This region harbors several important genes such as *CCND1*, *FGF4* and *FGF3* and other less studied ones such as *RPS6KB2*, which codes for the 40S ribosomal protein S6 kinase 2 (S6K2). However, most studies addressing this relationship have investigated *CCND1* as the major candidate gene in this region. Other genes, such as *RPS6KB2*, have been overlooked possibly due to the higher propensity of breast cancer tissue to exhibit copy number gains in such genes rather than amplifications [8].

S6 kinase (S6K) is activated downstream of the phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway [9]. It mediates the mitogen-induced phosphorylation of the 40S ribosomal protein S6 (rpS6), hence is believed to play an important role in protein translation [10,11]. Growth factor-induced activation of the PI3K/Akt pathway results in mTOR activation and subsequent phosphorylation and activation of S6K [12]. It is represented by two highly homologous proteins, S6K1 or

p70S6K and S6K2 or p54S6K encoded by two distinct genes, *RPS6KB1* and *RPS6KB2*, located on chromosomes 17q23 and 11q13, respectively. Gene amplification or copy number gain and overexpression of both genes have been reported in breast cancer [13,8,14-16]. In breast cancer tissues, S6K1 is localized primarily to the cytoplasm and S6K2 is predominantly localized in the nucleus [15,16]. The earliest report addressing the overexpression of S6K2 in breast cancer showed that *RPS6KB2* amplification occurred in 4.3% and gain in 21.3% breast cancers providing valuable information regarding the role of S6K2 as a prognostic indicator in ER-positive tumors [8]. While elegant studies have addressed the regulation of S6K1 in breast cancer cells [17], little is known about the causes and consequences for elevated S6K2 expression in breast cancer.

In the present study, we investigated the relationship between ER positivity and S6K2 overexpression in breast cancer cells and report for the first time that S6K2 is regulated by the estrogen signaling pathway to mediate breast cancer cell survival, and propose S6K2 as a potential novel target for the treatment of ER-positive breast cancers.

Materials and methods

Materials. Human recombinant TRAIL and goat polyclonal antibody against S6K2 was purchased from R&D Systems (Minneapolis, MN). Monoclonal antibody against cyclin D1 and GAPDH were obtained from Santa Cruz Biotechnology (Dallas, TX). 17-β-estradiol (E2), 4-hydroxytamoxifen (tamoxifen) and mouse monoclonal antibody against actin were purchased from Sigma-Aldrich (St Louis, MO). Mouse monoclonal antibody against ERα was purchased from Cell Signaling Technology (Danvers, MA). YO-PRO-1, Alexa-conjugated secondary antibodies and 4', 6-diamidino-2-phenylindole, dihydrochloride (DAPI) were purchased from Molecular Probes/Life Technologies (Carlsbad, CA). Horseradish peroxidase-conjugated goat anti-mouse, and mouse anti-goat antibodies were obtained from Jackson Immuno Research Lab. Inc. (West Grove, PA). Control non-targeting siRNA and siRNA specific for S6K2 were obtained from Dharmacon (Lafayette, CO). siRNA against ERα and compatible non-targeting control siRNA were purchased from Qiagen (Valencia, CA). Polyvinylidene difluoride membrane was from Millipore (Bedford, MA) and enhanced chemiluminescence detection kit was from Amersham (Arlington Heights, IL).

Cell Culture and Transfection. MCF-7 and T47D cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 2 mM glutamine. Cells were kept in a humidified incubator at 370C with 95% air and 5% CO2. For treatment with estradiol, cells were grown in phenol red-free medium supplemented with charcoal-stripped fetal bovine serum prior to the addition of estradiol. siRNA was transfected using Lipofectamine RNAi max transfection reagent according to the manufacturer's protocol (Life Technologies, Carlsbad, CA).

Immunoblot Analysis. Equivalent amounts of total cellular extracts were electrophoresed by SDS-PAGE and transferred electrophoretically to polyvinylidene difluoride membrane. Immunoblot analyses were performed as described before [18].

YO-PRO-1 staining of apoptotic cells. Cells were labeled with 0.5 μM YO-PRO-1 by incubating at 37°C for 15 min and visualized using a Zeiss Axiovert 40 inverted microscope with the AxioVision Rel 4.6 software (Zeiss, Göttingen, Germany).

Immunofluorescence. Cells were fixed with 3% paraformaldehyde, permeabilized with Triton X-100 and incubated overnight with primary antibodies against S6K2 and ERα. The unbound antibody was washed and the cells were incubated with Alexa 488-conjugated donkey anti-goat and Alexa 594-conjugated donkey anti-mouse secondary antibodies. DAPI was used to visualize nucleus and fluorescence images were obtained using a Zeiss Axiovert 40 inverted microscope with the AxioVision Rel 4.6 software (Zeiss, Göttingen, Germany).

Clonogenic cell survival assay. Cells transfected with or without control non-targeting or S6K2 siRNA were treated with or without estradiol and cultured until there were at least 50 cells per colony. At the end of the incubation, the cells were washed with PBS and incubated with 0.025% crystal violet solution for 15 min. Colonies were counted using ImageJ software (NIH) and the plate was photographed using the BioChemi System (BioImaging System, UVP, Upland, CA).

Reverse-transcriptase (RT) PCR analysis. Total RNA was isolated using TRI reagent RT from Molecular Research Center (Cincinnati, OH) as per the manufacturer's protocol and subjected to RT reaction using reverse transcriptase enzyme from Promega (Madison, WI). PCR amplifications were performed using the following primers: S6K2: forward, 5'- GTG

GAA CTG GCC TAT GCC TT-3'; reverse, 5'- GCA GAG TCC AAA GTC GGT CA-3'; GAPDH: forward, 5'-ACT GTG GTC ATG AGT CCT TC-3'; reverse, 5'-GAG CGA GAT CCC TCC AA -3' and ACTIN, forward, 5'-TAC AAT GAG CTG CGT GTG GCT-3'; reverse, 5'-ATC CAC ATC TGC TGG AAG GTG GA-3' For traditional PCR, cycle conditions were set up for 25 cycles as follows: denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, extension at 72°C for 1 minute, and final extension at 72°C for 10 minutes. PCR products were resolved on 2% agarose gel containing ethidium bromide. Quantitative real-time PCR analysis was performed using the SYBR green method in a Mastercycler Realplex 2 from Eppendorf (Hauppauge, NY).

Statistical analysis. Data are presented as mean ± S.D. and n = 3. Statistical significance was determined by paired Student's t-test using Microsoft Excel. A p-value < 0.05 was considered statistically significant.

Results

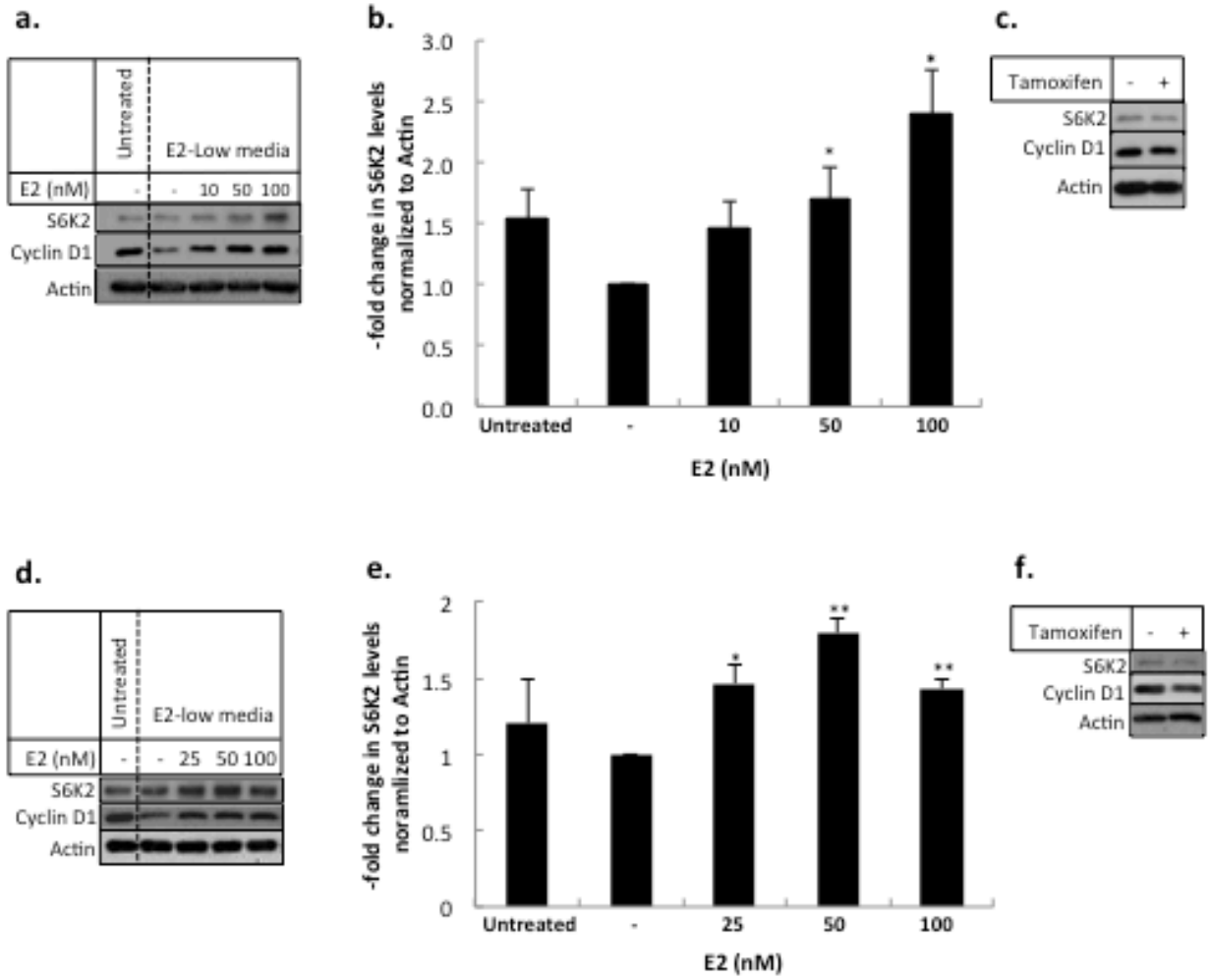
Estradiol upregulates S6K2 protein levels

Studies reporting the correlation between *RPS6KB2* amplification and ER positivity suggest that there exists a relationship between estrogen signaling and S6K2 at the cellular level. Therefore, we examined if the estrogen signaling pathway played a role in regulating S6K2 levels in breast cancer cells. Figure 1 shows that the treatment of ER-positive MCF-7 (Fig. 1a and 1b) and T47D (Fig. 1d and 1e) breast cancer cells with 17-β-estradiol (E2) increased cyclin D1, a protein previously reported to be regulated by estrogen [19]. E2 also led to a concentration-dependent increase in S6K2 levels with a maximum induction at 100 nM, the highest concentration tested in MCF-7 (Fig. 1a and 1b) and 50 nM in T47D (Fig. 1d and 1e) cells. The treatment of MCF-7 (Fig. 1c) and T47D (Fig. 1f) cells with 4-hydroxytamoxifen, the estrogen receptor antagonist, failed to increase S6K2 levels. These results suggest that S6K2 is positively regulated by the estrogen signaling pathway.

Figure 1

Estradiol upregulated S6K2 protein levels. MCF-7 (a, b, c) or T47D cells (d, e, f) were grown in either complete media (untreated) or in phenol red free media supplemented with charcoal-stripped FBS and treated with indicated concentrations of estradiol (E2) (a, d) or 4-hydroxytamoxifen (tamoxifen) (c, f). Western blot analysis was performed with indicated antibodies. b, e, densitometric quantification of results from 3 independent experiments. *, p-value < 0.05, **, p-value < 0.01.

Figure 1


a.
Untreated
E2-Low media
E2 (nM)
- - 10 50 100
S6K2
Cyclin D1
Actin
b.
-fold change in S6K2 levels normalized to Actin
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Untreated
-
10
50
100
E2 (nM)
c.
Tamoxifen - +
S6K2
Cyclin D1
Actin
d.
Untreated
E2-low media
E2 (nM)
- - 25 50 100
S6K2
Cyclin D1
Actin
e.
-fold change in S6K2 levels noramlized to Actin
2
1.5
1
0.5
0
Untreated
-
25
50
100
E2 (nM)
f.
Tamoxifen - +
S6K2
Cyclin D1
Actin

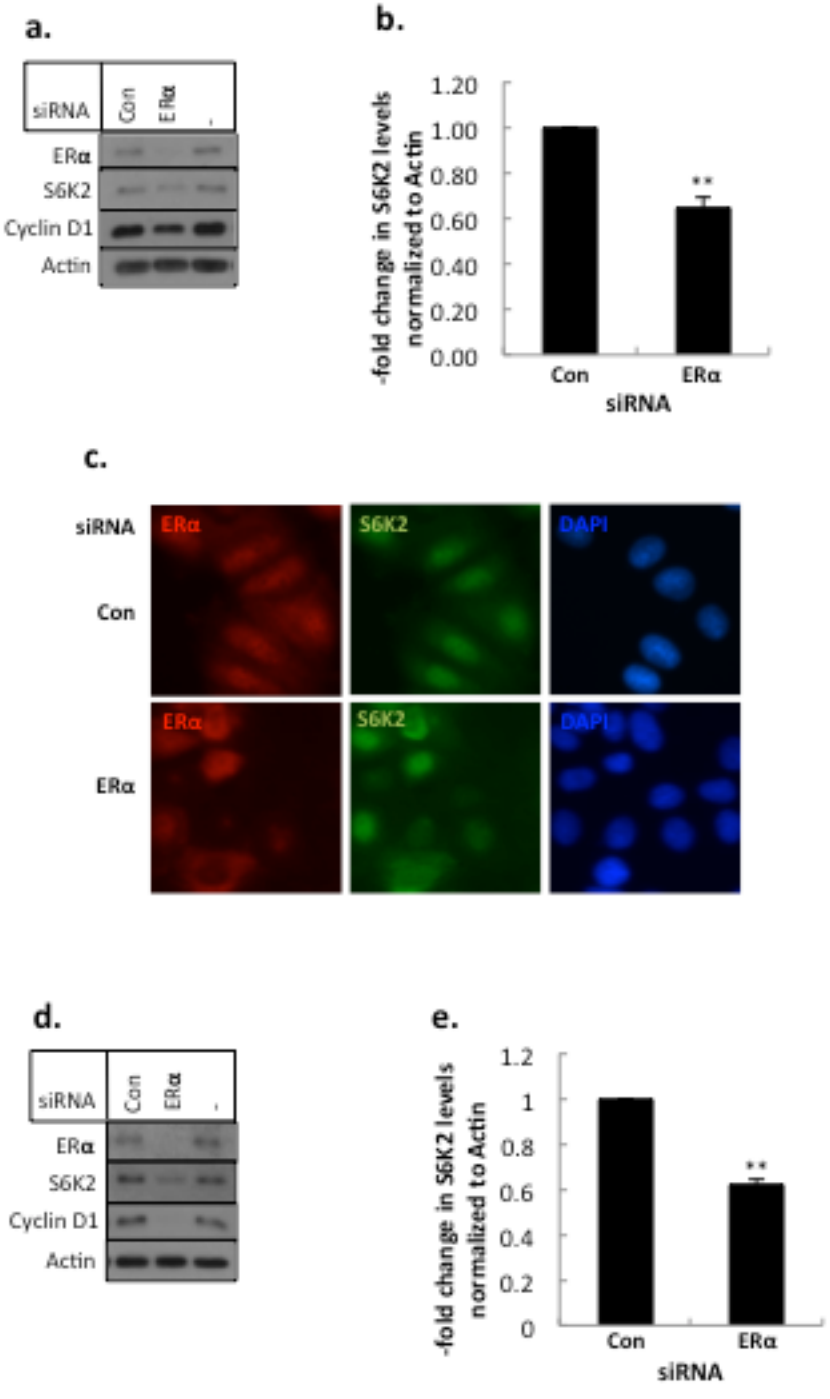
ERα depletion decreases S6K2 protein levels

Since S6K2 levels were increased by estradiol treatment, we next examined if the depletion of ERα would decrease S6K2 protein levels. Consistent with previous reports, the knockdown of ERα decreased cyclin D1 protein levels in MCF-7 (Fig. 2a) and T47D (Fig. 2d) cells. Furthermore, ERα depletion significantly decreased S6K2 protein levels in both MCF-7 (Fig. 2a, and 2b) and T47D (Fig. 2d and 2e) cells. Similar results were obtained by immunofluorescence studies of MCF-7 cells following ERα depletion (Fig 2c). ERα and S6K2 were detected using secondary antibodies conjugated to Alexa-594 (red) and Alexa-488 (green), respectively. Figure 2c shows that knockdown of ERα (decrease in red staining) in cells was accompanied by a decrease in S6K2 (green staining).

Figure 2

ERα depletion decreased S6K2 protein levels. MCF-7 (a, b, c) or T47D (d, e) cells were transfected with indicated siRNAs and Western blot analysis (a, d) or immunofluorescence staining (c) with indicated antibodies was performed. DAPI (blue) was used to visualize the nucleus. b, e, densitometric quantification of results from 3 independent experiments. **, p-value < 0.01.

Figure 2


a.
siRNA
Con
ERα
ERα
S6K2
Cyclin D1
Actin
b.
-fold change in S6K2 levels normalized to Actin
1.20
1.00
0.80
0.60
0.40
0.20
0.00
**
Con
ERα
siRNA
c.
siRNA
ERα
S6K2
DAPI
Con
ERα
d.
siRNA
Con
ERα
ERα
S6K2
Cyclin D1
Actin
e.
-fold change in S6K2 levels normalized to Actin
1.2
1
0.8
0.6
0.4
0.2
0
**
Con
ERα
siRNA

Regulation of S6K2 by estrogen is at the post-transcriptional level

ERα is a transcription factor known to regulate several proteins that promote cell cycle, cell growth and survival by binding to estrogen response elements on their promoters and inducing or inhibiting their expression [20]. Hence, we sought to determine if S6K2 was regulated by ERα at the transcriptional level. Figure 3 shows that the depletion of ERα failed to decrease S6K2 mRNA levels in MCF-7 (Fig. 3a and 3b) and T47D cells (Fig. 3d and 3e) as determined by RT-PCR (Fig. 3a and 3d) and quantitative real-time PCR (Fig. 3b and 3e). Similarly, the treatment with estradiol did not affect S6K2 mRNA levels in MCF-7 (Fig. 3c) and T47D (Fig. 3f) cells. Thus, S6K2 is regulated post-transcriptionally by the estrogen signaling pathway.

It has been reported that S6 kinases can be ubiquitinated and degraded via the proteasomal pathway [21]. Therefore, we examined if post-transcriptional regulation of S6K2 by ERα involves prevention of S6K2 degradation by the ubiquitin proteasome-mediated pathway. However, as seen in Figure 4, proteasomal inhibitor MG132 failed to rescue S6K2 levels in MCF-7 (Fig. 4a) and T47D (Fig. 4b) cells.

Figure 3

Regulation of S6K2 by estrogen is at the post-transcriptional level. MCF-7 (a) or T47D (d) cells were transfected with either control non-targeting siRNA or siRNA against ERα followed by reverse transcriptase PCR (RT-PCR) and quantitative real-time PCR analysis by the SYBR green method (b, MCF-7 and e, T47D). Results are representative of 3 independent experiments. MCF-7 (c) or T47D (f) cells were cultured in phenol-red free media supplemented with charcoal-stripped FBS and treated with estradiol (E2) followed by RT-PCR analysis. N.S, not significant.

Figure 3

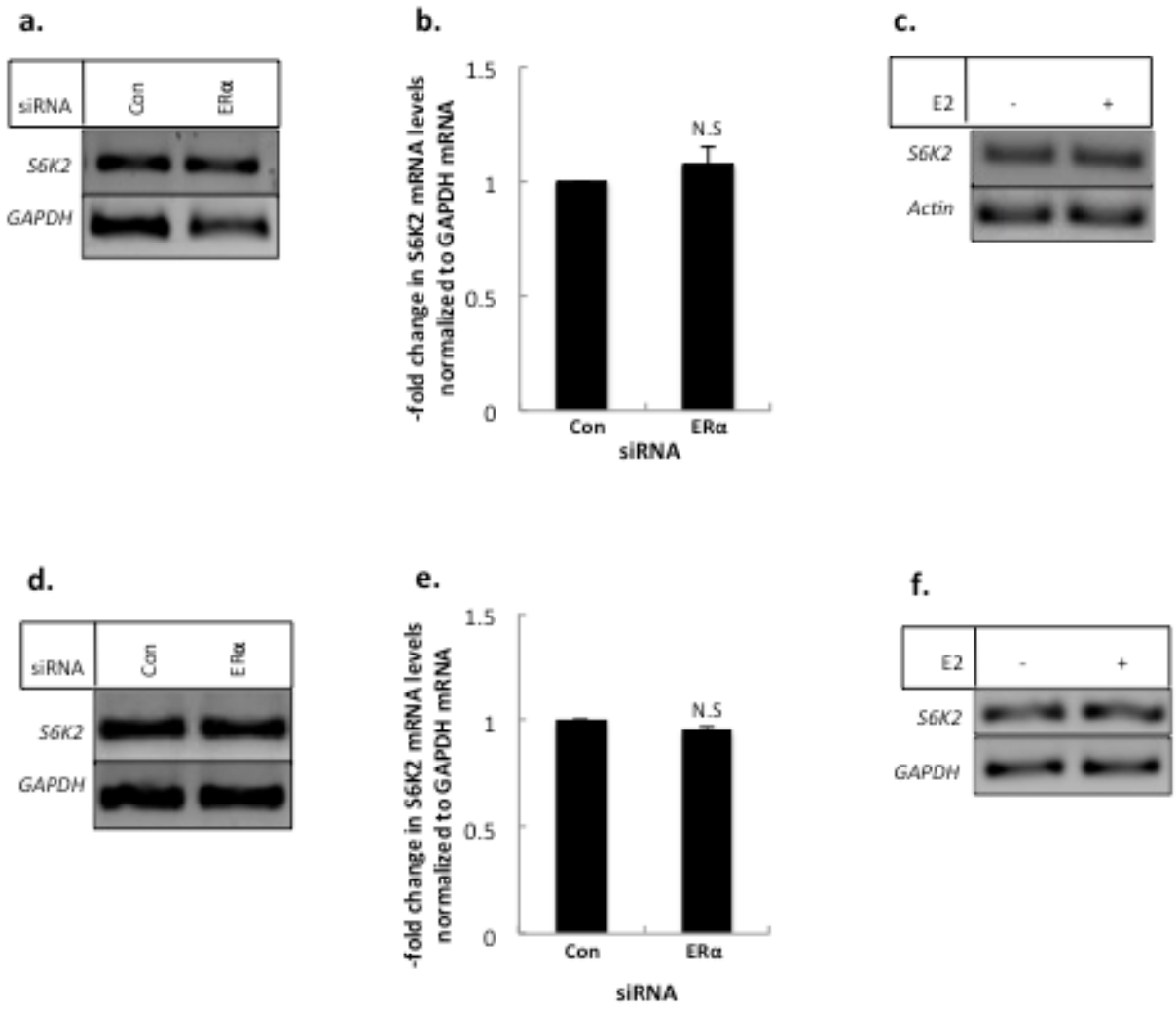
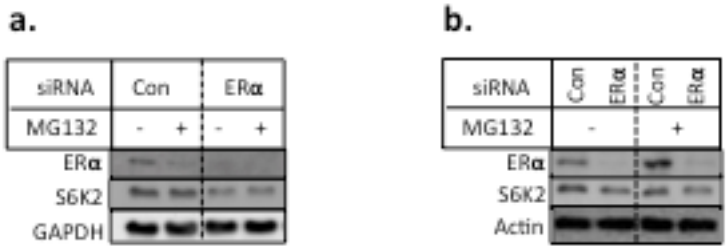

a.
siRNA
Con
ERα
S6K2
GAPDH
b.
-fold change in S6K2 mRNA levels normalized to GAPDH mRNA
1.5
1
0.5
0
N.S
Con
ERα
siRNA
c.
E2
-
+
S6K2
Actin
d.
siRNA
Con
ERα
S6K2
GAPDH
e.
-fold change in S6K2 mRNA levels normalized to GAPDH mRNA
1.5
1
0.5
0
N.S
Con
ERα
siRNA
f.
E2
-
+
S6K2
GAPDH

Figure 4

Regulation of S6K2 by ERα is independent of the proteasome pathway. MCF-7 (a) or T47D (b) cells transfected with either control non-targeting or ERα siRNA were treated with or without 10 μM MG132 and Western blot analysis was performed with indicated antibodies.

Figure 4


a.
siRNA
Con
ERα
MG132
- + - +
ERα
S6K2
GAPDH
b.
siRNA
Con
ERα
Con
ERα
MG132
- +
ERα
S6K2
Actin

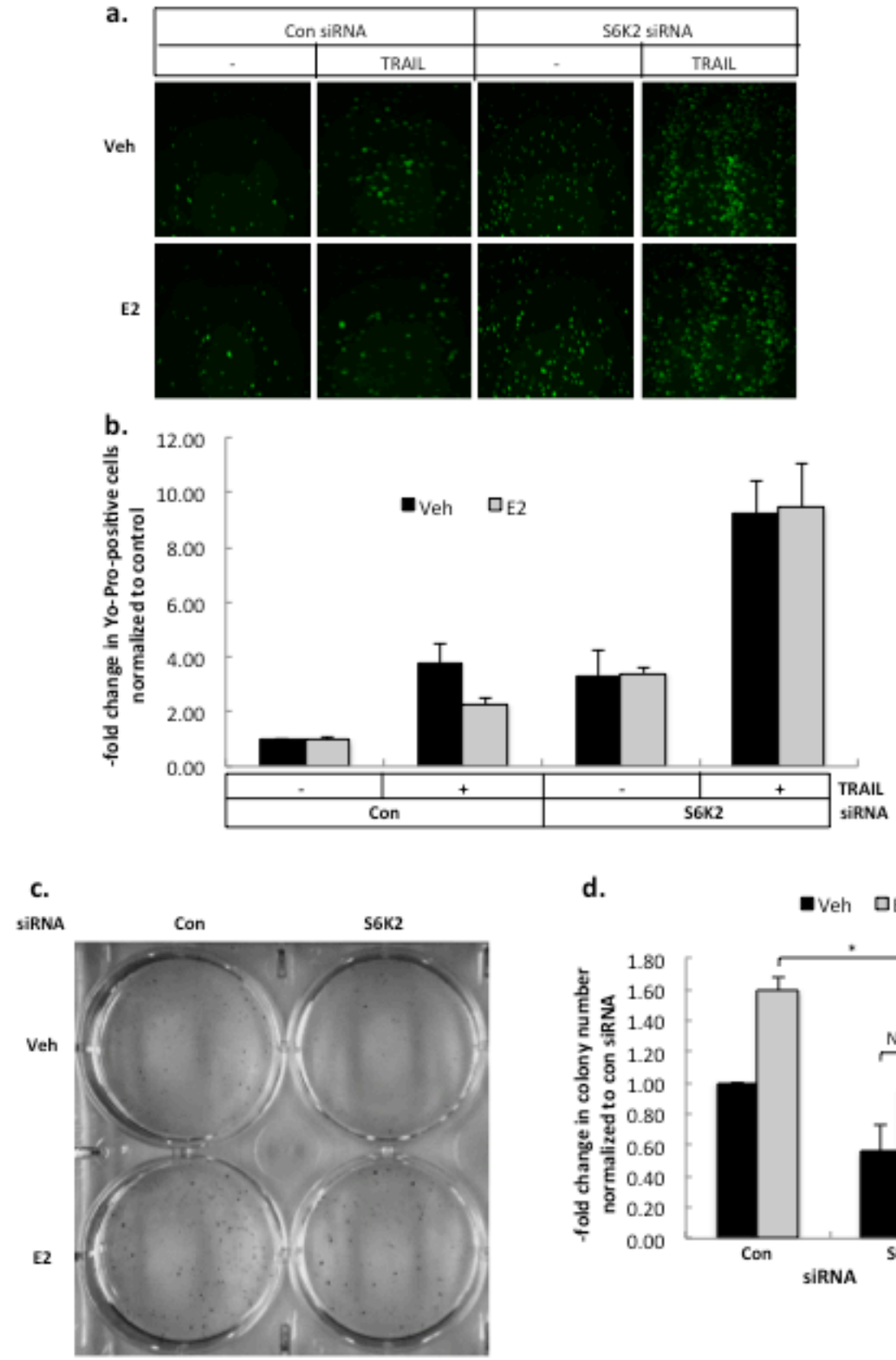
S6K2 depletion decreased estradiol-induced cell survival

We have previously reported that S6K2 promoted survival of breast cancer cells in response to apoptotic stimuli [22]. Therefore we sought to determine if estrogen could promote cell survival via S6K2. We treated T47D cells with TRAIL and stained with the dye YO-PRO-1, to which apoptotic cells are permeable. Figure 5a shows that the depletion of S6K2 enhanced apoptotic cell death in response to TNF-related apoptosis inducing ligand (TRAIL), and treatment of T47D cells with estradiol decreased apoptosis as determined by YO-PRO-1 staining. However, the depletion of S6K2 blunted the ability of estradiol to decrease cell death, suggesting that estradiol promoted cell survival via S6K2 (Fig. 5b). We also performed a clonogenic cell survival assay in MCF-7 cells to determine the effect of S6K2 depletion on estradiol-induced increase in clonogenicity. Figure 5c and 5d show that while E2 treatment substantially increased the number of colonies compared to untreated control, S6K2 knockdown significantly decreased it. These results suggest that estrogen mediates its prosurvival effects at least partly via S6K2.

Figure 5

S6K2 depletion decreased estradiol-induced cell survival. a, T47D cells were transfected with either control non-targeting siRNA or siRNA against S6K2 and treated with estradiol (E2) followed by TRAIL. Cells were then subjected to YO-PRO analysis to detect apoptotic cell death. b, quantification of results from a. Results are representative of 2 independent experiments. c, MCF-7 cells were transfected with either control non-targeting siRNA or siRNA against S6K2 and treated with estradiol (E2) followed by crystal violet staining to visualize colonies. d, quantification of results form c. *, p-value < 0.05, N.S, not significant.

Figure 5


a.
Con siRNA
S6K2 siRNA
-
TRAIL
-
TRAIL
Veh
E2
b.
-fold change in Yo-Pro-positive cells normalized to control
12.00
10.00
8.00
6.00
4.00
2.00
0.00
Veh
E2
-
+
-
+
TRAIL
Con
S6K2
siRNA
c.
siRNA
Con
S6K2
Veh
E2
d.
Veh
E2
-fold change in colony number normalized to con siRNA
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
*
N.S
Con
S6K2
siRNA

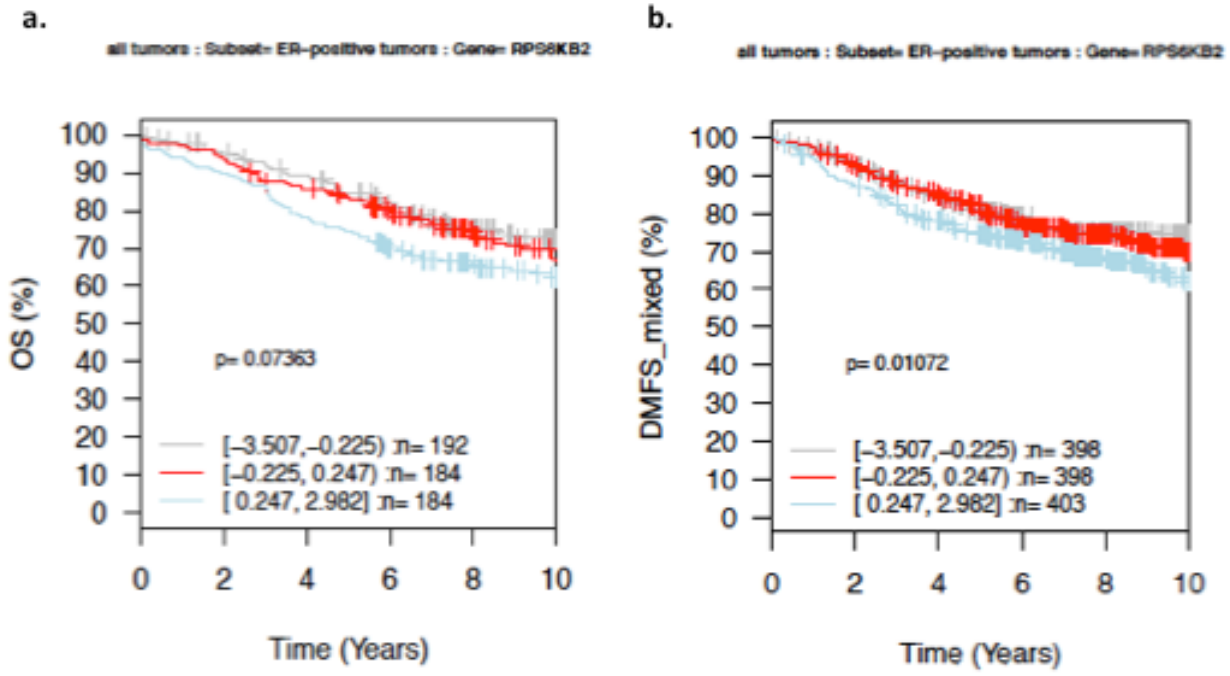
Higher RPS6KB2 expression in ER-positive breast cancer patients indicates poor prognosis

Since we observed that S6K2 is regulated via the estrogen signaling pathway in breast cancer cell lines, we examined if there existed a relationship between S6K2 mRNA levels and the outcome in patients presenting with ER-positive breast cancers. Using the Gene expression based Outcome for Breast cancer Online (GOBO) [23] database we observed that in ER-positive tumors, high S6K2 expression was associated with decreased overall survival (OS) (Fig. 6a) and distant metastasis-free and relapse-free survival (DMFS_mixed) (Fig. 6b). These observations suggest that S6K2 may play a role in anti-estrogen resistance, recurrence and metastasis downstream of ERα in breast cancer.

Figure 6

Higher RPS6KB2 expression in ER-positive breast cancer patients indicates poor prognosis. GOBO analysis showing Kaplan-Meier plots, using either overall survival (OS; a) or distant metastasis- and recurrence-free survival (DMFS_mixed; b) as endpoint, for ER-positive tumors stratified into the three levels based on *RPS6KB2* gene expression.

Figure 6


a.
all tumors : Subset= ER−positive tumors : Gene= RPS6KB2
OS (%)
p= 0.07363
[−3.507,−0.225) n= 192
[−0.225, 0.247) n= 184
[0.247, 2.982] n= 184
Time (Years)
b.
all tumors : Subset= ER−positive tumors : Gene= RPS6KB2
DMFS_mixed (%)
p= 0.01072
[−3.507,−0.225) n= 398
[−0.225, 0.247) n= 398
[0.247, 2.982] n= 403
Time (Years)

Discussion

The results of our present study demonstrate for the first time that S6K2 levels are regulated via the estrogen signaling pathway and that it promotes cell survival downstream of estrogen. While several studies have addressed the regulation of S6K2 activity, little is known about the causes for its elevated levels often observed in breast cancer cells and tissue specimens. Given the role of S6K2 as a prognostic marker in ER-positive tumors, we hypothesized that its levels were regulated via the ER signaling pathway in breast cancer and show that there exists a functional relationship between estrogen signaling and S6K2 in breast cancer cells.

While ERα depletion decreased S6K2 protein, we failed to see a change in its mRNA levels. This is consistent with a previous study, which reports that S6K2 mRNA levels do not correlate with ER-positive status [14]. Since S6Ks have been reported to be regulated via ubiquitination and proteasomal degradation [21], we also considered the possibility that estrogen promotes the stabilization rather than expression of S6K2. However, we failed to observe any effect of the proteasome inhibitor MG132 on S6K2 levels. This discrepancy is possibly due to the differences in the cell lines used and the status of mediators of ubiquitination and proteasomal degradation in cancer cells.

ERα is primarily a transcription factor, which regulates its targets by binding to estrogen response elements on their promoters [20]. However, there are reports that indicate that it interacts with translation factors implying its involvement in translational regulation. For example, ERα has been shown to interact with translation factors such as the members of the eukaryotic translation initiation complex [24,25]. Hence, it is conceivable that ERα regulates S6K2 at the translational level. Therefore, the non-genomic functions of estrogen

and ERα need to be considered in order to fully understand its role in mediating breast cancer pathogenesis.

Regardless of how estrogen signaling upregulates S6K2 level, our results suggest that targeting S6K2 may be a viable option for the treatment of ER-positive breast cancers. We have previously reported that S6K2 promotes cell survival in breast cancer cells via Akt [22], which has been associated with endocrine resistance [26,27], and knockdown of S6K2 enhanced breast cancer cell death in response to several chemotherapeutic agents ([22] and data not shown). Here, we show that the depletion of S6K2 decreased estradiol-induced increase in colony numbers and enhanced cell death in response to TRAIL, suggesting that S6K2 promotes breast cancer cell survival in response to estrogen. Nonetheless, in tumors that do not express ERα, it is possible that S6K2 levels are maintained by the activation of alternative pathways. Such redundancy has been well documented in different scenarios in breast cancer [28]. Thus, while S6K2 regulated cell survival downstream of estradiol in ER-positive breast cancer cells, it may also function independent of estrogen signaling to mediate its effects in breast cancer.

Although the availability of anti-estrogen therapy implies a favorable prognosis for ER-positive breast cancer patients, the development of resistance to such therapy poses a challenge in treating this subtype, which accounts for the majority of all breast cancers. While the lack of ER is the most common mechanism of de novo anti-estrogen resistance, a complete loss of ER is not common in acquired resistance, suggesting that signaling downstream of ER may contribute to endocrine resistance [29]. Also, a large number of ER-positive breast tumors exhibit resistance even prior to the time of initial diagnosis [29]. Based on GOBO database analysis, higher S6K2 expression was observed more frequently

after 5 years of initial diagnosis, which is typically the time associated with recurrence of ER-positive breast cancers. Recurrence, which is almost always associated with metastasis, remains the major cause of death [30]. Given the observation that among ER-positive breast cancers, those that exhibit 11q13 amplifications constitute a high-risk subtype [4,5] and the significant correlation between *CCND1* amplifications and *RPS6KB2* gains and amplifications [8], it is possible that S6K2 is one of the mediators of anti-estrogen resistance, relapse and metastasis.

Acknowledgements

We thank Ms. Smrithi Rajendiran for help with the quantitative real-time PCR experiments. This study was supported by the pre-doctoral bridge funding (SS) from the UNT Health Science Center.

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CHAPTER IV

Summary and conclusions

The results outlined in the present study address the role and regulation of S6K2 in breast cancer. While the overexpression of S6K2 in breast cancer tissues was reported (1-4), its causes and consequences remained unknown. The objective of this study was to address these gaps in the knowledge about S6K2 and to aid in characterizing it as a suitable target for breast cancer treatment.

Our results indicate that S6K2 plays an important role in mediating breast cancer cell survival. We observed that the depletion of S6K2 sensitized breast cancer cells to apoptotic agents such as TNF and TRAIL as shown here as well as several chemotherapeutic agents routinely used in the clinic such as paclitaxel, cisplatin and doxorubicin.

On exploring the mechanism by which S6K2 mediates its prosurvival effects, we observed that unlike S6K1 (5-8), S6K2 depletion decreased the activation of the prosurvival

protein Akt. This is in contrast to the widely accepted belief that S6K2 like S6K1 negatively regulates the PI3K/Akt signaling pathway. This is an important observation since the suppression of Akt activity is key to achieving cancer cell death and activation of Akt by inhibition of S6K1 limits the therapeutic efficacy of mTORC1 inhibitors. We further observed that S6K2 depletion enhanced receptor-mediated apoptotic cell death by TNF via the mitochondrial pathway by upregulating p53 and the pro-apoptotic Bcl-2 family member Bid. The engagement of the mitochondrial or intrinsic cell death pathway is critical in cancer cells since they require the activation of both pathways for effective killing by an extrinsic agent (9).

Following the observation that S6K2 is critical for the survival of breast cancer cells in response to apoptotic agents, we looked into the pathways that mediate its overexpression. Due to the correlation between positive ERα status and the amplification of chromosomal region that codes for S6K2 (10), and the observation that S6K2 gains in ER-positive breast cancers are associated with poor outcome (1), we studied the relationship between ER signaling and S6K2 levels. Our results demonstrate that S6K2 is indeed regulated by the estrogen signaling pathway via a post-transcriptional mechanism. We then determined the role of S6K2 in mediating survival downstream of estrogen signaling and observed that the depletion of S6K2 blunted estrogen-induced cell survival and clonogenicity. Furthermore, analysis of breast cancer tissue data revealed that the overexpression of S6K2 in ER-positive tumors indicated poor prognosis in patients. These findings suggest that S6K2 could serve as a potential target in the treatment of ER-positive breast cancers.

In summary, this study has demonstrated that S6K2 (i) is regulated via the estrogen signaling pathway in ER-positive breast cancers; (ii) plays an important role in mediating

breast cancer cell survival; (iii) is functionally divergent from S6K1 and (iv) mediates its effects on breast cancer cell survival via the regulation of the prosurvival protein Akt and proapoptotic proteins p53 and Bid as indicated in figure 1.

While inhibitors have been developed against mTOR and S6K1, there are no molecules that can selectively target S6K2 till date. This is possibly due to a dearth in studies that address its importance in cancer, the high degree of homology between S6K1 or S6K2 and the lack of structural information that is key for the development of novel and specific inhibitors. However, homology modeling of S6K2 based on the crystal structure of S6K1 bound to staurosporine identified a divergence in the region that comes in contact with the inhibitor (11). This region was narrowed down to cysteine 150 within the hinge region of S6K2, suggesting that it is indeed possible to develop S6K2-specific inhibitors. Furthermore, the differences between S6K1 and S6K2 in their N- and C-terminal domains could be exploited to develop S6K2-specific inhibitors.

Given the lack of obvious phenotypes in S6K2$^{-/-}$ mice (12), targeting S6K2 for breast cancer therapy will aid in enhancing chemotherapy-induced breast cancer cell death and is expected to be associated with minimal normal tissue effects. Moreover, this study has the potential of being extended to other cancers such as endometrial (13) and gastric cancers (14) which also show elevated S6K2 expression and gene amplification.

Figure 1

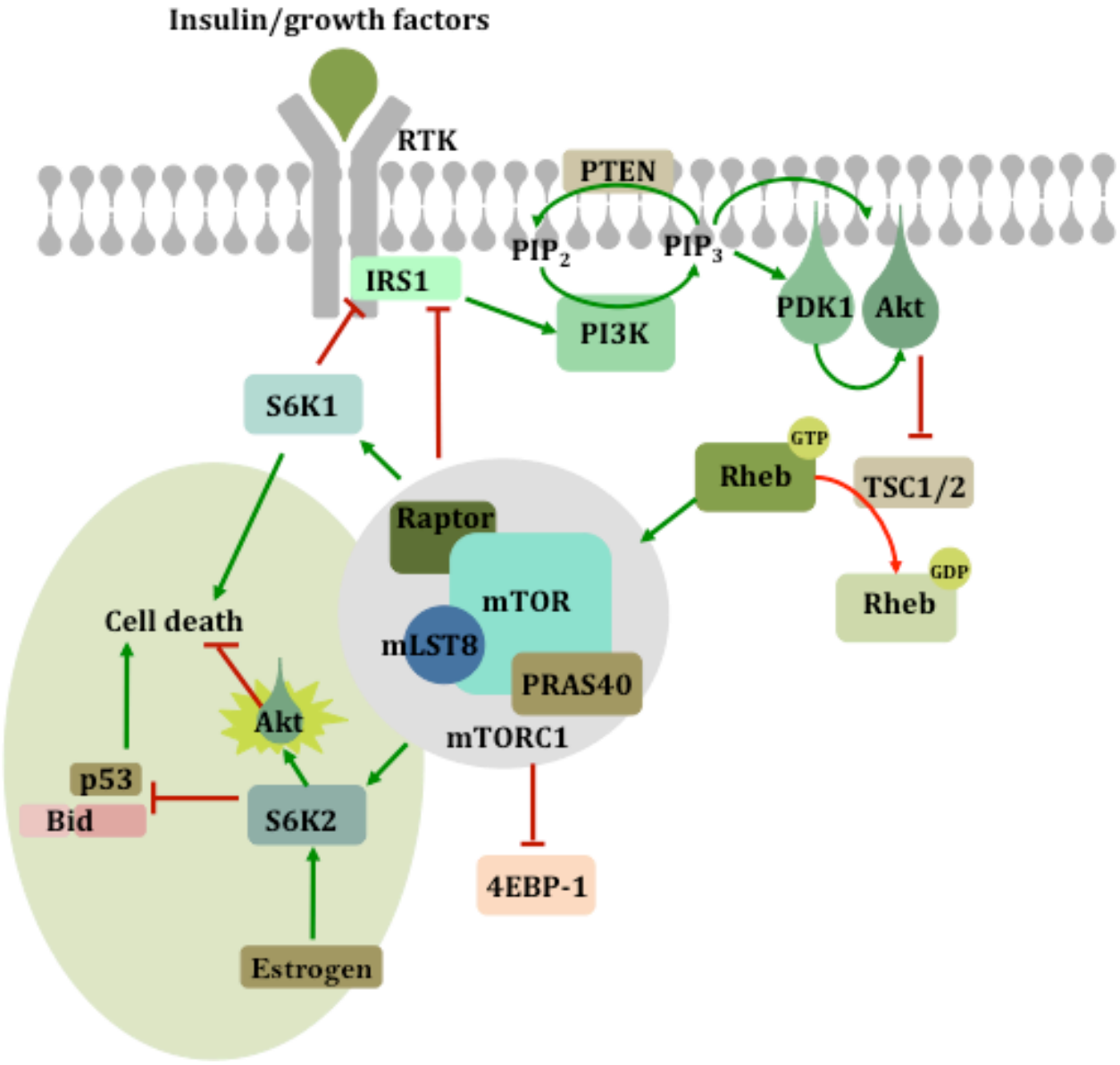

Fig. 1. Schematic summarizing the conclusions from the study.

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CHAPTER V

Future directions

We have reported here that S6K2 levels are regulated post-transcriptionally via the estrogen signaling pathway. It remains to be determined if it is upregulated by estrogen via a translational mechanism or a process involving micro RNAs, non-coding RNAs that regulate gene expression by translational repression or degradation of their target mRNAs.

Although highly homologous, S6K1 and S6K2 have opposing effects on breast cancer cell survival. The differences in their N- and C-terminal regions may result in interaction with distinct proteins ultimately leading to differential functions. Hence identifying unique binding partners of S6K2 may reveal the mechanisms by which it promotes cell survival.

While we have investigated the role of S6K2 in cell survival, it could potentially mediate other tumorigenic processes in breast cancer. For example, the interaction of S6K2 with heterogenous nuclear ribonucleoproteins (hnRNPs) is believed to play a role in cell proliferation. hnRNPs are important regulators of RNA processing, maturation and nuclear export which are global phenomena governing gene expression. However the role of S6K2 in this process is yet to be explored. Similarly, analysis of breast cancer tissue data suggests

that elevated S6K2 expression is associated with a decrease in distant metastasis- and relapse-free survival in patients. This indicates that S6K2 may play a role in mediating metastatic spread of breast cancer cells or maintenance of the metastases. It would be interesting to determine if S6K2 is involved in processes such as epithelial-to-mesenchymal transition (EMT), migration and invasion of breast cancer cells. These studies will advance our knowledge on the role and regulation of S6K2 and aid in unequivocally establishing it as a target for the treatment of breast cancer.