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The role of AKT isoforms in glioblastoma: AKT3 delays tumor progression

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Abstract

The growth factor receptor/PI3K/AKT pathway is an important drug target in many cancers including Glioblastoma. AKT, a key node in the pathway, has 3 isoforms, AKT1, AKT2 and AKT3. Here we investigate their role in GBM. We find each activated, ser473 phosphorylated isoform is present in some GBMs but expression patterns vary. There is a direct relationship between human GBM patient outcome and both AKT1 and AKT2 mRNA levels, but an inverse relationship with AKT3 mRNA. Furthermore, AKT3 mRNA levels were high in a less aggressive GBM subtype. Overexpressing AKT3 improves survival in a rodent model of GBM and decreases colony forming efficiency, but not growth rate, in glioma cells. Silencing AKT3 slows cell cycle progression in one cell line and increases apoptosis in another. Our studies of AKT3 substrates indicate (1) silencing both AKT2 and AKT3 reduces GSK3 phosphorylation (2) only AKT2 silencing reduces S6 phosphorylation. Since S6 phosphorylation is a marker of mTORC1 activity this indicates that AKT2 activates mTORC1, but AKT3 does not. Our results indicate AKT isoforms have different roles and downstream substrates in GBM. Unexpectedly, they indicate

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Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals *Ethical approval human* All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional review board and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.
Ethical approval animal All applicable international, national, and institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were approved by our Institutional Animal Care and Use Committee (IACUC) and were in accordance with the ethical standards of the institution at which the studies were conducted.

Informed consent For this type of study formal consent is not required.

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AKT3 delays tumor progression. Therefore strategies that inhibit AKT3 may be unhelpful in some GBM patients.

Keywords

Glioblastoma; GBM; AKT; AKT1; AKT2; AKT3

Introduction

The growth factor receptor/phosphatidyl-inositol-3-kinase/AKT (GFR/PI3K/AKT) pathway is hyper-activated in many human cancers including Glioblastoma (GBM). The serine/threonine kinase AKT is a key node in this pathway. It regulates many functions important for tumor initiation and progression including cell death, proliferation, invasion, angiogenesis and stem cell function [1] and is an important therapeutic target.

There are three isoforms of AKT with very similar sequences called AKT1, AKT2 and AKT3. Isoforms share some functions and substrates but can also have distinct roles. For example, AKT1 knockout mice have smaller overall size, AKT2 knockout mice have a type II diabetes-like phenotype, and AKT3 knockout mice have small brains [2–5]. Evidence supports an important role for AKT3 in the developing brain. It is the most abundant AKT isoform mRNA during neurogenesis [6] and increased gene dosage or activating mutations cause human brain overgrowth disorders [6–8]. Therefore, individual AKT isoforms have distinct functions and AKT3 has a unique and important role in brain development.

Many drugs against AKT inhibit all isoforms [9–13]. However, roles of individual AKT isoforms in GBM oncogenic signaling are not clear. Studies modulating isoform expression indicate their function depends on context. AKT2 was necessary for migration of glioma cell lines [14], but not transformed astrocytes [15] where only AKT3 was required [15]. AKT1 silencing improved survival in rodent models using transformed murine astrocytes [15] and a glioma cell line [16], while AKT2 silencing decreased survival in the former model and had little effect in the latter. Effects of AKT3 modulation were modest in both models [15]. Therefore, the role of AKT isoforms in transformation, progression and outcome of human GBM is unclear. Therefore, the effects of general AKT inhibition in tumors are also unclear.

Our investigations suggest AKT3 plays a surprising role in GBM. Human GBMs with high levels of AKT3 mRNA are less aggressive. Furthermore, our results indicate AKT3 overexpression improves survival in a rodent model of GBM. But it also indicates low concentrations are needed for progression through the cell cycle or suppression of apoptosis. We speculate a nonspecific AKT inhibitor is unhelpful in less aggressive GBM with high AKT3 levels.

Materials and methods

Reagents and cell culture

The following antibodies were from Cell Signaling (Danvers, MA): pan-AKT(#4685), AKT1(#2938), pAKT Ser-473(#9271, #4060), PARP (#9542), GSK3α (#9338), pGSK-3α

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ser21 (#9316), GSK3β (#9315), pGSK3β ser9 (#9323), pS6 ribosomal protein ser240/244 (#5364) all used at 1:1000 dilution. Antibodies against α-tubulin (#05-829) and AKT3 (#07-383) were from Millipore (Billerica, MA) and were used at 1 μg/ml and 1:500, respectively.

The GBM cell lines, U87 and U251, were obtained from ATTC (Manassas, VA) and maintained in DMEM (Waymouth medium, Life Technologies; Grand Island, NY) supplemented with 10 % fetal calf serum (Life Technologies), at 37 °C, 5 % CO_2 in a humidified incubator. They were routinely monitored for mycoplasma infection using fluorescence microscopy after DAPI staining to detect extranuclear nucleic acids.

Immunoprecipitation and immunoblot analysis

Tissue was lysed in 10 mM Tris–HCl, pH 7.4, 0.5 % Nonidet P-40, 150 mM NaCl containing protease and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 2 mM sodium vanadate, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). pAKT ser473 was immunoprecipitated from lysates containing 600 μg protein and washed with lysis buffer followed by S1 buffer (10 mM HEPES, pH 7.4, 0.15 M NaCl, 2 mM EDTA, 1.5 % Triton X-100, 0.5 % deoxycholate, 0.2 % SDS).

Cell lysates and immunoprecipitates were collected in 2 × SDS Page (0.1 M Tris pH 6.8, 4 % SDS, 20 % glycerol, 2 % bromophenol blue) or 4 × LDS sample buffer (106 mM Tris–HCL, 141 mM Tris base, 2 % LDS, 10 % glycerol, 0.51 mM EDTA, 0.22 mM SERVA Blue G250, 0.175 mM Phenol Red, pH 8.5) containing protease (1 μg/ml Pepstatin A, 10 ug/ml Leupeptin, 10 ug/ml Aprotinin, 1 mM PMSF) or for phosphorylated proteins, protease and phosphatase inhibitors (Halt protease and phosphatase inhibitor cocktail; Thermo Scientific, Waltham, MA). Lysates were resolved using Bis-Tris SDS–Page gels (Life Tecnologies, Waltham, MA), transferred onto nitrocellulose or PVDF membranes, membranes blocked with either 5 % BSA or dried nonfat milk and proteins detected with chemiluminescence using standard protocols.

siRNA silencing

Sequences for siRNA against AKT1, AKT2, AKT3 are listed in supplemental Table S1. Cells were transfected for 8–18 h with 20 nM siRNA using RNAiMAX (Life Technologies) according to manufacturer's protocols. We then exchanged siRNA-containing media for complete media and incubated cells for the time specified.

Orthotopic rodent model of GBM

All studies were approved by our institutional IACUC. Female 6–8 week old immunodeficient mice (nu/nu; Charles River) were stereotactically injected with 1×10^6 U87 cells in 10 μl phosphate buffered saline into the caudate/putamen. The endpoint was time to disease progression as determined by loss of >10 % of body weight or appearance of neurological symptoms.

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Plasmid and transfection

The two alternately spliced human AKT3 genes, AKT3V1 and AKT3V2, were purchased from Origene (Rockville, MD). They were subcloned into the PLXSN mammalian expression vector and a myc tag incorporated into the C terminus using PCR cloning as described [17]. Sequences of cloning primers are listed in supplemental Table S1. Purified plasmid was end sequenced to confirm gene identity and inframe insertion. Viral particles were generated after transfecting Phoenix A cells with AKT3 expressing plasmids using Lipofectamine according to manufacturers protocols. Cell lines were incubated with viral supernatants containing 8 μg/ml polybrene and stable transformants selected using antibiotics.

Growth curves and colony forming efficiency (CFE)

Equivalent numbers of cells were plated into 12 well plates for growth curves and triplicate wells counted for each treatment. To determine cell number at the indicated times, a single cell suspension was prepared using trypsin and cell concentration determined with a Z2 Beckman Coulter Counter. To evaluate colony forming efficiency, each well of a 6 well plate was seeded with 200 cells and treated with siRNA for 12 h. The siRNA containing media was gently exchanged for complete media and cells incubated till colonies formed. Colonies were fixed in methanol containing 0.1 % crystal violet and number of colonies with >50 cells counted.

Cell cycle analysis

To analyze DNA distribution, cells were fixed in 70 % ice cold ethanol, rinsed, then labeled with 50 μg/ml Propidium Iodide (PI) containing 60 U RNaseA. DNA content was measured using an Accuri C6 flow cytometer and data fit using FCS Express version 3. Debris, cell doublets and higher order aggregates were detected using plots of area vs. height of PI fluorescence as described [18]. We analyzed only single cells in G0/G1, S and G2/M that lay on the diagonal. Cell aggregates falling below the diagonal and cell debris with less PI fluorescence than the subG0 peak were excluded.

Associations between AKT isoform expression and tumor aggressiveness

We used GSE4290 data to find associations between AKT isoform expression and tumor grade. We used The Cancer Genome Atlas (TCGA) data to find associations between survival and AKT isoform expression. We downloaded AKT isoform expression information from the 166 TCGA GBM cases with RNA-Seq and survival information from cBIO (http://www.cbioportal.org/index.do) on 8/21/15. We downloaded days to death or last followup on 8/21/15 from the TCGA data portal at https://tcga-data.nci.nih.gov/tcgafiles/ftp_auth/distro_ftpusers/anonymous/tumor/gbm/bcr/biotab/clin/.

Statistics

Significant differences between normal and tumor tissue were evaluated with a two-sample, two-sided t test that assumed unequal variance. Survival comparisons between high and low expressers of AKT isoform mRNA were evaluated by plotting Kaplan Meier survival curves

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using Graph Pad Prism 5 software and significance determined using the Gehan-Breslow-Wilcoxon test.

Results

Activated isoforms in GBM tissue

Sequential phosphorylation of AKT at two key sites, the activation loop (thr308/309/305 in AKT1/AKT2/AKT3) and the hydrophobic motif (ser473/474/472 in AKT1/AKT2/AKT3) activates the kinase [19]. This report uses AKT1 terminology to identify phosphorylation sites. We investigated expression of activated isoforms in GBM tissue by immuno-precipitating ser473 pAKT and immunoblotting with isoform specific antibodies. As previously observed [20], pAKT is elevated in GBM vs. non-neoplastic tissue (Fig. 1a, lysate). Although AKT1 and AKT2 antibodies cross reacted (Fig. 1b) we could distinguish between the 2 isoforms since AKT1 had a higher apparent molecular weight (Fig. 1a, ippt). We find AKT2 is activated in each of the 5 GBMs tested, while AKT1 and AKT3 were activated in 3 of 5 GBM (60 %; Fig. 1a, ippt). The data suggest each activated isoform is present in some GBMs but expression patterns vary.

AKT isoform relationship to clinical outcome

We used public expression profiling databases to investigate associations between AKT isoform mRNA and clinical behavior. AKT1 and AKT2 mRNA were higher in higher grade tumors (Fig. 1c). AKT3 mRNA was lower in higher grade tumors (Fig. 1c; $p = 2 \times 10^{-9}$ and 2×10^{-7} for non-neoplastic vs. GBM and non-neoplastic vs. grade II and III, respectively). Patients with tumors expressing higher levels of AKT1 mRNA tended to shorter survival (Fig. 1d; p = .09, Gehan-Breslow-Wilcoxon test); patients with tumors expressing higher levels of AKT3 mRNA tended to longer survival (Fig. 1d; p = .06, Gehan-Breslow-Wilcoxon test).

Molecular profiling indicates there are 3–5 molecular subtypes of GBM [21–26]. An AKT pathway based classification suggested 5 AKT GBM subtypes [27]. Patients in one of the AKT-based subtypes, the SL subtype, were younger and lived approximately 3 years longer than patients in other subtypes [27]. This subtype also included tumors with the good prognostic factor, mutant IDH1. We find higher AKT3 mRNA in the less aggressive SL subtype (Fig. 1e). This occurred regardless of IDH1 mutation status (Fig. 1e, open symbols represent tumors with IDH1 mutation). Taken together these data suggest elevated AKT3 mRNA expression is directly associated with better clinical outcome.

Effect of AKT3 overexpression on tumor-associated functions

We did not expect to find elevated AKT3 mRNA associated with better clinical outcome. To further examine its role we overexpressed *AKT3* in GBM cell lines. There are two alternately spliced forms of *AKT3* called *AKT3V1* and *AKT3V2* [28]. The 28 amino acids at the C terminus of *AKT3V1* are absent in *AKT3V2* and replaced with a shorter sequence lacking the ser473 phosphorylation site (supplemental Fig S1a). AKT3V1 was the predominant alternately spliced form of AKT3 mRNA detected by qRT-PCR in 8 GBM cell lines (supplemental Fig S1b). We overexpressed myc-tagged *AKT3V1* and *AKT3V2* in U87

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and U251 cells. Increased immunodetection of AKT3 and myc at the appropriate molecular weight in infected U87 cells confirms overexpression of both alternately spliced gene products (Fig. 2a). Cells overexpressing *AKT3V1*, which retains the ser473 phosphorylation site, had increased pAKT, while those with *AKT3V2* did not (Fig. 2a). Overexpressing the two alternately spliced variants of *AKT3* had little effect on growth rate (Fig. 2b) but both decreased colony forming efficiency (CFE; Fig. 2c) of the 2 GBM cell lines. Their overexpression also increased survival of mice bearing intracranial xenografts of U87 cells (Fig. 2d). These activities were not dependent on ser473 phosphorylation since the form that lacks this site (AKT3V2) acts similarly to the form with the site (AKT3V1). These data show AKT3 overexpression in GBM cell lines decreases CFE in culture and increases survival in a rodent model. This supports a role for both alternately spliced forms of AKT3 in delaying tumor initiation or progression.

Characterization of AKT isoform protein in GBM cell lines

We investigated presence and activation of AKT isoforms in GBM cells. Each of 7 GBM cell lines expressed AKT1, AKT2 and AKT3 protein and ser473 pAKT by Western analysis (Fig. 3a). We chose U87 and U251 with intermediate and high basal AKT activation (ser473 phosphorylation) for further study.

We immunoprecipitated ser473 pAKT and immunoblotted with isoform specific antibodies to investigate activation of each isoform in cultured U87 GBM cells. Each isoform was ser473 phosphorylated (Fig. 3b; pAKT ippt). However, compared to the other isoforms, less AKT1 is phosphorylated since the relative amount of AKT1 in the immunoprecipitate to total lysate is lower (Fig. 3b; compare “pAKT ippt”/“lysate before” for each isoform). This is consistent with previous reports showing little AKT1 is phosphorylated in U87 cells [29]. Therefore cell lines may not be a robust model for investigating function of AKT1.

Effect of isoform silencing on glioma cells

We further investigated the role of each isoform in GBM cells using siRNA silencing. 3 and 4 days siRNA treatment specifically decreased each isoform’s protein (Fig. 3c). Silencing AKT1 had the least impact on total ser473 pAKT (Fig. 3d) supporting our conclusion that AKT1 is not efficiently ser473 phosphorylated (Fig. 3b). Silencing each isoform decreased growth rate (Fig. 3e) and colony forming efficiency (Fig. 3f) of U87 and U251 cells. These effects were more pronounced for AKT2 and AKT3 than AKT1. These data indicate each AKT isoform promotes growth.

AKT2 and AKT3 had a similar effect on cell cycle in U87 cells. Silencing them increased cell percentage in G0/G1 and decreased cell percentage in S (Fig. 4a and supplemental Fig S2a) consistent with a G1/S checkpoint role. No sub-G0 peak was detected (supplemental Fig S2a). Isoform silencing did not increase the number of U87 cells with DNA content > diploid (supplemental Fig S2a) indicating isoform inhibition does not destabilize the genome. These data indicate AKT2 and AKT3 promote progression through the G1 to S checkpoint in U87 cells.

In U251 cells silencing each isoform increased the subG0 peak (Fig. 4b and supplemental Fig S2b) indicating each suppresses apoptosis. The results were more pronounced for AKT3

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than AKT1 or AKT2. AKT3 silencing induced PARP cleavage further supporting AKT3's role in apoptosis suppression (Fig. 4c). The data indicate each isoform acts on apoptosis suppression in U251 cells but the effect is more robust for AKT3.

Downstream substrates of isoforms

We next investigated each isoforms role in phosphorylation of downstream substrates and found that AKT2 and AKT3 share some substrates but not others. Silencing both AKT2 and AKT3 decreased phosphorylated GSK3α and GSK3β in U87 cells and GSK3α in U251 cells (Fig. 5a). In contrast, only AKT2 silencing decreased pS6, a marker of mTORC1 activity (Fig. 5b; densitometric scan pS6/tubulin Fig. 5c). The data indicate AKT2 and AKT3 interact similarly with GSK3α and GSK3β but only AKT2 signals to mTORC1.

Discussion

Our evidence supports the idea that AKT3 delays GBM tumor progression. Three observations support this conclusion: (1) AKT3 mRNA is inversely associated with grade and survival in GBM (Fig. 1c, d), (2) AKT3 mRNA is elevated in the less aggressive subtype of GBM (Fig. 1e) and (3) AKT3 overexpression increases survival in an orthotopic rodent xenograft model of GBM (Fig. 2d) and decreases colony forming efficiency in glioma cells (Fig. 2c). The results indicate AKT3 inhibition worsens outcome for some GBM patients.

Overexpressing and silencing AKT3 had one phenotype in common; both decrease CFE (Figs. 2c, 3f). However mechanisms underlying this phenotype in the two situations differ. Silencing affected cell cycle and/or apoptosis (Figs. 4, S2), whereas overexpression does not (data not shown). A mechanism underlying AKT3 overexpression's effect on CFE is not yet clear. Flooding cells with overexpressed protein could sicken them decreasing CFE, however we believe this is not likely since their growth rate was unaltered (Fig. 2b). We conclude that AKT3 has a dual role dependent on its concentration. Tumor cells require a minimum amount of AKT3 to transit through cell cycle checkpoints or evade apoptosis, but higher amounts restrain tumorigenicity.

Our conclusions are different than those reached by Turner et al. who speculated AKT3 promotes progression [30]. They showed AKT3 is the isoform most often genomically amplified in many cancers including GBM [30]. We find AKT3 copy number gain does not correlate with increased AKT3 mRNA (R = .005, Spearmans correlation coefficient; TCGA level 3 data not shown). They also showed addition of myristoylated AKT3 increases progression in their low grade glioma model [30]. Myristoylation enforces membrane localization [31]. This may override differences in isoform regulation and protein interactions and disguise functional differences between wild-type isoforms.

What is the role of AKT1 and AKT2 in GBM? AKT1 mRNA is associated with poor patient outcome but was difficult to study in our models because of poor activation. Further study will require a different model. Overall, however, our data support a tumor promoting role for AKT2: (1) its mRNA is positively associated with grade, (2) it is activated in 5/5 of the GBM tumors examined and (3) it is the only isoform signaling to mTORC1 in our cell lines. This is consistent with literature indicating AKT2 promotes cell proliferation, invasion and

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chemoresistance in glioma cells [32, 33]. What might explain our study's failure to support an association between AKT2 mRNA and survival? One possible explanation is a mechanism that activates AKT2 but not the other isoforms at the post-translational level [34].

The serine/threonine kinase mTOR is an important component of the PI3K pathway [35–37]. It exists in 2 complexes called mTORC1 and mTORC2 [38]. mTORC1 links growth factor signaling to energy metabolism, growth, autophagy and neurogenesis. mTORC2 phosphorylates and regulates activity of AKT and other kinases. Our data indicates AKT2 activates mTORC1 but AKT3 does not (Fig. 5b, c). We need further studies to decipher the functional consequences of this difference but the data indicate AKT isoforms do not share all substrates in Glioblastoma cells.

Evidence is strong for AKT3's role in promoting proper brain development. For example, activating somatic mutations or increased gene dosage of AKT3 cause human developmental brain overgrowth disorders [6, 39]. Conversely, lack of AKT3 is associated with human microcephaly and complete or partial absence of the corpus callosum [5, 40, 41]. Furthermore, AKT3 knockout mice brains are small due to both smaller and fewer cells [4]. How can this evidence be reconciled with our data indicating a role for AKT3 in delaying tumor initiation and/or progression? We suggest AKT3 regulates neuronal progenitors and differentiation during cortical development. Supporting this are these facts: (1) AKT3 is the predominant AKT isoform mRNA expressed during human embryonic cortical development [6] and (2) levels of p-AKT ser473 [6] (which could include all 3 isoforms) and its direct substrates p-GSK3 Ser9 and p-beta catenin Ser552 [42] are high in the ventricular zone of the developing cortex of mouse brains and localized in apical progenitor cells. This supports a role for AKT3 in differentiation of the developing cortex. Indeed other genes promote both proliferation and differentiation depending on context. For example, the proneural gene, ASCL1 (MASH-1), promotes successive phases of proliferation, then cell cycle exit, and differentiation in the developing cortex [43]. We speculate AKT3 loss promotes dedifferentiation, increased stem cell-like character, and tumor progression of GBM. Further studies are required to test this hypothesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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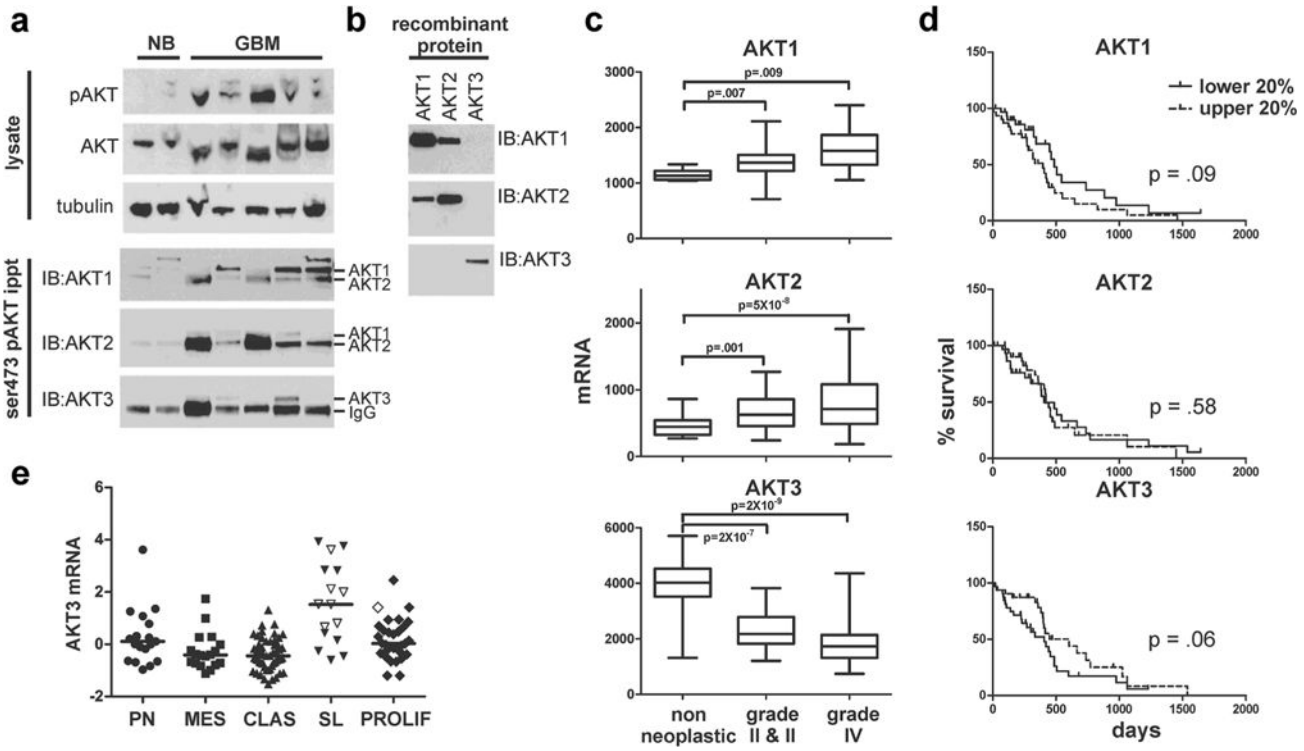

Fig. 1.

AKT isoforms in human GBM tissue. **a** p-AKT Ser473 was immunoprecipitated from 5 GBM and 2 non-neoplastic brain tissue (NB) lysates. Pre-immunoprecipitation lysates (lysate) and immunoprecipitate (ser473 p-AKT ippt) were analyzed by western blot using antibodies against AKT isoforms. **b** Specificity and cross-reactivity of AKT isoform antibodies were evaluated in immunoblots of recombinant, purified, tagged AKT1, 2 and 3. **c** Expression of AKT isoform mRNA in non-neoplastic tissue, WHO grade II and III and WHO grade IV astrocytic tumors is represented as a *box plot* (whiskers encompass the minimum and maximum; *box* represents the 25th to the 75th percentile; the *bar* is the median). **d** Kaplan–Meier survival curves for GBM patients with the highest and lowest 20 % AKT isoform mRNA expression (TCGA, RNA Seq). Statistical significance evaluated using the Gehan-Breslow-Wilcoxon test. **e** Expression of AKT3 mRNA in each AKT subtype. AKT3 RNA Seq Z scores relative to diploid tumors from 283 TCGA tumors downloaded from cBIO on 9/16/14. IDH1 mutant cases are identified by an *open symbol*

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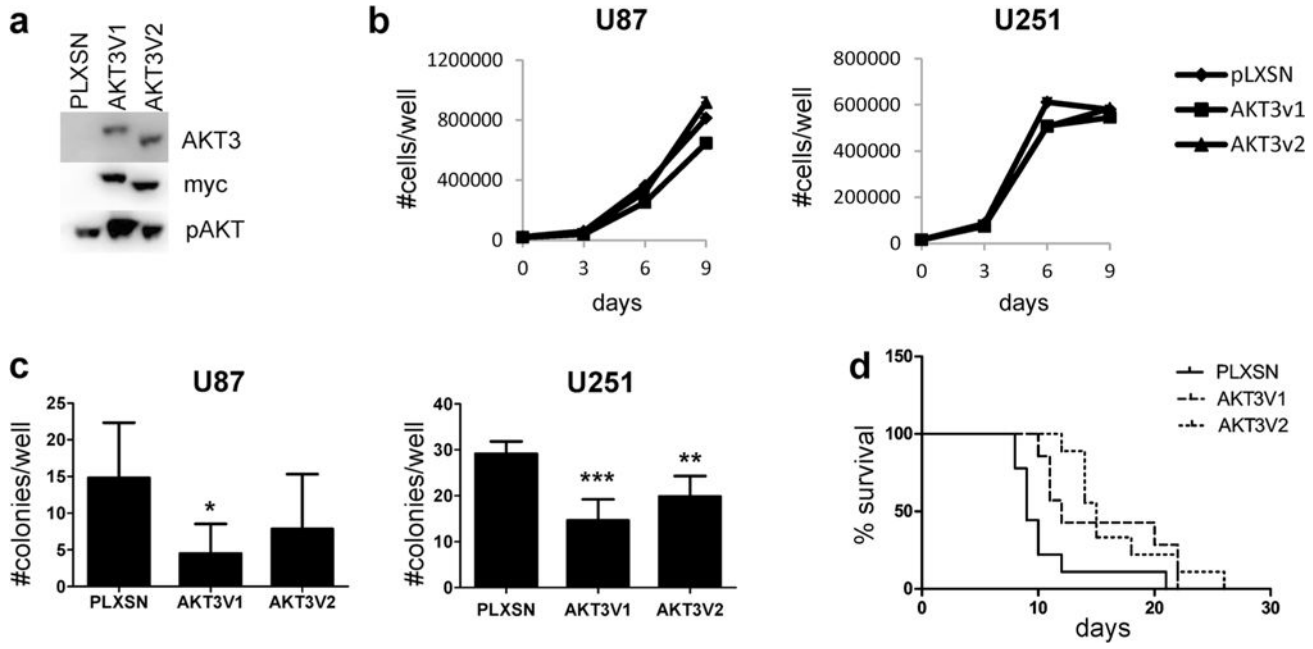

Fig. 2.

Akt3 overexpression decreases colony forming efficiency in glioma cells and increases survival in a rodent GBM model. **a** Western analysis for Akt3, myc-tag and ser473 pAkt in U87 cells stably transfected with myc-tagged Akt3v1 and Akt3v2 constructs or PLXSN vector control. **b** Growth rate of U87 and U251 cells stably infected with Akt3v1 and Akt3v2 expression constructs and PLXSN empty vector control. *Error bars* represent standard deviation for 3 replicates. **c** Colony forming efficiency of U87 and U251 cells overexpressing PLXSN vector control, AKT3V1 or AKT3V2 genes. *Error bars* represent standard deviation for at least 5 replicates. Significance determined using a two-sample, two-sided t test that assumed unequal variance, *p < .05, **p < .001, ***p < .0001 vs. PLXSN. **d** Kaplan–Meier curves for mice with intracranial implants of U87 cells stably expressing empty vector PLXSN control, AKT3V1 or AKT3V2 genes. p = .033 PLXSN vs. AKT3V1; p = .006 PLXSN vs. AKT3V2 log rank

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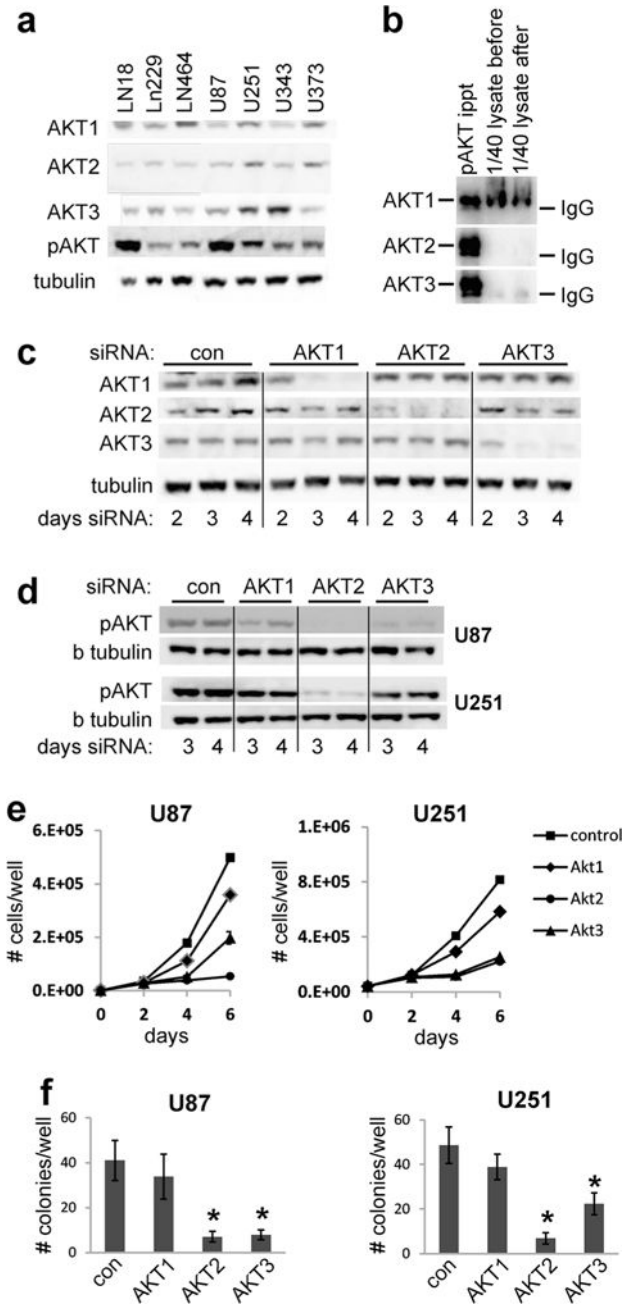

Fig. 3.

Akt2 and Akt3 silencing decreases growth rate and colony forming efficiency of glioma cell lines. **a** Western analysis for AKT isoforms and ser473 pAKT in GBM cell lines. **b** Ser473 pAKT was immunoprecipitated from U87 lysates then equivalent amounts of immunoprecipitate immunoblotted with isoform specific antibodies (ser473 AKT ippt). The pre-immunoprecipitation lysate (lysate before) and post-immunoprecipitation lysate (lysate after) were also immunoblotted in an amount equivalent to 1/40 of that used for immunoprecipitation. **c** Western analysis for Akt isoform protein in U87 cells 2, 3 and 4 days after exposure to AKT isoform siRNA. Tubulin serves as a loading control (**d**) western

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analysis for ser473 p-AKT 3 and 4 days after exposure to AKT isoform siRNA in U87 and U251 cells. Beta-tubulin serves as a loading control. **e** Effect of AKT isoform silencing on growth rate of U87 and U251 cells. *Error bars* represent standard deviation from 3 replicates. **f** Effect of AKT isoform silencing on colony forming efficiency of U87 and U251 cells. *Error bars* represent standard deviation from 6 replicates. Significance determined using a two-sample, two sided t test that assumed unequal variance; *$p < .01$

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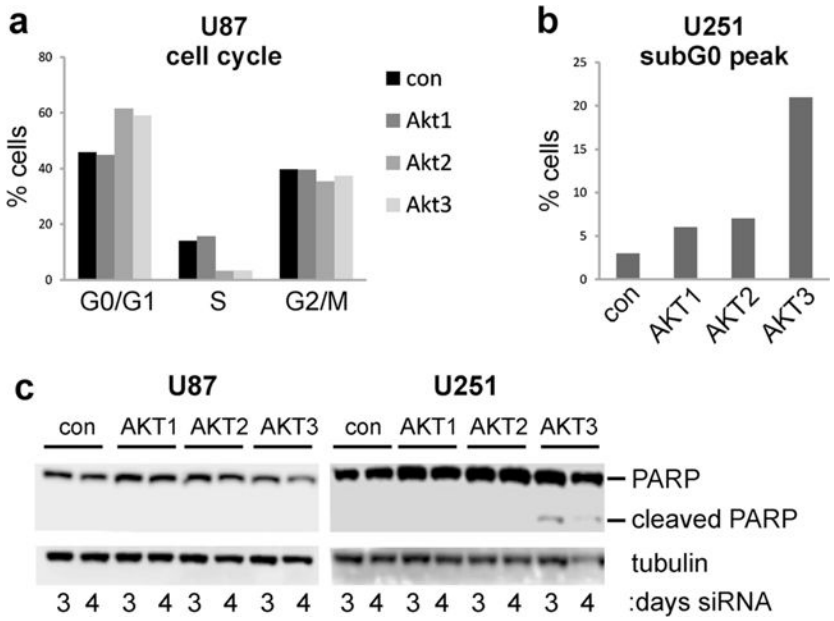

Fig. 4.

AKT2 and AKT3 silencing is cytostatic in U87 cells and induces apoptosis in U251 cells. **a** Effect of siRNA silencing of each AKT isoform on cell cycle distribution in U87 cells determined using flow cytometric analysis of PI labeled cells. Percent cells in each phase of the cell cycle was determined using FCS express and represented as a *bar graph*. **b** Effect of siRNA silencing of each isoform on % of cells in the subG0 peak in U251 cells. **c** Western analysis for PARP protein in U87 and U251 cells 3 and 4 days following siRNA silencing of each AKT isoform

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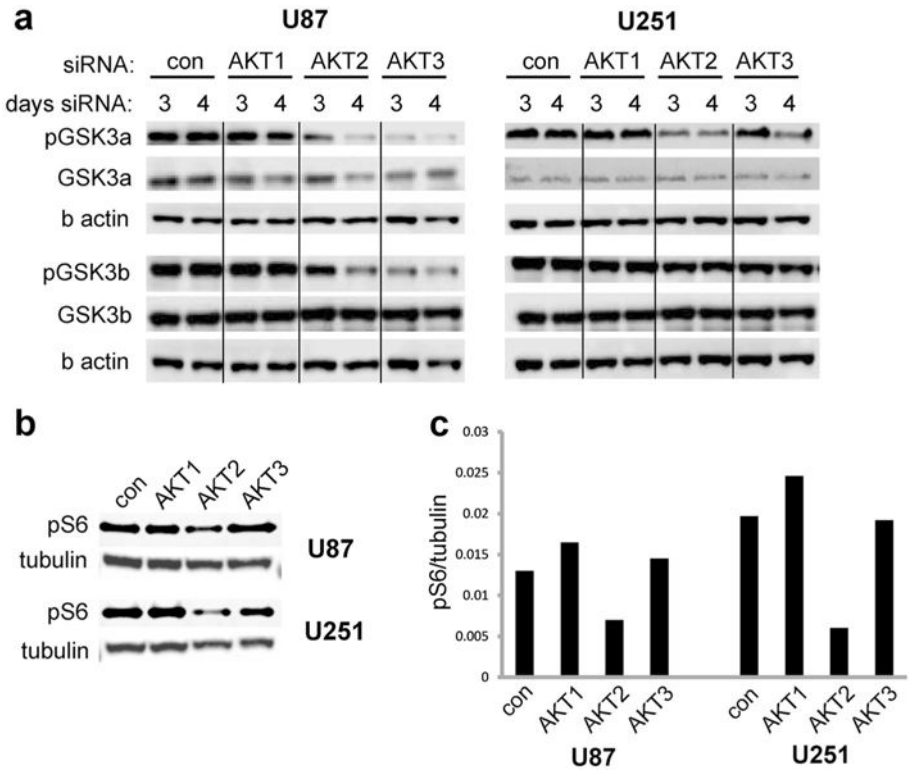

Fig. 5.

Evidence AKT2 but not AKT3 activates mTORC1. **a** Western analysis for total GSK3α and GSK3β and phosphorylated GSK3α (ser21) and GSK3β (ser9) protein 3 and 4 days following siRNA silencing of each AKT isoform. β-Actin serves as a loading control. **b** Western analysis for pS6 (ser 240/244) 3 days following siRNA silencing of AKT isoforms. Tubulin serves as a loading control. **c** Densitometric scan of (**b**) plotted as pS6/tubulin