S6K1 Plays a Key Role in Glial Transformation

Jean L. Nakamura, Edna Garcia, and Russell O. Pieper

Departments of Radiation Oncology and Neurological Surgery, University of California, San Francisco, California

Abstract

The mammalian target of rapamycin (mTOR) is a nutrient and ATP sensor suggested to play an important role in tumorigenesis, particularly in the setting of PTEN loss or activated Akt/PKB. Of mTOR's two known effectors, eIF4E has been implicated in tumorigenesis, whereas the role of S6 kinase (S6K1) in transformation is less understood. To assess the contribution of S6K1 to the transformed phenotype, we pharmacologically and genetically manipulated the mTOR-S6K pathway in glioma cells and monitored its effects on growth in soft agar, a hallmark of cellular transformation, and also assessed in vivo intracranial growth. Anchorage-independent growth by HRasV12-transformed human astrocytes as well as by U251 and U373 human glioma cells was inhibited by pharmacologic mTOR inhibition. Similarly, short hairpin RNA-mediated suppression of S6K1 in HRasV12-transformed human astrocytes, U251, and U373 cells failed to rescue colony formation, although expression of wild-type S6K1 or rapamycin-resistant S6K1 in rapamycin-treated U373 and U251 provided partial rescue. Consistent with the latter observation, small interfering RNA-mediated suppression of S6K1 in HRasV12-transformed human astrocytes, U251, and U373 cells resulted in a significant loss of anchorage-independent growth. Further, we found that in vivo short hairpin RNA-mediated suppression of S6K1 in HRasV12-transformed human astrocytes reduced intracranial tumor size, in association with reduced tumor levels of phosphorylated ribosomal protein S6. These findings implicate the mTOR-S6K6 pathway as a critical mediator of glial cell transformation. [Cancer Res 2008;68(16):6516–23]

Introduction

The mammalian target of rapamycin (mTOR) is an ATP and nutrient sensor that contributes to the control of cell size and cell cycle progression. mTOR's ability to control cell size and cell cycle is due at least in part to its ability to regulate the translation of specific classes of mRNAs. mTOR-mediated control of translation is a rapamycin-sensitive process accomplished by regulation of the downstream targets, S6K and eIF4E (1–3). mTOR phosphorylates S6K, leading to the phosphorylation of the ribosomal protein S6 and subsequent increased translation of mRNA with 5' terminal oligopyrimidine sequences (2). In contrast, inhibition of eIF4E by the translational repressor 4EBP1 is reversed when 4EBP1 is phosphorylated by mTOR, resulting in the release of eIF4E, which can associate with eIF4A, eIF4G, and eIF4B to initiate the translation of capped mRNA (4).

The rapamycin-sensitive translational functions mediated by S6K and 4EBP1 have recently been recognized to be a result of mTOR's interaction with raptor to form the mTORC1 complex, whereas rapamycin-insensitive functions are a result of mTOR's interaction with rictor, forming mTORC2 (5–9). It remains to be determined how regulation of mTOR by raptor and rictor is coordinated, although each seems to control distinct and mutually exclusive mTOR functions, mTORC1, but not mTORC2, activates S6K, which can then inhibit insulin receptor substrate-1 (IRS-1), thereby limiting insulin receptor-mediated signaling through phosphoinositide-3-kinase (PI3K), mTORC2, in contrast, has recently been shown to phosphorylate PKB at Ser473, thereby functioning as a PDK-2 (7).

Substantial indirect evidence indicates that mTOR fulfills a central role in tumor development and maintenance. Oncogenic signaling through a variety of molecules, such as the epidermal growth factor receptor, Ras, and PI3K, can up-regulate mTOR activity and promote neoplastic growth (10). Tumors lacking normal Akt control mechanisms have also been shown to be particularly vulnerable to mTOR inhibition (11) and evidence of elevated mTOR activity can be found in multiple types of tumors (12), including malignant gliomas (13). These findings have led to the idea that mTOR plays a role in tumor maintenance, and to the development of mTOR inhibitors as systemic therapy against a wide range of malignancies.

Despite evidence of a link between mTOR, S6K, and eIF4E in response to growth factor activation, it is unclear whether S6K and/or eIF4E connect mTOR to tumor development and growth. Evidence from model systems has implicated eIF4E and S6K in tumor development in specific oncogenic contexts. For example, overexpression of eIF4E has been shown to transform rat fibroblasts in cooperation with v-myc or E1A, and in vivo eIF4E overexpression causes the development of lymphomas, angiosarcomas, lung adenocarcinomas, and hepatocellular adenomas (14–17). Overexpression of eIF4E has been shown to transform rat fibroblasts in cooperation with v-myc or E1A, and in vivo eIF4E overexpression causes the development of lymphomas, angiosarcomas, lung adenocarcinomas, and hepatocellular adenomas (14–17). Inhibition of cap-binding by eIF4E also suppresses eIF4E-driven transformation (15). Although S6K has not been described as an oncoprotein, phosphorylated S6 protein levels are elevated in various tumor types, including malignant glioma (13, 18), and translational targets of S6K such as HIF1 are sensitive to mTOR inhibition, and expression of HIF1α seems to be critical in supporting tumor growth (19). Tumors with elevated HIF1α are sensitive to mTOR inhibition, and expression of HIF1α 5'-TOP sequences confers sensitivity to the mTOR inhibitor CCI-779 (20). Recent data also indicates that inhibition of angiogenesis by the tumor suppressor promyelocytic leukemia protein is in part dependent on its ability to inhibit mTOR and the synthesis of HIF1α (21). Although these data suggest that eIF4E and S6K may directly mediate transformation through mTOR, amplification or mutation of eIF4E or S6K has not been found in spontaneously arising tumors, nor is mTOR itself thought to be an oncogene. Thus, the contribution of eIF4E and S6K to mTOR-dependent glial transformation remains open.

Requests for reprints: Jean L. Nakamura, Department of Radiation Oncology, University of California, San Francisco, 505 Parnassus Avenue, Long Hospital L-75, San Francisco, CA 94143. Phone: 415-353-9694; Fax: 415-353-9883; E-mail: jnakamura@radonc.ucsf.edu. ©2008 American Association for Cancer Research. doi:10.1158/0008-5472.CAN-07-6188

In order to test whether mTOR-dependent transformation requires both eIF4E and S6K functions, we genetically and pharmacologically manipulated mTOR and its downstream effectors and monitored its effects on the transformation status of human glioma cell lines and transformed human astrocytes. We found that suppression of mTOR or raptor was sufficient to significantly reduce anchorage-independent growth in soft agar, an assay of transformation. Furthermore, S6K1, but not eIF4E, rescued glioma growth in soft agar from rapamycin-mediated suppression, and transient S6K1 inhibition was sufficient to significantly reduce glioma growth in soft agar. In vivo S6K1 suppression in intracranially implanted glioma xenografts reduced levels of phosphorylated S6 and also resulted in reduced intracranial tumor growth. This data is the first direct demonstration of S6K's importance in supporting tumor growth both in vitro and in vivo. Collectively, these findings define a significant role for the mTOR-raptor (mTORC1)-S6K pathway in supporting gliomagenesis.

Materials and Methods

Cells and cell culture. Human glioma cell lines were obtained from the Brain Tumor Research Center Tissue Bank at the University of California, San Francisco. Immortalized human astrocytes and HRasV12- and HRasV12/Akt-transformed human astrocytes (E6/E7/HRasV12 and E6/E7/HTert/HRasV12/Akt, respectively) were generated as previously described (22, 23). All cell lines were grown in DMEM (4,500 mg/L glucose; Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Life Technologies, Invitrogen), penicillin, and streptomycin. Cells were grown in a humidified incubator containing 8% carbon dioxide at 37°C.

Proliferation assay. To assess cell proliferation, the 3-(4-5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) assay was used (CellTiter96; Promega). Cells were plated in triplicate to 96-well plates at a concentration of 2,000 cells/well (100 μL/well). At specified time points, 20 μL of MTS reagent was added to each well and allowed to incubate for 1 h. Absorbance (490 nm) was then determined in a 96-well plate reader.

Plasmids, transfection, and selection of cells. A pCAN1 vector encoding wt-eIF4E was a gift from Frank McCormick (University of California, San Francisco, San Francisco, CA). To generate a construct permitting wt-eIF4E expression with a unique selectable marker in E6/E7/HTert/HRasV12 and E6/E7/HTert/HRasV12/Akt, respectively, were generated as previously described (22). All cell lines were grown in DMEM supplemented with 8% fetal bovine serum (Life Technologies, Invitrogen), penicillin, and streptomycin. Cells were grown in a humidified incubator containing 8% carbon dioxide at 37°C.

Immunoblot analysis. Cells were harvested by trypsinization and washed in cold PBS. Proteins were extracted from cells using RIPA buffer with protease and phosphatase inhibitors (Roche Applied Science). Lysates were centrifuged (12,000 g) for 10 min at 4°C. Equal amounts of protein were separated on SDS-PAGE 12% to 16% gels, then transferred to Immobilon-P membranes (Millipore). Membranes were blocked with 5% nonfat dry milk in TBS containing 0.1%Tween 20 and incubated with specific antibodies overnight at 4°C. Membranes were washed in TBS containing 0.05% Tween 20 and incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Signals were detected by chemiluminescence using ECL Western blotting detection reagent (GE Healthcare). Antibodies used included anti-phospho- S6 (Ser235/236; Cell Signaling), anti-S6, anti-4E-BP1, and anti-α-tubulin (Santa Cruz Biotechnology).
immunofluorescent staining as described previously (25). Images were captured and merged using Openlab (Improvision).

**Statistics.** Statistical analyses were performed using the GB-STAT statistical package (Dynamic Microsystems). Standard errors were calculated for each mean, and statistical differences between groups were determined by Student’s *t* test or ANOVA followed by Newman-Keul post hoc tests as indicated.

**Results**

**Anchorage-independent growth of human glioma cells is dependent on mTOR-raptor signaling.** Anchorage-dependent growth by tumors displaying activated PKB/Akt signaling, such as those lacking PTEN, is sensitive to mTOR inhibition (11), although mTOR’s role in maintaining anchorage-independent growth is less well defined. We used the mTOR inhibitor rapamycin to assess whether the anchorage-independent growth of two human glioma cell lines and two transformed human astrocytic cell lines was mTOR-dependent. Immortalized human astrocytes transformed by the expression of HRasV12 or HRasV12/Akt grew in soft agar as did the U251 and U373 glioblastoma cell lines. The addition of rapamycin in the agar, however, suppressed the colony-forming ability of all cells (data not shown). The anchorage-independent growth of HRasV12/Akt-transformed human astrocytes was also no more resistant to rapamycin than that of human astrocytes transformed by HRasV12 alone, indicating that activated Akt failed to rescue anchorage-independent growth from suppression by rapamycin.

To confirm these results, we also determined whether specific suppression of mTOR altered the growth of glioma cell lines in soft agar. To do so, we stably introduced lentivirus encoding shRNA targeting mTOR in U251 and U373, then assessed levels of mTOR and the downstream effectors phosphorylated p70S6K Thr389, phosphorylated Akt Ser473, and α-tubulin. Equal loading was verified by α-tubulin. B, cells expressing either shRNA targeting mTOR or raptor were plated at equal densities in 96-well plates, and proliferation was assessed over 96 h by the MTS assay. Proliferation slopes of each cell line are comparable, indicating that proliferation rates are not significantly altered by mTOR or raptor suppression. C, cells expressing either scrambled shRNA or shRNA targeting mTOR were plated in soft agar. Cells expressing scrambled shRNA were incubated with vehicle or 100 nmol/L of rapamycin, whereas cells expressing shRNA targeting mTOR were incubated without rapamycin. After 3 wk of incubation, cells were stained and the number of colonies was counted. Columns, mean number of colonies produced per cell line; bars, SE (*, *P < 0.05, Student's *t* test).
concomitant increase in phosphorylated Akt Ser473 (26). Although suppression of mTOR in cell lines did not significantly alter the proliferation rates of cells in culture (Fig. 1B), it did suppress the growth of cells in soft agar (relative to scramble control) to an extent comparable to the loss of growth observed with rapamycin exposure (Fig. 1C). These results show that mTOR plays a key role in maintaining anchorage-independent growth and transformation in glioma cells.

**eIF4E expression fails to reverse rapamycin-mediated suppression of anchorage-independent growth.** Because eIF4E has been shown to play a role in the transformation of 3T3 cells and in leukemogenesis, we expressed wild-type eIF4E in HRasV12-, transformed human astrocytes, HRasV12/Akt-transformed human astrocytes, and U373 cells, and assessed the ability of eIF4E to alter the effects of rapamycin on growth in soft agar. As shown in Fig. 2A, retroviral infection of HRasV12, HRasV12/Akt-transformed human astrocytes, and U373 cells increased eIF4E expression relative to vector control cells. eIF4E expression in HRasV12/Akt-transformed human astrocytes significantly increased colony formation in the absence of rapamycin relative to vector control cells (Fig. 2B). eIF4E expression did not, however, reverse rapamycin-mediated suppression of colony formation in soft agar (Fig. 2B). In contrast to the effects of eIF4E expression in HRasV12-, transformed human astrocytes, eIF4E expression in U373 did not alter baseline soft agar colony formation. However, as in the above cell lines, eIF4E expression in U373 failed to confer resistance to rapamycin exposure. These data indicate that whereas eIF4E introduction confers a growth advantage, it does not rescue cells from rapamycin-induced suppression of growth in soft agar.

eIF4E function is negatively regulated by 4EBP1, and we further substantiated the above finding by silencing 4EBP1 in HRasV12-, transformed human astrocytes and U251 using siRNA (Fig. 2C), then assessing the ability of 4EBP1 to alter the effects of rapamycin on growth in soft agar. Reduction of 4EBP1 levels in both cell lines failed to rescue cells from the effects of rapamycin in soft agar (Fig. 2D), providing further support that eIF4E does not significantly support mTOR-driven growth by gliomas.

**S6K supports anchorage-independent growth.** S6K has been shown to modulate the translation of messages possessing 5'-TOP sequences, but has not been implicated in tumorigenesis. To directly address the role of S6K in transformation, we expressed wild-type S6K, or a constitutively active, rapamycin-resistant mutant S6K (E389R) in U373 and U251, then assessed its effects on growth in soft agar with and without rapamycin present. As shown in Fig. 2C, retroviral infection of U373 cells with S6K-E389R increased S6K protein expression relative to vector control cells. eIF4E expression in U373 cells did not alter baseline soft agar colony formation. However, as in the above cell lines, eIF4E expression in U373 failed to confer resistance to rapamycin exposure. These data indicate that whereas eIF4E introduction confers a growth advantage, it does not rescue cells from rapamycin-induced suppression of growth in soft agar.

**Figure 2.** Neither eIF4E expression nor 4EBP1 suppression are sufficient to rescue anchorage-independent growth from rapamycin. A, human astrocytes transformed by HRasV12, or HRasV12/Akt, and human glioma U373 cells were infected with either an empty vector encoding GFP or vector encoding GFP and wild-type eIF4E. After cells were FACS-sorted to isolate GFP-positive cells, whole cell lysate from the cell lines were assessed by immunoblotting for total eIF4E protein. Equal loading was verified by α-tubulin. B, cells expressing either wild-type eIF4E or empty vector were plated in soft agar in the presence or absence of 100 mmol/L of rapamycin, then after 3 wk of incubation, cells were stained and colonies were counted. Columns, mean number of colonies produced per cell line; bars, SE (*, P < 0.05 and **, P < 0.01, two-way ANOVA followed by Neuman-Keuls post hoc test). C, U251- and HRasV12-transformed human astrocytes were transfected with either scramble control (scr) or siRNA against 4EBP1 (i4EBP1). Whole cell lysates collected at specified time points were assessed by immunoblotting for total 4EBP1 and α-tubulin. D, cells transfected with either 4EBP1 or scramble control were plated in soft agar 24 h after transfection (in the absence or presence of rapamycin) and scored for colony numbers after 3 wk of incubation. Columns, mean proportion of colonies produced per cell line, normalized to untreated controls; bars, SE (*, P < 0.05; Student’s t test).
shown in Fig. 3A, introduction of a vector encoding wild-type or constitutively activated S6K (E389R) increased levels of S6K and phosphorylated S6 Ser^235/236 2-fold to 3-fold in both cell lines relative to empty vector cells (pBabe). Having confirmed protein expression in viral transfectants, pooled transfectants were grown in soft agar in the presence or absence of rapamycin, and colonies were counted after 3 weeks. As shown in Fig. 3B, colony formation by U251 cells expressing wild-type S6K remained sensitive to rapamycin exposure, whereas colony formation by U251 cells expressing the mutant S6K E389R was resistant to the presence of rapamycin. In U373 cells, expression of either wild-type S6K or the mutant S6K E389R resulted in partial rescue of soft agar colony formation in the presence of rapamycin, as compared with the empty vector (Fig. 3B). The reduced expression of wild-type S6K, compared with the expression of mutant S6K E389R in the U251 cells, may explain the absence of rescue from rapamycin-mediated suppression of soft agar growth that was observed in the U251 as compared with the U373 cells which, in comparison, had more comparable protein levels of the wild-type and mutant forms of S6K. To further assess S6K’s importance in maintaining a transformed state, we performed the converse experiment by transiently transfecting U373, U251, and HRas^{V12}-transformed human astrocytes with siRNA targeting S6K1, plating cells 24 hours after transfection into soft agar and monitoring colony formation 3 weeks later. As shown in Fig. 4A, siRNA transfection produced an ~ 50% to 70% reduction of total S6K protein levels in all three cell lines by 96 hours after transfection, and a 50% to 70% reduction in phosphorylated S6 Ser^235/236 levels. Cells transfected with siRNA targeting S6K1 grew to confluence at a similar rate as cells transfected with scramble control (data not shown). Consistent with the idea that S6K1 maintains anchorage-independent growth, however, S6K1 suppression was associated with a significant loss of colony formation in soft agar by U373, U251, and HRas^{V12}-transformed human astrocytes (Fig. 4B).

Because transient S6K1 knockdown significantly reduced anchorage-independent growth in all three cell lines tested, we sought to determine whether S6K1 knockdown in vivo could similarly inhibit tumor growth. However, concerns regarding the appropriateness of transient RNAi, as performed above, for in vivo studies of orthotopic xenografts led us to favor a more stable and inducible system for RNAi-based experiments. To confirm our in vitro findings, we constructed a lentiviral construct encoding shRNA targeting S6K1 in a tetracycline-inducible fashion and expressed either this construct or control vector into HRas^{V12}-transformed human astrocytes. Pooled cells expressing the constructs were isolated by FACS for GFP expression, and HRas^{V12}-transformed human astrocytes expressing either control vector (Ras Tet) or vector targeting S6K1 (Ras SCT) were injected intracranially into rats. Based on previously described formulations for doxycycline-impregnated rodent feed (27), mice were exposed to either control feed or feed containing doxycycline. After a 14-day exposure to either control feed or feed containing doxycycline, animals were sacrificed and brains were fixed, sectioned and stained with H&E. We then assessed in vivo phosphorylated S6 Ser^235/236 levels by immunohistochemistry, and found that Ras SCT and Ras Tet tumors showed comparable levels of cytoplasmic phosphorylated S6 in the presence of control feed (Fig. 5B). Although exposure to doxycycline failed to alter levels of cytoplasmic phosphorylated S6 in Ras Tet tumors, Ras SCT tumors exposed to doxycycline had comparatively reduced cytoplasmic phosphorylated S6 (Fig. 5A). Animals injected with Ras SCT tumors and receiving doxycycline feed developed smaller tumors as compared with control animals (Fig. 5C, P < 0.05, Student’s t test). Similar to the degree of reduced soft agar colony formation after iS6K shown in Fig. 4B, we observed ~50% smaller tumors in the animals injected with Ras SCT tumor cells and receiving doxycycline feed relative to controls. These data indicate that HRas^{V12}-transformed human astrocytes showed significant cytoplasmic levels of phosphorylated S6 in vivo, and suggest that S6K1 knockdown occurred in a doxycycline-dependent manner. These results show that maintenance of S6K1 activity supports HRas^{V12} transformation in vivo, and that loss of S6K1 activity compromises tumor growth.
Discussion

mTOR and its downstream effectors, eIF4E and S6K, have been implicated in cellular transformation, although their contribution to glial transformation remains undefined. In this work, we show that growth of glioma cells in soft agar, a stringent assay for transformation, is blocked by down-regulation of mTORC1 and that signaling through S6K, but not eIF4E, maintains glial transformation. We also show that in vivo suppression of S6K results in reduced intracranial glioma growth. These findings indicate that the S6K arm may have special significance in glial transformation.

Our data suggest that mTORC2 function is less significant in mTOR-dependent anchorage-independent growth for a few reasons. Rapamycin has been reported to have alternate effects on Akt phosphorylation; prolonged rapamycin exposure has been shown to inhibit the assembly of mTORC2, thereby inhibiting Akt (28), and mTOR inhibition has also been described to induce IRS-1, leading to Akt activation (26). In the human glioma cell lines U251 and U373, we found that suppression of mTOR resulted in a significant increase in phosphorylated Akt Ser 473 as compared with cells expressing scramble control. Despite the increase in phosphorylated Akt Ser 473, mTOR knockdown nonetheless significantly compromised these cells' anchorage-independent growth. These data suggest that in gliomas, mTORC2 is not the dominant arm supporting mTOR-dependent transformation, although it is possible that mTORC2 has effects on tumorigenesis that are Akt-independent.

Our data also suggest that S6K and eIF4E have distinct roles in gliomagenesis. Although prior findings have shown that eIF4E overexpression, however, increased colony formation in HRasV12/Akt-transformed human astrocytes, suggesting that eIF4E plays a positive role in transformation, and it is possible that eIF4E's effects on transformation require other mTOR-dependent pathways such as S6K1. Another reason we cannot fully exclude a role by eIF4E in glial transformation is that superphysiologic levels of eIF4E beyond the 2-fold to 4-fold increases generated in this study may be required for transformation. Malignant gliomas have been described immunohistochemically to express more eIF4E as compared with normal neuroglial cells (29), although the degree of eIF4E overexpression in malignant gliomas remains undefined.

Although S6K has not been shown to be an oncoprotein, in the human gliomas assessed in this study, S6K seemed to be a key factor in maintaining anchorage-independent growth. The actions of S6K shown in human astrocytes may indicate that S6K has differing roles in various tissue types: for example, S6K1 deletion blocks growth factor–stimulated hypertrophy in muscle but not in neurons (30). S6K has numerous downstream targets, among them mRNAs with 5'-TOP sequences, the protein products of which are starting to be understood. The critical targets of S6K are not well defined, although the present data clearly suggest that these targets may be distinct from those influenced by eIF4E, and may represent better therapeutic targets. It should be noted that whereas the mTOR-S6K pathway seems to be critical for the growth of cells in soft agar, transformation of glial cells requires a series of events (p53 inactivation, retinoblastoma inactivation, telomerase reactivation, Ras activation) to which the mTOR-S6K pathway is merely a contributor (22, 31). This observation is consistent with the finding that supplying S6K to rapamycin-treated cells only partially rescues growth. The present findings suggest that S6K, but not eIF4E, plays a key, although not sufficient, role in glial transformation.

Figure 4. Reduction of S6K1 reduces tumor growth in vitro and in vivo. A, U251, U373, and HRasV12-transformed human astrocytes were transfection with either scramble control (scr) or siRNA against S6K (iS6K). Whole cell lysates collected at specified time points were assessed by immunoblotting for total S6K, phosphorylated S6 Ser 235/236, and α-tubulin. B, cells transfected with either iS6K or scramble control were plated in soft agar 24 h after transfection (in the absence of rapamycin) and scored for colony numbers after 3 wk of incubation. Columns, mean proportion of colonies produced per cell line, normalized to untreated control; bars, SE (*, P < 0.05; Student's t test).
In addition to our data, which shows an important role for S6K1 in supporting gliogenesis in vitro and in vivo, recently published data describe ribosomal S6 kinase 2 (RSK2) as supporting anchorage-independent growth induced by tumor-promoting agents such as epidermal growth factor and 12-O-tetradecanoylphorbol-13-acetate (32). RSK2, a homologue of S6K1, is similarly activated by mitogens and is inhibited by rapamycin (33). Although both RSK2 and S6K1 phosphorylate S6 in vivo, these kinases do not seem to be functionally redundant for a few reasons. S6K1 knockout mice have a small-body phenotype, despite the finding that mouse embryo fibroblasts from these animals show normal S6 phosphorylation in vivo, suggesting that RSK2 does not completely duplicate S6K1’s functions (34). Comparisons of amino acid sequences and localization between the two S6 kinases also suggest distinct functional differences (33, 35). It remains possible that S6K1 and RSK2 support tumor growth through similar mechanisms, and further studies defining the transformation-promoting effects common or specific to these kinases are needed.

Defining the role of the mTOR-S6K pathway in glial transformation may have an effect on the design and implementation of glioma therapies. Current targeted therapies are based on our knowledge of pathways thought to be critical for tumorigenesis and proliferation. This rationale has led to the clinical testing of signaling inhibitors such as Tarceva and CCI-779. Despite this mechanistic approach to drug development, these agents have shown only modest effects, and combinatorial strategies that inhibit multiple kinases (for example PI3K or Akt in combination with mTOR) show more promise than strategies employing single kinase inhibition (36, 37). In the case of Akt/mTOR combinatorial therapy, the fact that mTOR inhibition can induce Akt activation through IRS-1 may explain why targeting the same pathway at multiple sites is associated with better efficacy. Concerns have been raised that Akt activation with mTORC1

![Image](https://example.com/figure5.png)

**Figure 5.** Reduction of S6K1 reduces tumor growth in vivo. A, short hairpin sequence targeting firefly luciferase (control) or S6K1 in a tetracycline-inducible lentiviral construct was expressed in HRasV12-transformed human astrocytes (Ras). Cells were exposed to medium containing vehicle alone or doxycycline (5 μg/mL) for 4 d. Whole cell lysate from treated cells were assessed by immunoblotting for levels of phosphorylated S6 Ser235/236. Equal loading was verified by α-tubulin. B, HRasV12-transformed human astrocytes expressing either control vector (Ras Tet) or vector targeting S6K1 (Ras SCT) were injected intracranially in rats. After a 14-d exposure to either control feed or feed containing doxycycline (−DOX or +DOX), animals were sacrificed and brains were fixed and sectioned. Representative 5-μm sections through tumors were immunolabeled for phosphorylated S6 Ser235/236 and stained with 4,6-diamidino-2-phenylindole (DAPI). Fluorescence microscopy was used to visualize 4’,6-diamidino-2-phenylindole (blue) and phosphorylated S6 Ser235/236 (green). Images were taken at ×20 magnification, and individual channels are displayed. C, 5-μm-thick coronal sections were taken at 0.5-mm intervals through the entire tumor, then stained with H&E. Columns, sum of maximum cross-sectional areas of tumors measured on consecutive coronal sections for each tumor; bars, SE (*, P < 0.05, Student’s t test). Ras Tet (n = 6), Ras Tet +Dox (n = 7), Ras SCT (n = 5), and Ras SCT +Dox (n = 6).
inhibition could represent a mechanism for drug resistance and sustained tumor growth, although in our model, Akt activation did not rescue tumor growth from mTORC1 inhibition. Our observation that the mTOR-S6K pathway plays a key role in glial transformation suggests that targeting the Akt-mTOR-S6K pathway at a more distal point may be as effective as dual inhibition at a more proximal point. Selective S6K inhibitors are, at present, not available at the clinical or preclinical level, although the present study suggest that such agents, alone or in combination with other agents, might be rational choices for glioma therapy, and perhaps other tumors dependent on mTOR-S6K signaling for maintenance of the transformed phenotype.

Disclose of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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