CANCER GENOMICS & PROTEOMICS *6*: 255-262 (2009)

# Inhibition of mTOR Activates the MAPK Pathway in Glioblastoma Multiforme

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**Abstract.** *Tumorigenesis of glioblastoma multiforme (GBM), the most aggressive primary intracranial neoplasm, is associated with aberrant PI3K/AKT/mTOR signaling. Inhibitors of mTOR, such as rapamycin (RAPA) or its analogs, have provided limited benefit. Here, we aim to decipher the signaling pathways involved in RAPA resistance. We found that RAPA induced a time-dependent activation of MAPK (pERK1/2) and MEK1/2. Inhibition of upstream kinase MEK1/2 by U0126 partially suppressed RAPA-induced ERK1/2 activation. Small interfering RNA suppression of mTOR resulted in higher pERK1/2 levels and pre-treatment with RAPA potentiated PDGF-induced activation of ERK1/2. Furthermore, nuclear localization of pERK1/2 was evident following RAPA, which was MEK1/2-dependent. Cell proliferation was significantly suppressed by combined MEK1/2 and mTOR inhibition compared to mTOR inhibition alone. These results demonstrate activation of a mitogenic pathway involving a feedback mechanism between mTOR and PI3K/ERK1/2 and support the basis for combined inhibitors in GBM treatment.*

Glioblastoma multiforme (GBM), the most aggressive and frequently occurring primary intracranial neoplasm, is uniformly fatal (1). Several genetic pathways are shown to be abnormal in GBM including amplification of epidermal growth factor receptor (EGFR), loss of chromosome 10q, mutation in phosphatase and tensin homolog (PTEN), mutation of p53, and concominent loss of p16 and p18 (2, 3). Furthermore, mutations of the tumor suppressor gene *PTEN* occur frequently and have supported the investigation of downstream proteins as therapeutic targets (4, 5). PTEN loss results in the up-regulation of the phosphoinositide 3-kinase (PI3K)/AKT pathway (6, 7). AKT (protein kinase B), a serine/threonine protein kinase, regulates cell growth and survival by activating multiple downstream targets, including GSK-3β, p21, p27, and NF-κB (6), and activation of AKT plays a crucial role in gliomagenesis as shown in animal models (8).

Notably, the downstream signaling target of AKT, mammalian target of rapamycin (mTOR), is a critical effector of cell signaling pathways generally deregulated in many cancers including GBM (9-11). mTOR integrates several signals including growth factors, nutrition, and metabolic stimuli to regulate cell growth and proliferation. mTOR exists in two distinct multiprotein complexes, namely mTORC1 and mTORC2 (11). mTORC1, which is nutrient and growth factor sensitive, regulates protein translation by its downstream targets, p70S6K and 4EBP1 (6). The mTORC1 complex is composed of proteins such as regulatory associated protein of mTOR (RAPTOR), which is sensitive to rapamycin (RAPA) treatment. It has been shown that mTORC1 function is tightly regulated by PI3K/AKT. In contrast to mTORC1, the mTORC2 complex is sensitive to growth factors but not nutrients, and is associated with the Rapamycin-insensitive companion of mTOR (RICTOR) along with other proteins (11). mTORC2 activation has been associated with AKT phosphorylation at Serine473 (12). Our recent studies have demonstrated that suppression of mTORC1 can activate mTORC2, and this activation modulates cellular proliferation and motility discretely (13).

Mutations along the PI3K/AKT/mTOR pathway can create the potential for novel chemotherapeutic approaches to control or abate tumorigenesis (14). Drugs that inhibit both mTOR and PI3K have enhanced activity in GBM models (13, 15). Thus far, the molecular mechanisms of drug failure have not been fully understood. Studies have shown that RAPA selectively inhibits the mTORC1 complex, and in some cells this appears to block mTORC2 activity by preventing the assembly of a new complex (15, 16). In recent years, various therapeutic potentials for this drug in the treatment of GBM and other cancers have encouraged the development of derivatives for use in clinical trials. However, RAPA and its analogs have provided very little clinical response in patients. For example, the response rate of a RAPA analog, CCI-779, in breast cancer and neuroendocrine carcinoma were lower than 10% (14).

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*Key Words:* ERK, GBM, MAPK, mTOR, RAPA.

Similarly, recent studies demonstrated low efficacy of RAPA-induced mTOR inhibition in the reduction of PTEN-deficient tumor cell growth in GBM patients (17). Understanding the reduced efficacy of these drugs would provide insight into the feedback loop associated with downstream targets of mTOR and offers a molecular basis for treatment (18). This study is aimed at deciphering the feedback loop involved with RAPA. We found that the activation of another mitogenic pathway, Ras/ERK1/2, occurs as a result of RAPA treatment.

## Materials and Methods

*Cell lines*. The GBM cell line LN18 (ATCC, Manassas, VA, USA) was used to investigate the involvement of the PI3K/AKT/mTOR signaling pathway in GBM progression. This cell line has an underlying mutation of *p53* at codon 213 and wild-type *PTEN*.

*Cell culture*. Cells were maintained in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS and 1% penicillin/streptomycin/amphotericin in a humidified 5% $CO_2$ incubator at 37˚C. Cells were made quiescent by serum deprivation 24 h prior to treatment with various combinations of rapamycin (RAPA, mTOR inhibitor, 10 nM), LY294002 (LY, PI3K inhibitor, 10 μM), platelet-derived growth factor (PDGF, 25 ng/ml) (EMD Chemicals, Gibbstown, NJ, USA), U0126 (MEK1/2 inhibitor, 10 μM) fibronectin (FN, extracellular matrix, 20 ng/ml) (Sigma-Aldrich, St. Louis, MO, USA).

*Isolation of protein*. Protein extraction was performed with whole cell lysis buffer 1% Triton X-100, 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA containing phosphatase and protease inhibitors (Sigma-Aldrich). Protein concentrations were determined by the modified Lowry Method (Bio-Rad Laboratory, Hercules, CA, USA).

*Western blot analysis*. Equal amounts of protein were resolved on a 10% SDS-PAGE gel and then electrotransferred onto nitrocellulose membrane. Membranes were processed according to the manufacturers' instructions (Santa Cruz Biotechnology, Santa Cruz, CA, USA; Cell Signaling Technology, Danvers, MA, USA; EMD Chemicals) using primary antibodies for activated and total ERK1/2 as well as activated and total MEK1/2, and bands were detected by chemiluminescence (Cell Signaling Technology). Blots were stripped with reagent (EMD Chemicals) and re-probed with actin or respective total antibodies to ensure equal loading. Experiments were conducted at least 3 times.

*Small interfering RNA (siRNA)*. Cells were transfected with siRNA according to manufacturer's instructions (Qiagen, Valencia, CA, USA). siRNA duplex target sequence was generated for mTOR (FRAP, NM_004958; CAGGCCTATGGTCGAGA TTTA). AllStar Hs Cell Death Control and non-specific AllStar Negative Control (Qiagen) were used as positive and negative controls, respectively. Following 48-h incubation, proteins were collected and analyzed. Successful transfection was determined by the presence of apoptotic cells in positive siRNA treatment.

*Immunofluorescence*. Cells were made quiescent by serum free media for 24 h and subjected to the following treatments: i) RAPA (24 h); ii) RAPA (24 h) followed by U0126 (24 h); iii) U0126 (24 h); iv) U0126 (24 h) followed by RAPA (24 h), and v) RAPA and U0126 combined (24 h). This was followed by fixation with 4% paraformaldehyde/0.3% Tween. Cells were blocked with 5% goat serum, incubated overnight with pERK1/2 antibody (1:100; Cell Signaling) and subsequently incubated with Rhodamine-conjugated antibody (1:200; Jackson ImmunoResearch, West Grove, PA, USA) according to the manufacturers' instructions. DAPI counterstain was performed (Sigma-Aldrich).

*Cell proliferation assay*. Approximately 3,000 LN18 cells/well were seeded in 96-well culture plates. Quiescent cells were treated with RAPA (10 nM), U0126 (10 μM) and in combination for various time points. Cell proliferation was evaluated by MTT Assay (Sigma-Aldrich) for up to 4 h. This assay is based on the conversion of the yellow tetrazolium dye MTT to purple formazan crystals by metabolically active cells. Absorbance was measured on an ELISA plate reader at 570 and 630 nm.

*Statistics*. The results presented reflect the mean±SEM and were compared using Student's *t*-test (unpaired, 2-tailed, $p<0.05$).

## Results

*Inhibition of mTOR activates ERK1/2 and MEK1/2*. In order to assess whether the MAPK pathway is activated following inhibition of mTOR by RAPA, we subjected cells to timed RAPA treatment (1, 3, 6, 12 and 24 h) and measured the levels of phosphorylated ERK1/2 $Thr^{202/Tyr204}$ (pERK1/2) and total ERK1/2 (tERK1/2). The activation of ERK1/2 by RAPA treatment occurred in a time-dependent manner as determined by the increased levels of pERK1/2 (Figure 1A). The bottom panel represents the same blot stripped and probed with tERK1/2 depicting relatively unchanged levels across treatments. Bar graphs represent ratios of pERK1/2:tERK1/2 for corresponding treatments. The highest increase in the pERK1/2:tERK1/2 ratio was observed at 12 h following RAPA treatment. At that time, RAPA induced activation of ERK1/2 increased by 352%. At 24 h, ERK1/2 activation, while still up-regulated compared to control, was noticeably lower than at 12 h. In order to establish that suppression of mTOR activates the Ras/MAPK pathway, we determined the levels of activated MEK1/2 and observed that timed treatments of RAPA enhanced the levels of pMEK1/2 (Figure 1B). The levels of pMEK1/2 peaked at 3 h and decreased gradually until 24 h. Also shown in this figure are the levels of total MEK1/2 (tMEK1/2). The relative values of ERK1/2 and MEK1/2 activation by RAPA are represented in graphed form (Figure 1C). Furthermore, we tested whether upstream inhibition of MEK1/2 by U0126 had any influence on RAPA-induced activation of ERK1/2. Twenty-four hours of U0126 treatment suppressed ERK1/2 activation but partially suppressed RAPA-induced activation of ERK1/2 (Figure 1D). The ratio of pERK1/2:tERK1/2 confirms this trend presented in bar graph form.

*RAPA potentiated PDGF-induced activation of ERK1/2*. In order to establish that RAPA-induced ERK1/2 activation was associated with growth factor or ECM regulated Ras/MAPK

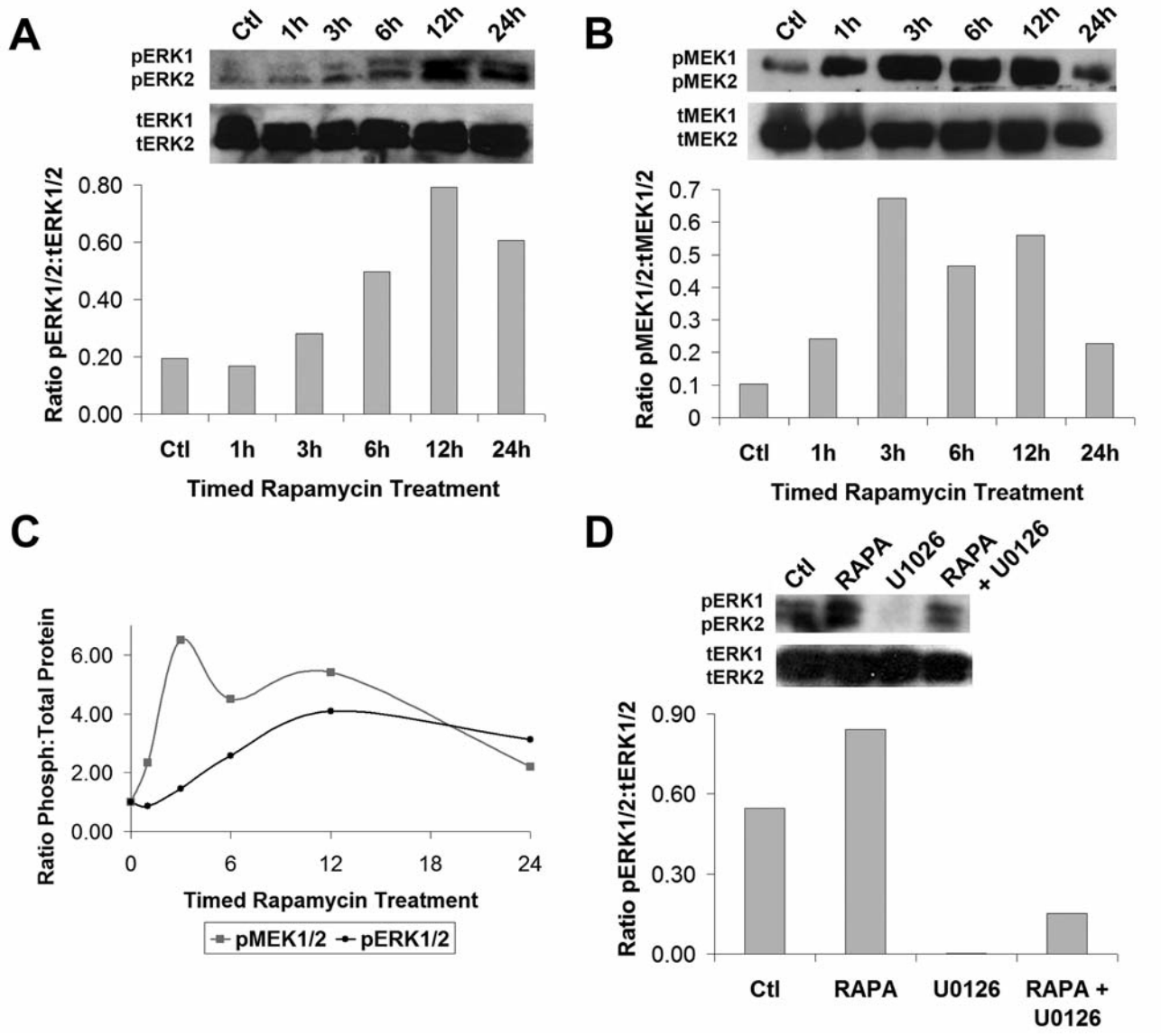


Figure 1. *Inhibition of mTOR activates ERK1/2 and MEK1/2. A) Timed treatment with mTOR-inhibitor RAPA (10 nM) caused a progressive activation of ERK1/2 as evident by higher expression of pERK1/2$^{Thr202/\ Tyr204}$, with maximum activation at 12 h. Total ERK1/2 displayed similar levels across the treatments. The bottom panel represents the ratio of pERK1/2:tERK1/2 density calculated from the Western blot. B) RAPA-induced activation was believed to be through the activation of upstream kinase, MEK1/2$^{Ser217/Ser222}$, which showed a time-dependent activation of MEK1/2. The bottom panel represents the ratio of pMEK1/2:tMEK1/2. C) ERK1/2 and MEK1/2 activation occurred in a coordinated manner following RAPA treatment where MEK1/2 activity preceded pERK1/2 up-regulation. D) RAPA-induced activation of ERK1/2 was partially suppressed by pre-treatment with upstream kinase inhibitor U0126 (10 μM). The bottom panel represents the ratio of pERK1/2:tERK1/2.*

signaling, we subjected RAPA or PI3K inhibitor (LY) pre-treated GBM cells to PDGF or FN. As expected, pERK1/2 levels were noticeably higher in PDGF-treated cells. Treatment with PDGF in RAPA pretreated cells dramatically induced pERK1/2 levels demonstrating a profound synergistic activation of the Ras/MAPK pathway. In contrast, inhibition of PI3K reduced PDGF-induced activation of ERK1/2 (Figure 2A). This figure also presents the findings of GBM cells treated with extracellular matrix FN where FN failed to induce ERK1/2 activation singly as well as when given with LY or RAPA.

*Effect of siRNA suppression of mTOR on ERK1/2 activation*. To confirm our findings that activation of ERK1/2 is due to inhibition of mTOR, we treated cells with siRNA for mTOR for 48 h. Our data demonstrated that mTOR siRNA treatment up-regulated ERK1/2 activation as compared to control or negative siRNA (Figure 2B). The levels of tERK1/2 remained unchanged across treatments. The values presented in the bar graph represent the

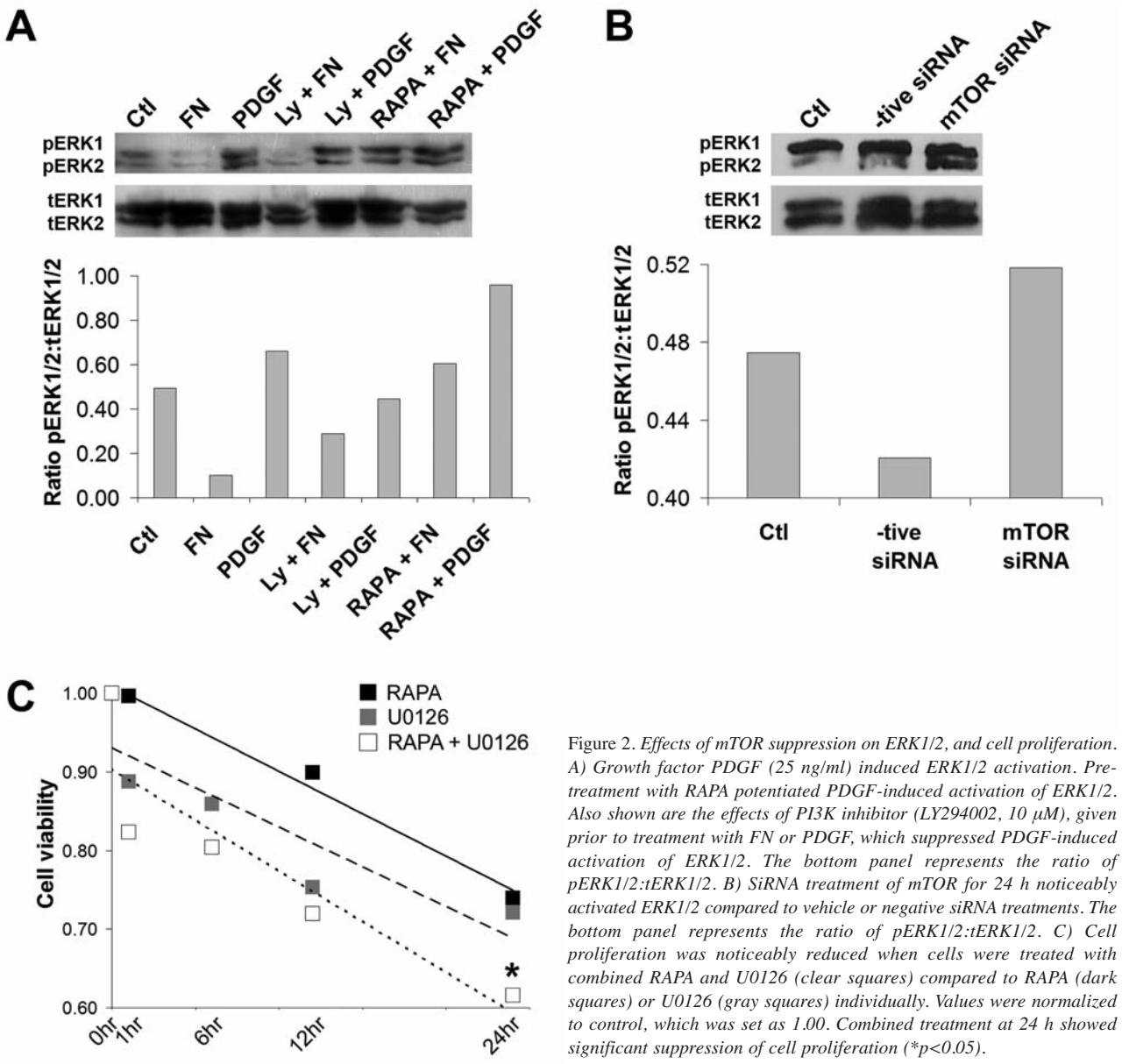


Figure 2. *Effects of mTOR suppression on ERK1/2, and cell proliferation. A) Growth factor PDGF (25 ng/ml) induced ERK1/2 activation. Pre-treatment with RAPA potentiated PDGF-induced activation of ERK1/2. Also shown are the effects of PI3K inhibitor (LY294002, 10 μM), given prior to treatment with FN or PDGF, which suppressed PDGF-induced activation of ERK1/2. The bottom panel represents the ratio of pERK1/2:tERK1/2. B) SiRNA treatment of mTOR for 24 h noticeably activated ERK1/2 compared to vehicle or negative siRNA treatments. The bottom panel represents the ratio of pERK1/2:tERK1/2. C) Cell proliferation was noticeably reduced when cells were treated with combined RAPA and U0126 (clear squares) compared to RAPA (dark squares) or U0126 (gray squares) individually. Values were normalized to control, which was set as 1.00. Combined treatment at 24 h showed significant suppression of cell proliferation (*$p<0.05$).*

pERK1/2:tERK1/2 ratio.

*Combined inhibition of mTOR and MEK1/2 suppressed proliferation*. Over a 24 h period, timed RAPA treatment resulted in suppression of cell proliferation as assessed by MTT assay. Also, U0126 caused a time dependent inhibition in cell proliferation, although more pronounced than RAPA treatment alone. Interestingly, when given in combination (RAPA and U0126), an additive suppression of cell growth occurred as evident in Figure 2C, where the combined treatment showed a noticeable decline in cell proliferation compared to the other two groups. Treatment with RAPA and U0126 for 24 h resulted in significant suppression of proliferation compared to control (Student's 2-tailed *t*-test, $p<0.05$).

*RAPA influences cellular localization of pERK1/2*. In vehicle treated cells, pERK1/2 was observed in the cytoplasm (Figure 3A, a and b). In cells treated with RAPA, the expression of pERK1/2 was found in the nucleus (Figure 3A, c and d). A combined treatment of RAPA and U0126 for 24 h showed pERK1/2 expression in cytoplasm as well as nucleus (Figure 3A, e and f). RAPA treatment for 24 h followed by U0126 treatment for 24 h totally suppressed pERK1/2 expression in both cytoplasm and nucleus (Figure 3A, g and h). On the other hand, U0126 treatment for 24 h followed by RAPA treatment for 24 h showed a similar pattern as a combined

treatment of RAPA and U0126, that is, pERK1/2 expression in cytoplasm and nucleus (Figure 3A, i and j). It is worth mentioning that when pERK1/2 was localized in the nucleus, it presented in a speckled, granular pattern.

## Discussion

The results of this study demonstated that inhibition of mTOR resulted in ERK1/2 activation (Figure 1A). We further showed that the selective inhibitor of MEK1/2, U0126, abrogated RAPA-induced up-regulation of ERK1/2 (Figure 1B, D). In addition, growth factor PDGF induced activation of ERK1/2 which was potentiated in the presence of RAPA (Figure 2A). The siRNA inhibition of mTOR confirms the finding that physiological suppression of mTOR activated ERK1/2 (Figure 2B). Since this activation relies on upstream Ras/Raf/MEK1/2, RAPA treatment significantly up-regulated an upstream kinase of ERK1/2, MEK1/2, in a time-dependent manner (Figure 1B, C). Cell proliferation was noticeably reduced when cells were treated with combined inhibitors (Figure 2C). In addition, we observed that RAPA treatment induced nuclear localization of pERK1/2 (Figure 3A-I), which was MEK1/2 dependent.

The mTOR pathway is known to be activated in various cancers including GBM. It influences protein synthesis *via* its regulation of ribsosomes through S6K, which is downstream from mTOR (10). Inhibitors of mTOR have been considered for treatment of GBM, however, clinical trials have only produced modest results (19). We observed MEK1/2 and ERK1/2 activation in a coordinated manner following RAPA treatment (Figure 1C). The Ras/ERK1/2 pathway has also been shown to be activated in GBM and appears to have a role in the malignant phenotype of GBM (20). The MAPK cascade integrates various signalling components to elicit multiple physiological functions (21). The mTOR and Ras/ERK1/2 pathways appear to interact through Ras, influencing growth and differentiation (22). However, the exact nature of the interaction of these signaling pathways has not been well defined. Several studies have suggested the existence of a crosstalk between Ras/ERK1/2 and PI3K/AKT pathways, where Ras has been shown to bind to and activate PI3K (23, 24).

Some studies have shown that the MAPK pathway regulates the activation of mTORC1 through mTORC2 phosphorylation (18). mTORC1 function is regulated by PI3K/AKT signaling pathway through the function of TSC2, which has been shown to be associated with TSC1 (25, 26). Furthermore, recent studies have suggested mTORC1 inhibition as a signal by which mTORC1 controls tumor growth and cancer progression *via* PI3K modulation (27). In addition, the proximal mTORC1 activator, RHEB1, directly interacts with and inhibits B-RAF and RAF1, which underscores the complexity of the connection between mTORC1, PI3K, and MAPK. On the other hand, we and others have provided evidence that prolonged RAPA treatment in fact activates the mTORC2 complex, *via* the AKT pathway, and may suggest another mechanism of ERK1/2 activation, perhaps *via* AKT activation (13). So far, potentiation of a feedback loop by mTORC1 inhibitors has shown to influence the PI3K/AKT pathway (11); however, the impact of mTORC1 inhibition in other mitogenic pathways has not been demonstrated in GBM.

The findings of this study demonstrate that mTORC1 inhibition activated MAPK. These findings are significant for GBM and certain other cancers since they provide a basis for the limited benefit of clinical trials using RAPA or its analogs. While RAPA treatment suppressed mTOR activity, it also suppressed downstream pS6K levels to a threshold that initiates a negative feedback loop to activate upstream IRS-1 (11). This negative feedback can directly activate the Ras/ERK1/2 pathway. The activation of the MAPK pathway occurs upon mTORC1 inhibition in GBM cells since the physiological and pharmacological suppression of mTOR activated ERK1/2 (Figure 2B). In association with these molecular observations, we found that a combined treatment with RAPA and U0126 has a dramatic effect on cell suppression as compared to RAPA alone (Figure 2C). Several studies have suggested that these inhibitors are involved in growth suppression and cell cycle regulation (28; 29). These findings not only provide insight into the molecular basis for the complex mTOR signaling network but also provide a reasoning for including dual inhibitors in treatment.

Another interesting finding of this study is that nuclear localization of activated ERK1/2 occurs upon inhibition of mTOR (Figure 3A, C, D). Several studies have shown that a translocation of activated ERK1/2 to the nucleus is necessary for growth factor induced gene expression as well as cell cycle entry (21). However, it is not clear from this study whether the nuclear localization of ERK1/2 is increased due to translocation or nuclear retention, since the inhibitor of MEK1/2, U0126, partially suppressed this effect. Future studies would determine the significance of cyto-nuclear localization, however, some studies have indicated that AKT may influence nuclear localization of activated ERK1/2 (30).

These results further our knowledge and provides a molecular basis for the ineffectiveness of mTOR inhibitors in GBM treatment and underscores therapeutic strategies with combined inhibitors of ERK1/2 along with mTORC1 and mTORC2. Consequently, combinatory treatments may abrogate the activation of cellular pathways which are a result of induced feedback loops.

## Acknowledgements

We gratefully acknowledge The American Research Foundation for their support.

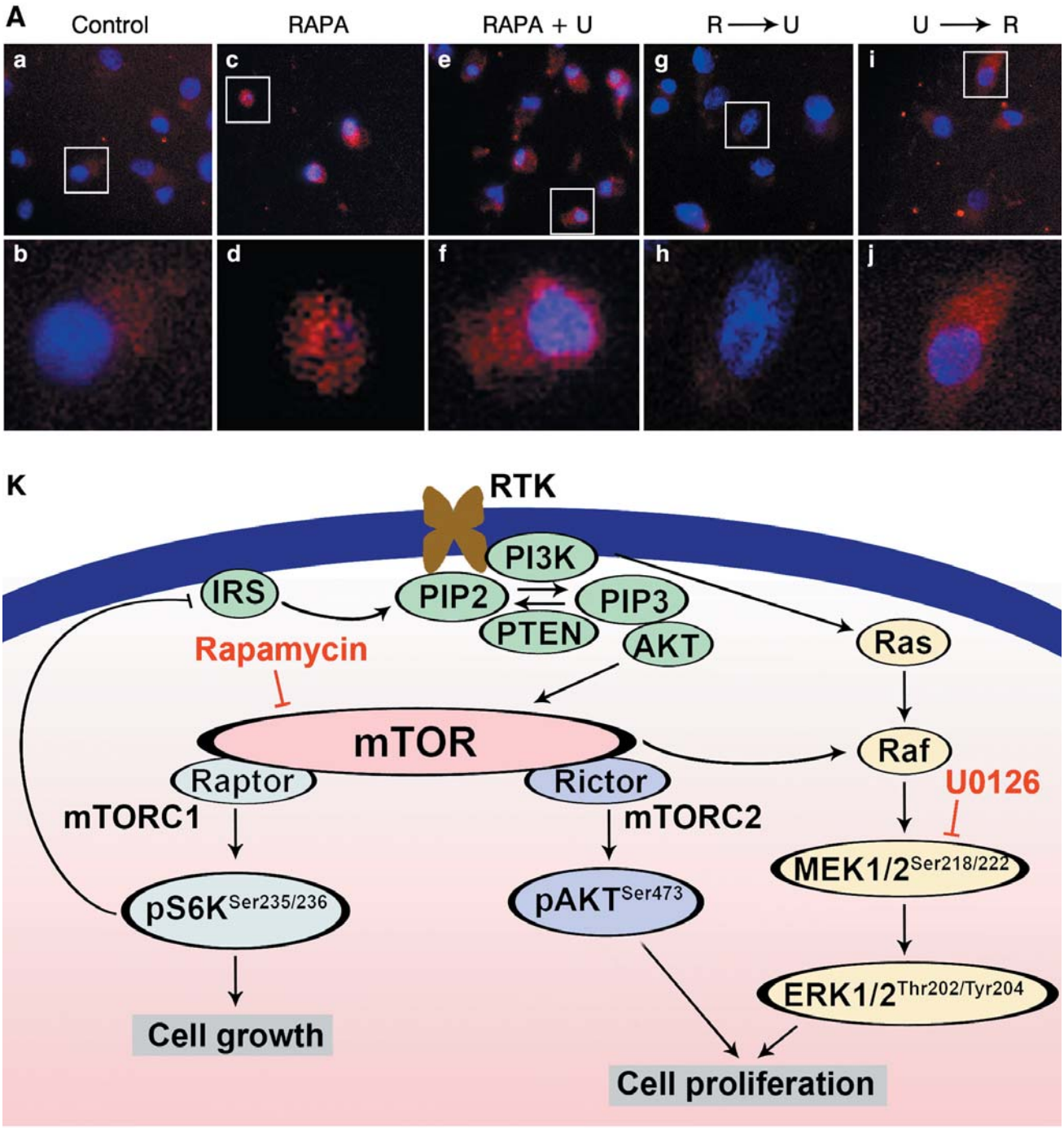


Figure 3. *Suppression of mTOR Influences the cellular location of pERK1/2. A) The expression of pERK1/2 was seen in the cytoplasm of untreated GBM cells (a,b). RAPA treatment induces pERK1/2 translocation into the nucleus (c,d). Both cytoplasmic and nuclear expression of pERK1/2 was seen following a combined treatment with RAPA and U0126 (e,f). RAPA (24 h) followed by U0126 treatment (24 h) suppressed pERK1/2 expression (g,h). U0126 (24 h) followed by RAPA treatment (24 h) displayed nuclear and cytoplasmic pERK1/2 expression (i,j). B) A schematic representation of the PI3K/AKT/mTOR pathway is shown. A feedback inhibition stemming from sustained inhibition of mTORC1 signaling promotes activation of the MAPK (ERK1/2) pathway. RAPA treatment inhibited pS6K that caused the activation of IRS1 leading to the activation of PI3K which in turn stimulated the Ras/ERK1/2 and AKT pathways. Sustained inhibition of pS6K by RAPA can stimulate IRS1 through negative feedback. RTK: Tyrosine receptor kinase, PI3K: phosphoinositide 3-kinase, PIP2: phosphoinositol-2-phosphate, PIP3: phosphoinositol-3-phosphate, PTEN: phosphatase and tensin homolog, AKT: protein kinase B, mTOR: mammalian target of rapamycin, Raptor: regulatory associated protein of mTOR, Rictor: rapamycin-insensitive companion of mTOR, IRS: insulin response signal, ERK1/2: extracellular regulated kinase/mitogen-activated protein kinase, MEK1/2: MAPK kinase.*

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*Received September 15, 2009*
*Accepted September 21, 2009*