Therapeutic Strategy for Targeting Aggressive Malignant Gliomas by Disrupting Their Energy Balance*

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Although abnormal metabolic regulation is a critical determinant of cancer cell behavior, it is still unclear how an altered balance between ATP production and consumption contributes to malignancy. Here we show that disruption of this energy balance efficiently suppresses aggressive malignant gliomas driven by mammalian target of rapamycin complex 1 (mTORC1) hyperactivation. In a mouse glioma model, mTORC1 hyperactivation induced by conditional Tsc1 deletion increased numbers of glioma-initiating cells (GICs) in vitro and in vivo. Metabolic analysis revealed that mTORC1 hyperactivation enhanced mitochondrial biogenesis, as evidenced by elevations in oxygen consumption rate and ATP production. Inhibition of mitochondrial ATP synthetase was more effective in repressing sphere formation by Tsc1-deficient glioma cells than that by Tsc1-competent glioma cells, indicating a crucial function for mitochondrial bioenergetic capacity in GIC expansion. To translate this observation into the development of novel therapeutics targeting malignant gliomas, we screened drug libraries for small molecule compounds showing greater efficacy in inhibiting the proliferation/survival of Tsc1-deficient cells compared with controls. We identified several compounds able to preferentially inhibit mitochondrial activity, dramatically reducing ATP levels and blocking glioma sphere formation. In human patient-derived glioma cells, nigericin, which reportedly suppresses cancer stem cell properties, induced AMPK phosphorylation that was associated with mTORC1 inactivation and induction of autophagy and led to a marked decrease in sphere formation with loss of GIC marker expression. Furthermore, malignant characteristics of human glioma cells were markedly suppressed by nigericin treatment in vivo. Thus, targeting mTORC1-driven processes, particularly those involved in maintaining a cancer cell's energy balance, may be an effective therapeutic strategy for glioma patients.

Abnormal metabolic regulation is critical for malignant transformation leading to cancer (1, 2). In particular, a tumor cell must maintain a proper energy balance between ATP production and its consumption to support the cell’s heightened proliferation, survival, and undifferentiated status. Historically, it has been believed that, even in the presence of oxygen, cancer cells generate energy mainly by glycolysis rather than through mitochondrial oxidative phosphorylation (OXPHOS), a concept known as the Warburg effect (3). However, several previous studies using tumor cells lacking mitochondrial DNA challenged the Warburg hypothesis, because these data showed that tumors depend on mitochondrial respiration for the maintenance of fully transformed malignant phenotypes (4–7). Supporting this idea, recent studies have demonstrated that mitochondrial activity is essential for malignant properties, such as metastasis and multidrug resistance (8–10). Accordingly, several small molecule compounds targeting mitochondrial function have been investigated for their anti-cancer effects (11). For example, numerous clinical studies of metformin, which inhibits mitochondrial complex I, have established the efficacy of this agent for cancer treatment. Other compounds that decrease mitochondrial bioenergetic capacity also have anti-tumor effects; therefore, targeting mitochondrial energetics is deemed to be a promising basis for new cancer therapies.

The mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase that belongs to the PI3K-related protein kinase family. mTOR participates in two complexes, desig-

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nated mTOR complex 1 (mTORC1) and 2 (mTORC2), both of which phosphorylate multiple substrates (12–14). Activation of PI3K via receptor tyrosine kinases leads to activation of AKT. AKT phosphorylates tuberous sclerosis complex 2 (TSC2) and blocks the GAP activity of the TSC. The TSC exhibits GAP activity toward the small G protein Rheb and inhibits its ability; therefore, TSC is a negative regulator of mTORC1. Among the substrates of mTORC1 are the p70 ribosomal protein S6 kinases (p70S6Ks), eukaryotic initiation factor (eIF), 4E-binding proteins (4E-BPs), Ulk1, Lipin1, and growth factor receptor-bound protein 10 (Grb10). Phosphorylation of 4E-BP1 by mTORC1 leads to its dissociation from eIF4E, allowing recruitment of eIF4G to the 5′ cap and translation initiation. p70S6Ks phosphorylate ribosomal protein S6, eukaryotic translation elongation factor 2 kinase (eEF2K), cap-binding protein 80 (CBP80), and eukaryotic translation initiation factor 4B (eI4FB), all of which stimulate protein synthesis. On one hand, mTORC1 activation induces aerobic glycolysis by up-regulating pyruvate kinase isoenzyme type M2 (PKM2). On the other hand, mTORC1 activation stimulates several pathways that contribute to mitochonrdial activation and OXPHOS. For example, mTORC1 is crucial for the mitochondrial activation mediated by PPAR-γ coactivator 1α (PGC1α) and the transcription factor Ying-Yang 1 (YY1) (15, 16). Another study has demonstrated that mTORC1 stimulates the synthesis of nucleus-encoded mitochondrial proteins via 4E-BPs, resulting in increased mitochondrial ATP production (17). Because mRNA translation is the most energy-consuming process in the cell, mTORC1 coordinates both energy consumption and production, contributing to malignant progression.

Glioblastoma (GBM) is the most common high grade malignant glioma in humans. GBM is categorized as a World Health Organization grade IV astrocytoma, a very aggressive, invasive, and destructive brain tumor (18). Numerous studies have identified a tumor cell population that can initiate glioma development. These cells are called glioma-initiating cells (GICs) and are conceptually recognized as “glioma stem cells” (19). It is the behavior of these GICs that determines the malignant phenotypes of GBM. Alterations in several signaling cascades are known to affect gliomagenesis, including the receptor tyrosine kinase/RAS/EGFR (EGFR, PDGF receptor, NFI, and PTEN), the p53 pathway (p53, CDKN2A/ARF, and MDM2), and the RB pathway (RB1, CDKN2A/p16INK4A, CDKN2B, and CDKN2C) (20). Consistent with the fact that activation of most oncogenic signals triggers mTORC1 activation, the phosphorylation status of substrates of mTORC1 is a prognostic indicator for glioma patients (21–23). However, although the mTORC1 pathway is clearly a major player in gliomagenesis and malignant progression, mTORC1 inhibitors, such as rapamycin and its analogs, have failed to successfully treat GBM patients in the clinic. It is speculated that a feedback loop may exist in which mTOR inhibition by the allosteric inhibitors stimulates PI3K activation, supporting survival of tumor cells. Moreover, although mTOR ATP-competitive inhibitors and PI3K/mTOR inhibitors that fully inhibit substrate phosphorylation have been developed (24), these compounds are likely to have unwanted side effects and may cause serious damage to normal tissues. Therefore, there is a pressing need to devise novel approaches to providing effective GBM therapy.

Previously, we reported that hyperactivation of mTORC1 in a murine inducible Tsc1 gene deletion resulted in early tumor onset in an EGFRVIII-driven mouse glioma model (p16\textsuperscript{hofka} p19\textsuperscript{Arf-deficient background}) (25). In this glioma model, Tsc1 deletion increased tumor mass and enhanced microvascular formation, leading to intracranial hemorrhage, indicating that mTORC1 hyperactivation promotes malignant phenotypes of glioma in vivo. In the present study, we further investigate the molecular mechanism by which mTORC1 hyperactivation causes the malignant phenotypes of glioma cells. We show that mTORC1 hyperactivation promotes mitochondrial energy production, which in turn supports GIC expansion. Importantly, we use our unique mTORC1-driven glioma model and drug screening system to identify small molecule compounds that may be effective for GBM therapy.

### Results

#### mTORC1 Hyperactivation Expands Mouse GICs in Vitro and in Vivo

To investigate the role of mTORC1 in GIC expansion, we used our previously described mouse glioma model in which mTORC1 is activated by a tamoxifen (TAM)-inducible system (25). Briefly, we injected neural stem/progenitor cells (NSPCs) of Tsc1\textsuperscript{floxed}; Rosa26-CreERT\textsuperscript{2} mice (p16\textsuperscript{hofka}\textsuperscript{−/−} p19\textsuperscript{Arf−/−} background) with retrovirus carrying EGFRVIII gene plus the huKO gene as a marker and injected these infected cells into the basal ganglia of immunocompromised mice. To activate mTORC1 in glioma tissue in vivo, we administrated TAM to NSPC-bearing recipient mice on day 5 post-transplantation. After gliomas had developed (at about 3 weeks post-transplantation), we collected huKO\textsuperscript{+} cells from digested brain tissues of recipient mice and isolated glioma cells by flow cytometry (Fig. 1A). Efficient deletion of the Tsc1 gene in this system has been confirmed previously by genomic DNA analysis (25). To determine whether Tsc1 deficiency affected sphere formation, we cultured.huKO\textsuperscript{+} cells in ultra-low attachment dishes under standard NSPC culture conditions (i.e. in the absence of serum but in the presence of the growth factors EGF and FGF2). Tsc1 deficiency significantly increased the number of spheres formed (Fig. 1B), indicating that the sphere-forming cells had expanded upon mTORC1 activation. To evaluate the tumor-initiating capacity of glioma cells in vivo, we inoculated recipient mice with 100, 1,000, or 10,000 freshly isolated huKO\textsuperscript{−} glioma cells. We found that Tsc1 deficiency promoted tumor development and accelerated the death of recipients compared with Tsc1-competent glioma cells (Fig. 1C). When as few as 10 huKO\textsuperscript{−} cells were transplanted, only Tsc1-deficient glioma cells were capable of producing gliomas and not control cells. Thus, GIC frequency is increased in vivo by mTORC1 activation.

#### mTORC1 Activation Causes Growth Factor-independent Proliferation of Mouse GICs

To investigate how mTORC1 activation affects the proliferation and survival of murine GICs, we analyzed the effect of Tsc1 deletion on sphere formation in vitro. We allowed Tsc1\textsuperscript{floxed}; Rosa26-CreERT\textsuperscript{2} glioma cells to form spheres in culture and then added 4-hydroxytamoxifen (4-OHT) to delete the Tsc1 gene. First, we confirmed that 4-OHT efficiently induced Tsc1 deletion in these sphere cells,
as evidenced by the disappearance of TSC1 protein from lysates of sphere cells that had been cultured with 4-OHT (Fig. 2A). However, unexpectedly, there was no difference in the number of spheres formed by control and Tsc1-deficient cells cultured in the presence of EGFR (Fig. 2B). This discrepancy may be due to differences between culture conditions in vitro and microenvironmental conditions in vivo. Although the level of phosphorylation in 4E-BP1 was slightly up-regulated, those of S6 and p70S6K were almost normal in Tsc1-deficient glioma cells cultured under these conditions (Fig. 2A). We speculated that mTORC1 is fully activated when cytokines are abundant and that levels of these factors are much higher in vitro than in vivo; therefore, Tsc1 deletion might not be able to further enhance such signaling in this culture condition. When we cultured control and Tsc1-deficient glioma cells in the absence of EGFR (FGF2, the size and number of spheres formed in these control glioma cell cultures was decreased compared with those in control cultures containing growth factors; however, Tsc1-deficient glioma cells showed comparable sphere-forming capacity in the presence and absence of these growth factors (Fig. 2B). Thus, mTORC1 hyperactivation maintains sphere-forming capacity even when growth factors are withdrawn. Consistent with this observation, although levels of S6 and 4E-BP phosphorylation in control glioma cells cultured without growth factors were lower than those with growth factors, such down-regulation of phosphorylation due to growth factor deprivation was not observed in Tsc1-deficient cells (Fig. 2A). Because we did not observe a remarkable change in the expression of Olig2, a glioma stem cell marker, in Tsc1-deficient glioma cells (Fig. 2C), we assume that Tsc1 deficiency promotes the proliferation and/or survival of GICs. Finally, whereas the addition of gefitinib, an EGFR inhibitor, inhibited sphere formation by control cells, it had much less effect on sphere formation of Tsc1-deficient cells (Fig. 2D). Thus, mTORC1 hyperactivation induces GIC expansion that is independent of growth factors.

Increased Sensitivity of Tsc1-deficient Glioma Cells to Glucose Depletion—We next wanted to dissect the mechanism by which mTORC1 activation affects GIC growth in our mouse glioma model. Although the metabolic status of whole glioma cells might not necessarily be identical to that of GICs due to tumor heterogeneity, we assessed metabolite levels in control and Tsc1-deficient glioma cells in culture in vitro using capillary electrophoresis TOF-MS (26, 27). Several metabolites in the glycolytic pathway, including glucose 6-phosphate (G-6-P), fructose 1,6-bisphosphate (F-1,6-BP), glycero-3-phosphoglycerate (3-PGA), and phosphoenolpyruvate (PEP), were significantly up-regulated (Fig. 3A). The same was true for components of the pentose phosphate pathway, including 6-phosphogluconolactone (6-PGL), ribulose 5-phosphate (Ru-5-P), and sedoheptulose 7-phosphate (S-7-P) (Fig. 3A). These findings suggested that glucose metabolism might be stimulated in Tsc1-deficient cells. When we analyzed gene expression levels, we found that mRNAs encoding glycolytic enzymes, such as glucose transporter 1 (Glut1), hexokinase 2 (Hk2), and pyruvate kinase M2 (Pkm2), were all elevated by mTORC1 activation (Fig. 3B). Consistent with the observed increase in Glut1 expression, glucose uptake, which was evaluated by incorporation of the fluorescent glucose analog 2-NBDG, was promoted by Tsc1 deficiency (Fig. 3C). In addition, Tsc1-deficient cells were markedly more sensitive to glucose deprivation compared with controls and showed decreased viability (Fig. 3D). In contrast, a reduction in glutamine, which is an alternative carbon source utilized by several cancer cell types, had a comparable effect on the viability in both control and Tsc1-deficient cells (Fig. 3E). These data indicate that mouse...
glioma cells experiencing mTORC1 hyperactivation show increased dependence on glucose for survival.

Enhanced Mitochondrial ATP Production Supports mTORC1-driven GIC Expansion—Our metabolomic analysis showed that lactate levels in glioma cells were not significantly affected by Tsc1 deletion (Fig. 3A). These data suggested that the increased glucose uptake exhibited by Tsc1-deficient cells might contribute to enhanced mitochondrialOXPHOS rather than to the production of lactate via typical glycolysis. To determine OXPHOS in these cells, we evaluated the OCR and found that it was significantly increased in Tsc1-deficient glioma cells (Fig. 4A). In addition, the mitochondrial copy number (Fig. 4B) and expression levels of mitochondria-associated genes were up-regulated by Tsc1 deletion (Fig. 4C). Consistent with this enhanced mitochondrial activity, ATP levels were increased in Tsc1-deficient cells compared with controls (Fig. 4D, left). To assess whether this increase in ATP in Tsc1-deficient cells was in fact due to enhanced OXPHOS, we treated the cells with oligomycin, an ATP synthetase inhibitor. Interestingly, whereas oligomycin had only a modest effect on ATP levels in control cells, it dramatically reduced ATP levels in Tsc1-deficient cells treated with/without EGF and FGF2 (Fig. 4D, right). Glucose starvation also led to a striking reduction in ATP in Tsc1-deficient cells but not in control cells (Fig. 4E), indicating that mTORC1 hyperactivation drives reliance on mitochondrial activity as the cell’s primary energy source. Consistent with the marked ATP reduction in oligomycin-treated Tsc1-deficient glioma cells, oligomycin also profoundly suppressed sphere formation by Tsc1-deficient cells compared with controls (Fig. 4F). These results indicate that mTORC1 hyperactivation stimulates mitochondrial ATP production that is vital for the vigorous expansion of GICs.

Drug Screening to Identify Small Molecule Compounds That Can Suppress Sphere Formation by Tsc1-deficient Mouse Glioma Cells—The new application of a known drug, called drug repositioning or drug repurposing, has been a beneficial approach for developing novel therapies for human diseases. With this in mind, we assessed whether our mouse glioma model would be useful for drug screening to identify known compounds able to specifically inhibit the aggressive phenotypes of glioma cells. To this end, we evaluated the effects of numerous small molecule compounds from commercially available existing drug libraries (a total of 1,301 compounds) on the proliferation/survival of control and Tsc1-deficient mouse glioma cells. To compare the efficacy of an individual compound on control versus Tsc1-deficient cells, we first estimated the inhibitory effect of each compound on both types of cells and then calculated the ratio of the inhibitory effect on Tsc1-deficient cells compared with its effect on control cells; we termed this ratio the “index for drug sensitivity of Tsc1-deficient cells” (see “Experimental Procedures”). Most compounds screened exhibited an index of about 1.0 ± 0.5 (Fig. 5A), indicating that they had equal effects on control and Tsc1-deficient cells. Several compounds showed low Index values, suggesting that these drugs were less effective in inhibiting the growth of Tsc1-deficient cells than that of control cells. For example, we found that the EGFR inhibitors gefitinib and erlotinib showed less efficacy in Tsc1-deficient cells than in control cells (Fig. 5B, left) (data not shown), consistent with our findings in Fig. 2D. Several genotoxic reagents, including mitoxantrone and topotecan, were also less efficacious in Tsc1-deficient cells (Fig. 5B, right) (data not shown), suggesting that mTORC1 hyperactivation allows glioma cells to resist conventional chemotherapy. In contrast to the above, we identified several compounds that were highly effective in inhibiting the growth of Tsc1-deficient glioma cells compared with that of control cells (Fig. 5C).

From our first screening, we selected 13 drugs (nigericin, amoxapine, A23187, auranofin, rottlerin, valinomycin, minocycline, nifedipine, pentamidine, cyclosporine, clodronic acid, clindamycin, and moxifloxacin) that showed reproducible increased efficacy in Tsc1-deficient cells compared with controls. The concentra-
The behavior of human GBM cells as well as mouse Tsc1-deficient cells, we applied each agent to human patient-derived GBM cell lines (TGS-01 and TSG-04 cells). Although it is unclear exactly which GBM subtype these cells represent, they appear to have characteristics similar to the proneural type. A recent proteomics analysis has demonstrated that, compared with mesenchymal GBMs, proneural GBMs show elevated expression and activation of elements of the PI3K-AKT-mTORC1 pathway. Therefore, like our Tsc1-deficient mouse glioma cells, human TGS-01 and TSG-04 GBM cells exhibit relatively high levels of mTORC1. We found that all five of our drug compounds reduced ATP in TGS-01 cells compared with untreated GBM cells (Fig. 7A). All compounds also induced abnormality in mitochondrial membrane potential (Fig. 7B). Nigericin (a K⁺/H⁺ ion exchanger) induced mitochondrial membrane hyperpolarization, as previously reported (29), whereas A23187 (Ca²⁺ ionophore), rottlerin (K⁺ ionophore), and valinomycin (K⁺ ionophore) trig-
Energy Balance in mTORC1-driven Malignant Gliomas

FIGURE 4. Effects of Tsc1 deficiency on mitochondrial function and sphere formation in mouse glioma cells. A, quantitation of OCR by control and Tsc1-deficient cells cultured without EGF + FGF2. Data are the mean ± S.D. (error bars). B, qRT-PCR analysis of mitochondrial DNA content in the cells in A. Data were normalized to genomic DNA and are presented as the mean -fold change ± S.D. relative to control cells. C, qRT-PCR analysis of Atp5g1, Cox5a1, and cyclophilin mRNA levels in the cells in A. Data are the mean -fold change ± S.D. relative to control cells. D and E, quantitation of intracellular ATP levels in control and Tsc1-deficient cells that were cultured without EGF + FGF2 and treated with the ATP synthetase inhibitor oligomycin (D) or glucose withdrawal (E) for 12 h. Data are the mean -fold change ± S.D. relative to control cells. F, quantitation of sphere formation by control and Tsc1-deficient cells that were cultured without EGF + FGF2 and treated with the indicated concentrations of oligomycin. Data are the mean ± S.D. **, p < 0.01; ***, p < 0.001.

Nigericin Suppresses Malignant Phenotypes of Human Patient-derived GBM Cells—We next investigated whether our selected compounds might have therapeutic potential for human GBM. Among our candidates, nigericin has previously been selected by a drug screening program as being capable of targeting cancer stem cell properties, which are induced by the epithelial-mesenchymal transition. Therefore, we focused on nigericin to determine whether this compound could have an advantage in suppression of malignant phenotypes of human GBM cells in vitro and in vivo. We found that nigericin could indeed effectively reduce sphere formation by human GBM cells in culture (Fig. 8A). Whereas nigericin treatment blocked the cell cycle, specifically S-phase entry, as determined by BrdU incorporation (Fig. 8B), it did not induce significant apoptosis (data not shown). Mitochondrial reactive oxygen species (ROS) were up-regulated in glioma cells, as detected by MitoSOX (Fig. 8C), indicating that nigericin induces mitochondrial dysfunction. Moreover, expression levels of the glioma stem cell markers, Olig2 and CD133, were dramatically down-regulated during culture with nigericin (Fig. 8D and E). These data indicate that nigericin suppresses proliferation of GBM cells, associated with the loss of stem cell properties. Interestingly, nigericin clearly triggered AMPK phosphorylation that was associated with marked inhibition of phosphorylated S6K and 4E-BP1 (Fig. 8F), suggesting that down-regulation of ATP levels stimulates an anti-tumor signaling cascade that includes AMPK activation and mTORC1 inactivation. mTOR inhibition and AMPK activation are both known to induce autophagy (12), and, as expected, nigericin dramatically induced autophagy in glioma cells, as determined by an observed increase in the LC3-II/LC3-I ratio (Fig. 8F). To investigate whether nigericin inhibits its sphere formation due to abnormality in energy control, we...
increased the concentration of sodium pyruvate in culture media, because pyruvic acid supplies energy to cells through the OXPHOS in the presence of oxygen. As a result, the addition of sodium pyruvate mitigated the inhibitory effect of a low concentration, but not a higher concentration (\(0.1\) M), of nigericin on sphere formation (Fig. 8). These data suggest that a low dose of nigericin inhibits sphere formation due to partial, not complete, impairment of mitochondrial energy production. Although remarkable changes of energy signals were not observed with such a low dose of nigericin, presumably due to subtle changes below detectable limits, these data support the idea that energy imbalance causes dysfunction of GICs.

Last, we determined whether nigericin administration could inhibit glioma growth in vivo. Immunocompromised mice were injected with human GBM cells, and tumor development was monitored. Indeed, tumor volume was greatly reduced in nigericin-treated recipient mice (Fig. 9A). Histological analyses showed that important histological hallmarks for GBM malignancy, such as remarkable vasculature formation and pseudopalisading necrosis, were observed in control tumor tissues (Fig. 9B). In contrast, these malignant characteristics dramatically disappeared, associated with down-regulation of Ki67 staining, by nigericin treatment in vivo (Fig. 9B). When we evaluated the effect of nigericin on tumor cell growth in recipient mice bearing \(Tsc1^{Δ/Δ}\) or control mouse glioma cells, we found that nigericin profoundly suppressed the growth of \(Tsc1\)-deficient tumors in vivo, consistent with our in vitro results (Fig. 9C). In addition, when we evaluated the effects of our other candidate agents on human GBM cells, we found that all of these compounds suppressed sphere formation (data not shown). We selected auranofin to perform an in vivo experiment because this agent has been clinically approved for treatment of rheumatoid disease, as mentioned above. We found that auranofin treatment of glioma-bearing mice resulted in a significant reduction in GBM growth in vivo (Fig. 9D). These data clearly indicate that our screening system based on an mTORC1-driven glioma model is useful for selecting compounds able to target aggressive malignant gliomas.

**Discussion**

There is much debate over the metabolic status of cancer stem cells in several types of tumors, including gliomas. One
study reported that GICs are primarily glycolytic and that pharmacological inhibition of glycolysis decreases the tumorigenicity of these cells (32). However, another study found that undifferentiated glioma cells show predominantly mitochondrial activity (33). These discrepancies may be due to fact that metabolic status is dynamic and easily influenced by factors critical for the determination of cancer cell fate (e.g. the cell of origin of the tumor, the types of gene mutations it bears, and the nutrient conditions in the surrounding microenvironment). This complexity makes it difficult to precisely define the role of metabolic regulation in a given cancer. In our study, we focused on the relationship between metabolic status and mTOR signaling in glioma because mTORC1 hyperactivation correlates well with GBM patient prognosis (21–23). We found that mTORC1 hyperactivation increased energy dependence by mitochondrial OXPHOS. Although hyperactivation of mTORC1 is known to stimulate not only mitochondrial activity but also the metabolism of lipids, nucleotides, and other cellular components, our data clearly indicate that enhanced bioenergetic metabolism of lipids, nucleotides, and other cellular components is crucial for expansion of GICs, supporting glioma because mTORC1 hyperactivation correlates well with GBM patient prognosis (21–23). We found that mTORC1 hyperactivation increased energy dependence by mitochondrial OXPHOS. Although hyperactivation of mTORC1 is known to stimulate not only mitochondrial activity but also the metabolism of lipids, nucleotides, and other cellular components, our data clearly indicate that enhanced bioenergetic metabolism of lipids, nucleotides, and other cellular components is crucial for expansion of GICs, supporting glioma because mTORC1 hyperactivation correlates well with GBM patient prognosis (21–23). 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crisis in mTORC1-driven glioma cells. It remains possible that nigericin and the other drug candidates selected by our screening process have effects in addition to their reduction of ATP levels that result in suppression of mTORC1-driven malignant phenotypes. For example, nigericin reportedly affects inflammasome activation (37), and such additional effects could conceivably contribute to suppression of tumor growth in vivo. Although we are not currently able to exclude this possibility for individual agents, the fact that our system efficiently selected several compounds that induce mitochondrial abnormalities supports our hypothesis that targeting the altered energy balance in glioma cells has the potential to bring therapeutic benefits to glioma patients.

The biguanide metformin was originally used for treatment of diabetes mellitus but was recently found to have a potent tumor-suppressive effect that is independent of its anti-hyperglycemic function (11). A key outcome of metformin treatment is inhibition of mitochondrial complex I in the electron transport chain, which leads to an increase in the AMP/ATP ratio. This imbalance in turn induces activation of AMPK, a critical energy sensor that integrates multiple signaling pathways. In our study, we found that, like metformin, nigericin induces AMPK activation associated with mTORC1 inhibition. We also uncovered the interesting possibility that disruption of a cell’s energy balance may induce glioma cell differentiation mediated by AMPK activation. On the other hand, more detailed evaluations of our observations are required for their translational application because nigericin administration did not greatly extend the survival of recipient mice bearing human GBM cells in the brain (data not shown), presumably due to the inability to penetrate the blood–brain barrier. Therefore, the characterization of individual compounds for their pharmacokinetics/pharmacodynamics in brain tissues is also important for any drug repositioning or drug repurposing designed to treat GBM patients. Further investigation is required to determine how these compounds exert tumor suppression and to develop efficient anticancer therapeutics.

**Experimental Procedures**

**Mice**—Rosa26-CreER<sup>T2</sup> mice were the kind gift of Dr. Tyler Jacks (Massachusetts Institute of Technology). p16<sup>Indk<sup>+/−</sup> p19Arf<sup>+/−</sup> mice were obtained from the Mouse Models of...
Human Cancers Consortium of NCI-Frederick, National Institutes of Health (38, 39). Tsc1f/f mice were purchased from the Jackson Laboratory. For transplantation experiments using mouse and human glioma cells, BALB/c nu/nu mice (4-week-old females) were purchased from Sankyo Laboratory Service. All animal experiments were approved by the Committee on Animal Experimentation of Kanazawa University and performed following the university’s guidelines for the care and use of laboratory animals.

**Mouse Glioma Model**—Glioma-bearing mice were established as described previously (25). Briefly, primary NSPCs were isolated from subventricular zone regions of Tsc1f/f; Rosa26-CreER<sup>T2</sup> mice (p16<sup>loxp-loxp</sup>−/−; p19<sup>Arf</sup>−/− background) and cultured in Coaster Ultra-Low attachment plates (Corning Inc.) in serum-free NSPC medium, which contained DMEM/F-12, B27, and 50 units/ml penicillin, 0.5% streptomycin (all from Life Technologies), plus 20 ng/ml human FGF2 (Wako) and 20 ng/ml human EGF (Sigma). For preparation of retrovirus carrying EGFRvIII, Plat-E cells, provided by Dr. Toshio Kitamura (Institute of Medical Science, University of Tokyo), were transfected with pGCDN-EGFRvIII-IRESS-humanized Kusabira-Orange (huKO), provided by Dr. Masafumi Onodera, National Research Institute for Child Health and Development (40). Retrovirus-containing supernatants were concentrated by centrifugation at 6,000 × g for 16 h. Cultured primary NSPCs were infected with pGCDN-EGFRvIII-IRESS-huKO-expressing retroviruses for 24 h and maintained in culture until transplantation. EGFRvIII-transduced NSPCs were dissociated into single cells and resuspended in 5% FBS/PBS, and 1 × 10<sup>6</sup> cells were inoculated into the brains of anesthetized BALB/c nu/nu mice. For nigericin treatment in vivo, 1 × 10<sup>6</sup> huKO<sup>+</sup> cells (Tsc1f/f; Rosa-CreER<sup>T2</sup>) were subcutaneously transplanted into female BALB/c nu/nu mice. To delete the Tsc1 gene in tumor cells in vivo, recipient mice were injected i.p. with 1 mg/day TAM (Sigma) or vehicle control (corn oil, Sigma) for 4 days. To isolate glioma cells, tumor tissues were dissociated with the Brain Tumor Dissociation Kit (Miltenyi Biotec), and huKO<sup>+</sup> cells were sorted using a BD FACSaria III instrument (BD Biosciences). For transplantation of glioma cells, 100, 1,000, or 10,000 huKO<sup>+</sup> cells were inoculated into the brains of recipient mice.

**Cell Culture**—To delete the Tsc1 gene in mouse glioma cells in vitro, huKO<sup>+</sup> cells (Tsc1f/f; Rosa-CreER<sup>T2</sup>) were cultured for 3 days in complete NSPC medium (containing EGF + FGF2) plus 0.1 μM 4-OHT (Sigma). Cultures were washed clean of 4-OHT and cultured for another 2 days in complete NSPC medium. For sphere formation assays, single-cell suspensions were prepared using Accutase (Innovative Cell Technologies, Inc.) and filtered through a 40-μm cell strainer (BD Biosciences), followed by culture for 7 days in NSPC medium with or without EGF + FGF2 and containing 1% methylcellulose (Wako). Human patient-derived GBM cells, termed TGS-01 and TGS-04, were established as described previously (41). Use of these human materials and protocols was approved by the ethics committees of Kanazawa University and the University...
of Tokyo. For glucose or glutamine starvation assays, cells were cultured in DMEM/F-12 without glucose (Nacalai Tesque, Inc.) or glutamine (Life Technologies) containing B27 and penicillin/streptomycin, plus glucose (Sigma) or glutamine (Life Technologies) at the concentrations specified in the figures.

Drug Screening—Libraries used for drug screening were as follows: Food and Drug Administration-approved drug library (ENZO; CB-BML-2841J0100), ICCB known bioactives library (ENZO; CB-BML-2840J0100), kinase inhibitor library (ENZO; CB-BML-2832J0100), fatty acid library (ENZO; CB-BML-2803J0100), and phosphatase inhibitor library (ENZO; CB-BML-2834J0100). To confirm the effects of individual compounds, we assayed nigericin (Sigma-Aldrich), amoxapine (Wako), A23187 (Sigma-Aldrich), valinomycin (Sigma-Aldrich), rottlerin (Abcam), auranofin (Abcam), clodronic acid (Cayman), moxifloxacin (Sigma-Aldrich), nifidipine (Sigma-Aldrich), minocycline (Santa Cruz Biotechnology), clindamycin (Sigma-Aldrich), and pentamidine (Sigma-Aldrich). Briefly, control and Tsc1-deficient glioma cells were treated with a compound at three doses (1:500, 1:2,000, and 1:10,000 dilution of provided compounds in the library) in 384-well plates (Corning) for 48 h, followed by analysis of cell viability as mentioned below. The “index for drug sensitivity of Tsc1-deficient glioma cells” was calculated as the ratio of value 2/value 1 at a specific dose of a compound, where value 1 was for the drug efficacy in Tsc1-deficient glioma cells (e.g., 0.2 means 80% reduction), and value 2 was for the drug efficacy in control cells (e.g., 0.8 means 20% reduction). An index >1.0 means that Tsc1-deficient glioma cells were more sensitive than control glioma cells to the drug. An index <1.0 means that loss of Tsc1 induced drug resistance.

Western Blotting—Proteins were extracted with lysis buffer (0.1 M Tris (pH 6.7), 4% SDS, phosphatase inhibitor (Thermo Fisher Scientific), complete mini (Roche Applied Science)) and quantified using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific). Proteins (5 μg) were fractionated by SDS-PAGE and transferred onto 0.45-mm PVDF membranes (Millipore). Membranes were blocked in 5% (w/v) BSA, 0.02% (v/v) Tween 20, PBS and incubated with primary Abs overnight at 4 °C, followed by incubation with HRP-conjugated secondary Abs (GE Healthcare) and detection with ECL Prime (GE Healthcare). Primary Abs recognizing the following proteins were used: Tsc1 (catalog no. 4906), pp70S6K (Thr-389) (catalog no. 9234), p70S6K (catalog no. 2708), pS6 (Ser-235/236) (catalog no. 4858), S6 (catalog no. 2217), p4E-BP1 (Thr-37/46) (cat-

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**Figure 9. Therapeutic potential of nigericin and auranofin for treatment of human GBM in vivo.** A, quantitation of volumes of tumors in mice that had been subcutaneously inoculated with TGS-01 cells and treated with DMSO (control) or nigericin. Data are the mean volume ± S.D. (error bars) for tumors from control (n = 6) and nigericin-treated (n = 8) mice. Statistical analyses were performed to detect differences between treated and untreated mice. B, immuno-histochemistry analyses of tumors that were isolated from the mice in A and subjected to H&E staining or immunostaining with Ki67 antibody. Data are representative of 6 tumors examined/group. C, quantitation of volumes of tumors in mice subcutaneously inoculated with huKO3 glioma cells (Tsc1+/−; Rosa-CreERT2) and treated with/without TAM, which was administered on day 1 after tumor cell inoculation. Nigericin (4 mg/kg/day, i.p. injection, every 2 days) was administered on day 6 postinoculation. Data are the mean volume ± S.D. for tumors from control (n = 10) and nigericin-treated (n = 8) mice. Statistical analyses were performed to detect differences between treated and untreated mice. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001; ns, not significant.
Energy Balance in mTORC1-driven Malignant Gliomas

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Energy Balance in mTORC1-driven Malignant Gliomas

Abstract—Metabolic reprogramming is essential for the maintenance of tumor cell viability. Glucose uptake and the Warburg effect are important properties of cancer cells, but their role in gliomas has not been well characterized. We used cell lines and primary cultures of human glioblastoma multiforme (GBM) cells to investigate the role of the tumor suppressor protein Tsc1 in the regulation of energy metabolism. mTORC1-driven GBMs were more sensitive to mitochondrial respiratory inhibitors than were control cells, suggesting an increase in mTORC1-driven mitochondrial respiration. We found that mTORC1 inhibition decreased glucose uptake and increased oxygen consumption, whereas mTORC1 activation increased glucose uptake, ATP content, and mitochondrial mass. The mTORC1 inhibitor rapamycin decreased the O2 consumption of GBM cells, whereas the mTORC1 activator FGFR2 increased it. These results indicate that mTORC1 activity is required for maintaining the metabolic rate and survival of GBMs.

Keywords—mTORC1, glioblastoma, glucose uptake, gluconeogenesis, respiration.

Introduction—The mTORC1 complex (mammalian target of rapamycin complex 1) regulates cell growth and proliferation, survival, autophagy, and metabolism in response to extracellular signals. mTORC1 is activated by growth factors and growth factor receptors, such as the insulin-like growth factor receptor IGF-I, and by nutrient availability, including glucose and amino acids. mTORC1 activity is also influenced by the tumor suppressor gene Tsc1, which encodes tuberin, a Ras-GAP that negatively regulates mTORC1. Tsc1 deficiency results in excessive mTORC1 activity, cell proliferation, and tumor formation.

Materials and Methods—Cell lines and primary cultures of human glioblastoma multiforme (GBM) cells were used to investigate the role of the tumor suppressor protein Tsc1 in the regulation of energy metabolism. mTORC1-driven GBMs were more sensitive to mitochondrial respiratory inhibitors than were control cells, suggesting an increase in mTORC1-driven mitochondrial respiration. We found that mTORC1 inhibition decreased glucose uptake and increased oxygen consumption, whereas mTORC1 activation increased glucose uptake, ATP content, and mitochondrial mass.

Results—mTORC1 inhibition decreased the O2 consumption of GBM cells, whereas the mTORC1 activator FGFR2 increased it. These results indicate that mTORC1 activity is required for maintaining the metabolic rate and survival of GBMs.

Discussion—mTORC1 activity is required for maintaining the metabolic rate and survival of GBMs.
stained with H&E. For immunostaining, sections were treated with target retrieval solution (Dako) and stained with anti-Ki67 (BD Biosciences, 550609; 1:100), followed by visualization with an HRP-conjugated secondary Ab (GE Healthcare) and the DAB Peroxidase Substrate Kit (Vector Laboratories). Stained sections were counterstained with hematoxylin and viewed using a microscope (Axio ImagerA1, Carl Zeiss).

Statistical Analyses—Student's t test was used when comparing two groups, and one-way analysis of variance followed by Bonferroni's post hoc test was used when comparing more than two groups. For the survival analysis in Fig. 1C, differences in survival rate were analyzed by the log-rank test. Calculations of significance were performed using Prism6 software: *, p < 0.05; **, p < 0.01; *** p < 0.001; **** p < 0.0001.

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for CD44-high and CD133-low glioblastoma initiating cells. J. Neurooncol. 121, 239–250
29. Nicholls, D. G. (2006) Simultaneous monitoring of ionophore- and inhibitor-mediated plasma and mitochondrial membrane potential changes in cultured neurons. J. Biol. Chem. 281, 14864–14874
30. Rigobello, M. P., Scutari, G., Boscolo, R., and Bindoli, A. (2002) Induction of mitochondrial permeability transition by auranofin, a gold(II)-phosphine derivative. Br. J. Pharmacol. 136, 1162–1168
31. Managò, A., Leanza, L., Carraretto, L., Sassi, N., Grancara, S., Quintana-Cabrera, R., Trimarco, V., Sorrentino, L., Trentin, L., Semenzato, G., Gulbins, E., Zoratti, M., and Szabò, I. (2015) Early effects of the antineoplastic agent salinomycin on mitochondrial function. Cell Death Dis. 6, e1930
32. Zhou, Y., Zhou, Y., Shingu, T., Feng, L., Chen, Z., Ogasawara, M., Keating, M. J., Kondo, S., and Huang, P. (2011) Metabolic alterations in highly tumorigenic glioblastoma cells: preference for hypoxia and high dependency on glycolysis. J. Biol. Chem. 286, 32843–32853
33. Vlashi, E., Lagadec, C., Vergnes, L., Matsutani, T., Masui, K., Poulou, M., Popescu, R., Della Donna, L., Evers, P., Dekmezian, C., Reue, K., Christofk, H., Mischel, P. S., and Pajonk, F. (2011) Metabolic state of glioma stem cells and nontumorigenic cells. Proc. Natl. Acad. Sci. U.S.A. 108, 16062–16067
34. Gupta, P. B., Onder, T. T., Jiang, G., Tao, K., Kuperwasser, C., Weinberg, R. A., and Lander, E. S. (2009) Identification of selective inhibitors of cancer stem cells by high-throughput screening. Cell 138, 645–659
35. Heid, M. E., Keyel, P. A., Kamga, C., Shiva, S., Watkins, S. C., and Salter, R. D. (2013) Mitochondrial reactive oxygen species induces NLRP3-dependent lysosomal damage and inflammasome activation. J. Immunol. 191, 5230–5238
36. Deng, C. C., Liang, Y., Wu, M. S., Feng, F. T., Hu, W. R., Chen, L. Z., Feng, Q. S., Bei, J. X., and Zeng, Y. X. (2013) Nigericin selectively targets cancer stem cells in nasopharyngeal carcinoma. Int. J. Biochem. Cell Biol. 45, 1997–2006
37. Muñoz-Planillo, R., Kuffa, P., Martínez-Colon, G., Smith, B. L., Rajendiran, T. M., and Núñez, G. (2013) K+ efflux is the common trigger of NLRP3 inflammasome activation by bacterial toxins and particulate matter. Immunity 38, 1142–1153
38. Tuveson, D. A., Shaw, A. T., Willis, N. A., Silver, D. P., Jackson, E. L., Chang, S., Mercer, K. L., Grochow, R., Hock, H., Crowley, D., Hingorani, S. R., Zaks, T., King, C., Jacobetz, M. A., Wang, L., Bronson, R. T., Orkin, S. H., DePinho, R. A., and Jacks, T. (2004) Endogenous oncogenic K-ras(G12D) stimulates proliferation and widespread neoplastic and developmental defects. Cancer Cell 5, 375–387
39. Serrano, M., Lee, H., Chin, L., Cordon-Cardo, C., Beach, D., and DePinho, R. A. (1996) Role of the INK4a locus in tumor suppression and cell mortality. Cell 85, 27–37
40. Tamase, A., Muraguchi, T., Naka, K., Tanaka, S., Kinoshita, M., Hoshii, T., Ohmura, M., Shugo, H., Ooshio, T., Nakada, M., Sawamoto, K., Onodera, M., Matsumoto, K., Oshima, M., Asano, M., et al. (2009) Identification of tumor-initiating cells in a highly aggressive brain tumor using promoter activity of nucleostemin. Proc. Natl. Acad. Sci. U.S.A. 106, 17163–17168
41. Ikushima, H., Todo, T., Ino, Y., Takahashi, M., Miyazawa, K., and Miyazono, K. (2009) Autocrine TGF-β signaling maintains tumorigenicity of glioma-initiating cells through Sry-related HMG-box factors. Cell Stem Cell 5, 504–514
42. Hoshii, T., Tadokoro, Y., Naka, K., Ooshio, T., Muraguchi, T., Sugiyama, N., Soga, T., Araki, K., Yamamura, K., and Hirao, A. (2012) mTORC1 is essential for leukemia propagation but not stem cell self-renewal. J. Clin. Invest. 122, 2114–2129