Molecular mechanisms of metabolic regulation of glioma stem cells by mTORC1 activation

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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 the results show that disruption of this energy balance efficiently suppresses aggressive malignant gliomas driven by mTOR 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 (OCR) 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, I screened drug libraries for small molecule compounds showing greater efficacy in inhibiting the proliferation/survival of Tsc1-deficient cells compared to controls. Several compounds able to preferentially inhibit mitochondrial activity, dramatically reducing ATP levels and blocking glioma sphere formation were identified. 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.

mTORC1 hyperactivation expands mouse GICs *in vitro* and *in vivo*

To investigate the role of mTORC1 in GIC expansion, the previously described mouse glioma model in which mTORC1 is activated by a TAM-inducible system was used. After gliomas had developed, huKO$^+$ cells were collected and glioma cells isolated by flow cytometry. huKO$^+$ cells were cultured under standard NSPC culture conditions, Tsc1 deficiency significantly increased the number of spheres formed (**Fig.1**), indicating that the sphere-forming cells had expanded upon mTORC1 activation. To evaluate the tumor-initiating capacity of glioma cells *in vivo*, recipient mice were inoculated with 100, 1,000 or 10,000 freshly isolated huKO$^+$ glioma cells. Tsc1 deficiency promoted tumor development and accelerated the death of recipients compared to Tsc1-competent glioma cells (**Fig.2**). When as few as 10 huKO$^+$ cells were transplanted, only Tsc1-deficient glioma cells were capable of producing gliomas, but not control cells. Thus, GIC frequency is increased *in vivo* by mTORC1 activation.

mTORC1 activation causes growth factor-independent proliferation of mouse GICs

First, 4-OHT efficiently induced Tsc1 deletion in these sphere cells was confirmed by western blotting(**Fig.3**). However, unexpectedly, there was no difference in the number of spheres formed by control and Tsc1-deficient cells cultured in the presence of EGF+FGF2 **(Fig.4)**. This may be due to differences between conditions *in vitro* and *in vivo*. Although level of phosphorylation in 4E-BP1 was slightly up-regulated, those of S6 and p70S6K were almost normal in Tsc1-deficient glioma cells (**Fig.3**). It is 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 control and Tsc1-deficient glioma cells were cultured in the absence of EGF+FGF2, the size and number of spheres formed in these control glioma cell cultures was decreased compared to those in control cultures containing growth factors, however, Tsc1-deficient glioma cells showed comparable sphere forming capacity in presence and absence of these growth factors (**Fig.4**). Thus, mTORC1 hyperactivation maintains sphere-forming capacity even when growth factors are withdrawn. In addition, 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 depletion was not observed in Tsc1-deficient cells (**Fig.3**). Results did not show a remarkable change in the expression of Olig2, a glioma stem cell marker, in Tsc1-deficient glioma cells (**Fig.5**), which assume that Tsc1 deficiency promotes the proliferation and/or survival of GICs.

Increased sensitivity of Tsc1-deficient glioma cells to glucose depletion

metabolite levels were assessed in control and Tsc1-deficient glioma cells in

culture *in vitro* using CE-TOFMS. Several metabolites in the glycolytic pathway, including (G6P), (F1,6BP), (3PGA), and (PEP), were significantly up-regulated (Fig.6). The same was true for components of the pentose phosphate pathway, including (6PGL), (Ru5P), (S7P) (**Fig.6**). These findings suggested that glucose metabolism might be stimulated in Tsc1-deficient cells. Gene expression levels showed that mRNAs encoding glycolytic enzymes such as glucose transporter 1, hexokinase2, and pyruvate kinase M2 were all elevated by mTORC1 activation (**Fig.7**). These data indicate that mouse glioma cells experiencing mTORC1 hyperactivation show increased dependence on glucose.

Enhanced mitochondrial ATP production supports mTORC1-driven GIC expansion

Metabolomics analysis showed that lactate levels in glioma cells were not significantly affected by Tsc1 deletion (**Fig.6**). These data suggested that the increased glucose uptake exhibited by Tsc1-deficient cells might contribute to enhanced mitochondrial OXPHOS rather than to the production of lactate via typical glycolysis. To determine OXPHOS in these cells, OCR was evaluated and it was significantly increased in Tsc1-deficient glioma cells (**Fig.8**). In addition, the expression levels of mitochondria-associated genes were up-regulated by Tsc1 deletion (**Fig.9**). ATP levels were increased in Tsc1-deficient cells compared to controls (**Fig.10, left**). To assess whether this increase in ATP in Tsc1-deficient cells was in fact due to enhanced OXPHOS, the cells were treated with oligomycin, an ATP synthetase inhibitor. Although, oligomycin had only a modest effect on ATP levels in control cells, it dramatically reduced ATP levels in Tsc1-deficient cells (**Fig.10, right**). oligomycin also profoundly suppressed sphere formation by Tsc1-deficient cells compared to controls (**Fig.11**).

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. I assessed whether this 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, I 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. First the inhibitory effect was estimated for each compound on both types of cells, and then the ratio of the inhibitory effect on Tsc1-deficient cells compared to its effect on control cells was calculated; this ratio was termed the "Index for drug sensitivity of Tsc1-deficient cells". Most compounds screened exhibited an Index of about 1.0 ± 0.5 (**Fig.12**), 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, EGFR inhibitors gefitinib and erlotinib showed less efficacy in Tsc1-deficient cells than in control cells. Several genotoxic reagents, including mitoxantrone and topotecan, were also less efficacious in Tsc1-deficient cells. In contrast to the above, several compounds were identified that were highly effective in inhibiting the growth of Tsc1-deficient glioma cells compared to that of control cells. From the first screening, 13 drugs were selected that showed reproducible increased efficacy in Tsc1-deficient cells compared to controls.

Next, I screened the selected compounds for those that caused a greater reduction in intracellular ATP levels in Tsc1-deficient cells, based on the previous observation that oligomycin treatment or glucose starvation triggered a significant reduction in ATP in the former. I found that 5 drugs (nigericin, A23187, auranofin, rottlerin and valinomycin) clearly reduced intracellular ATP levels when used at less than 20 μM (**Fig.13**). I confirmed that these 5 compounds also had a greater suppressive effect on sphere formation by Tsc1-dificent glioma cells than on that by control cells (**Fig.14**), suggesting that the screening system could efficiently identify drug candidates in therapeutic approach for mTORC1-driven glioma.

To investigate the selected compounds effect on human GBM cells, I applied each agent to human patient-derived GBM cell lines (TGS-01 and TSG-04 cells). I found that all the 5 drug compounds reduced ATP in TGS-01 cells compared to untreated GBM cells (**Fig.15**). All compounds also induced abnormality in mitochondrial membrane potential (**Fig.16**). This means that treatment with most of these selected compounds might drive down intracellular ATP levels by interfering with mitochondrial ATP production.

Since it was previously reported that nigericin and valinomycin affect mitochondrial respiratory chain, Conferment of their effects was performed. After downregulation of OCR by ATP synthase inhibition (oligomycin treatment), it was recovered by valinomycin (as expected) (**Fig.17**). In contrast, nigericin treatment blocked the respiratory chain and maintained its suppression even after addition of FCCP (an uncoupler), indicating that nigericin is an efficient inhibitor of mitochondrial bioenergetics.

Nigericin suppresses malignant phenotypes of human patient-derived GBM cells

Next I investigated whether the selected compounds might have therapeutic potential for human GBM. Among the candidates, nigericin has previously been selected by a drug screening program as being capable of targeting cancer stem cell properties. Therefore, I focused on nigericin to determine if this compound could have advantage in suppression of malignant phenotypes of human GBM cells *in vitro* and *in vivo*. I found that nigericin could indeed effectively reduce sphere formation by human GBM cells in culture (**Fig.18**). While nigericin treatment blocked the cell cycle, (**Fig.19**), it did not induce significant apoptosis

(**Fig.20**). Mitochondrial ROS were up-regulated in glioma cells (**Fig.21**), indicating that nigericin induces mitochondrial dysfunction. Moreover, expression levels of the glioma stem cell markers, Olig2 and CD133, were dramatically downregulated during culture with nigericin (**Fig.22, 23**). These data indicate that nigericin suppresses proliferation of GBM cells, associated with the loss of stem cell properties. nigericin clearly triggered AMPK phosphorylation that was associated with marked inhibition of phosphorylated S6K and 4EBP1 (**Fig.24**), suggesting that downregulation 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, as determined by an observed increase in the LC3-II/LC3-I ratio **(Fig.24)**. To investigate whether nigericin inhibits sphere formation due to abnormality in energy control, concentration of sodium pyruvate was increased in culture media, because pyruvic acid supplies energy to cells through the OXYPHOS in the presence of oxygen. As a result, addition of sodium pyruvate mitigated the inhibitory effect of low concentration, but not higher concentration (>0.1 μM), of nigericin on sphere formation (**Fig.25**). These data suggest that low dose of nigericin inhibits sphere formation due to partial, but not complete, impairment of mitochondrial energy production.

Lastly, I 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.26**). Histological analyses showed that important histological hallmarks for GBM malignancy were observed in control tumor tissues. In contrast, these malignant characteristics dramatically disappeared by nigericin treatment *in vivo* (**Fig.27**). Also, down-regulation of Ki67 staining, by nigericin treatment *in vivo* (**Fig.28**). When the effect of nigericin on tumor cell growth in recipient mice bearing $Tsc1^{\Delta/\Delta}$ or control mouse glioma cells were evaluated, I found that nigericin profoundly suppressed the growth of Tsc1-deficient tumors *in vivo*, consistent with *in vitro* results (**Fig.29).** In addition, when I evaluated the effects of other candidate agents on human GBM cells, I found that all of these compounds suppressed sphere formation (**Fig.30,31**). Then, auranofin was selected to perform an *in vivo* experiment. auranofin treatment of glioma-bearing mice resulted in a significant reduction in GBM growth *in vivo* (**Fig.32**). These data clearly indicate that this screening system based on an mTORC1-driven glioma model is useful for selecting compounds able to target aggressive malignant gliomas.

Fig. 1

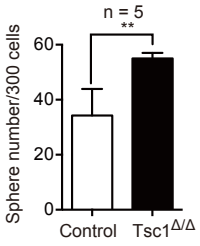


Fig. 2

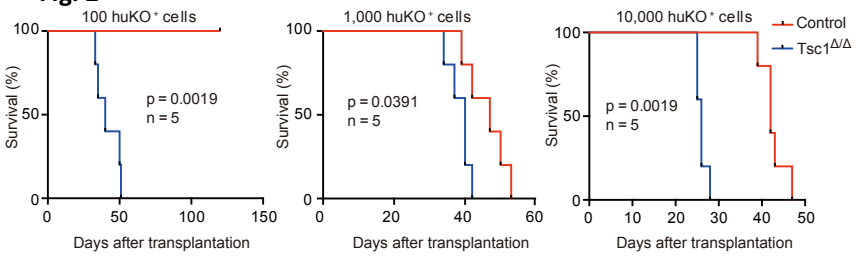


Fig. 3

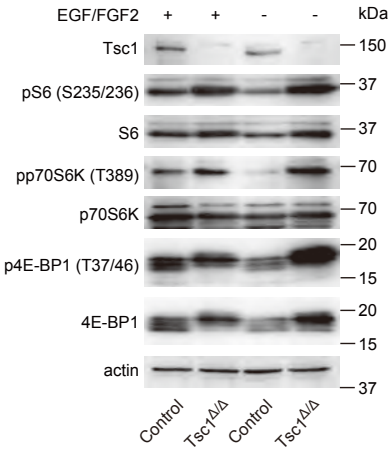


Fig. 4

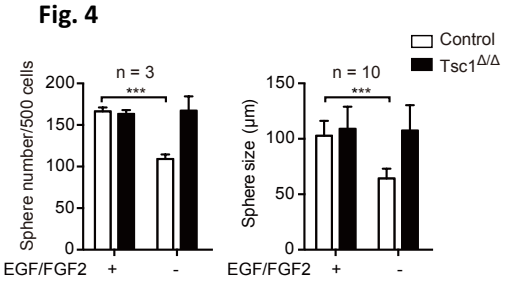
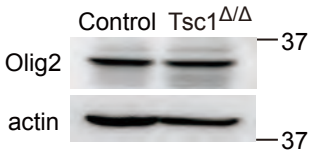


Fig. 5



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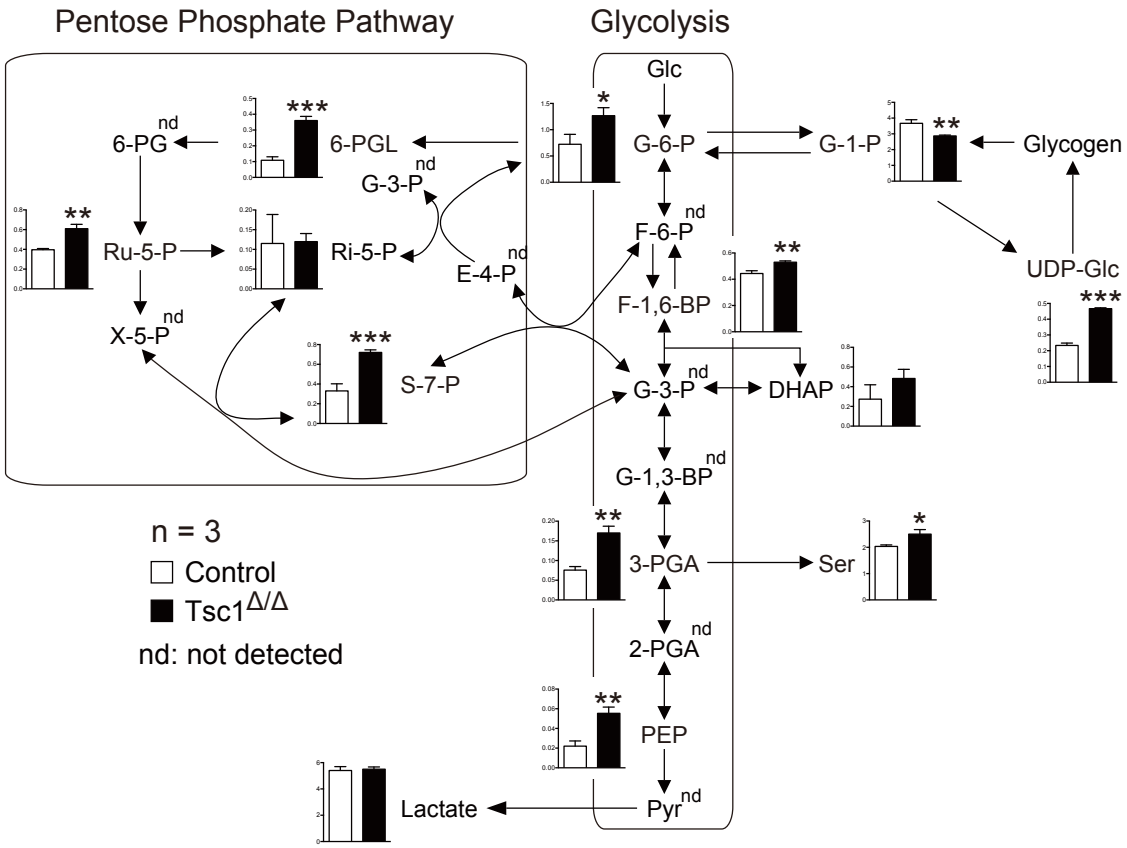

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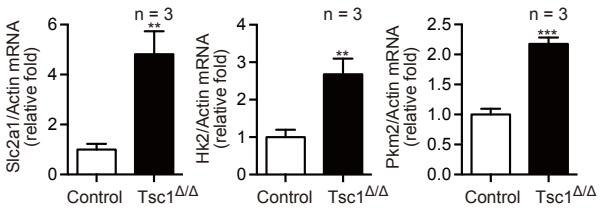

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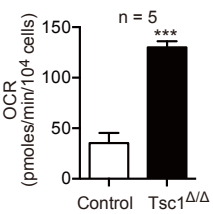

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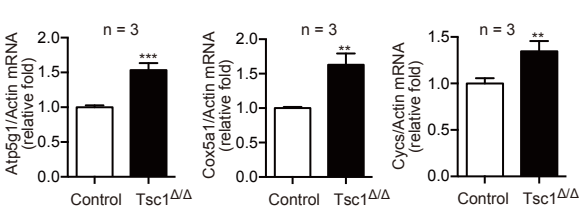
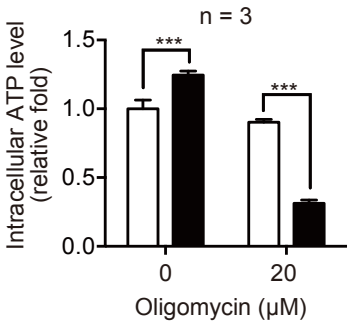


Fig.10



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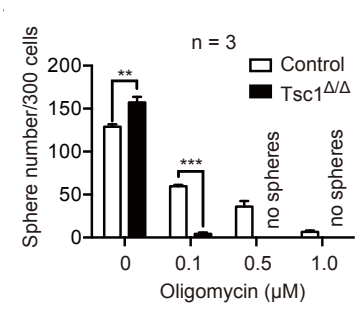

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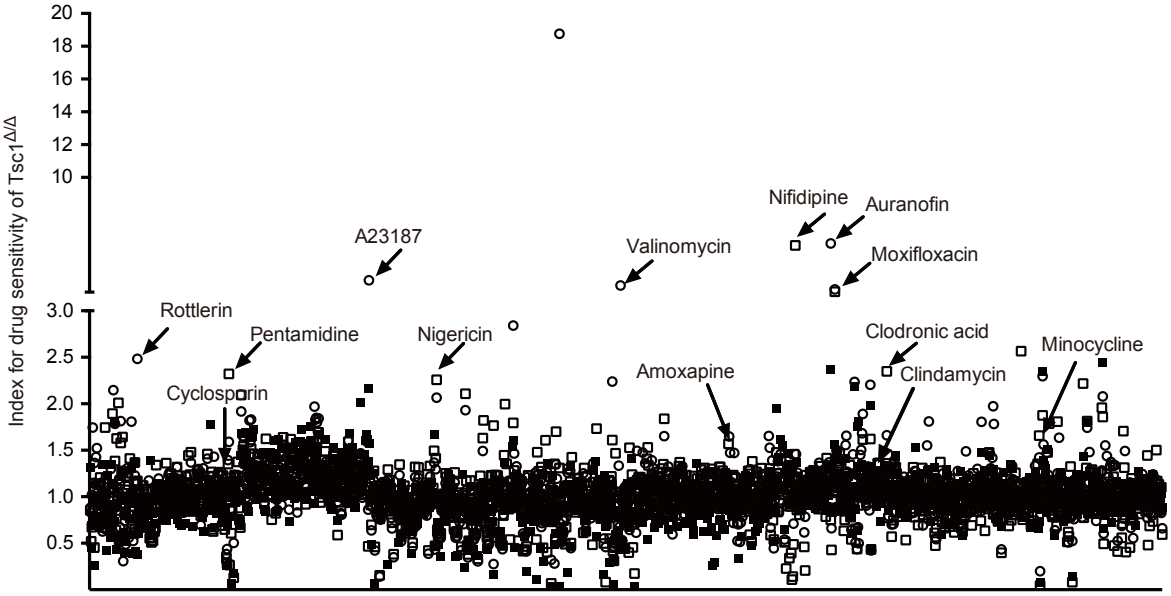
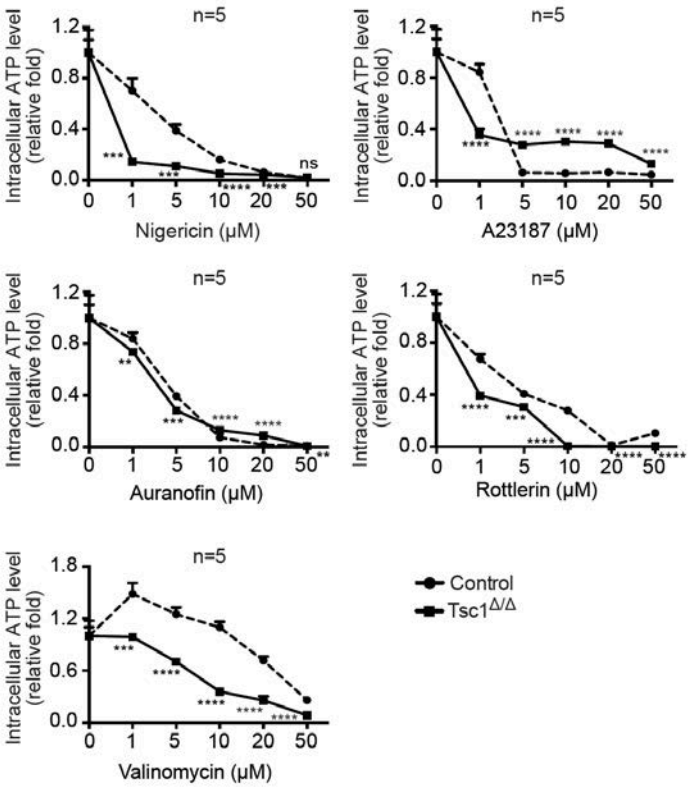


Fig.13



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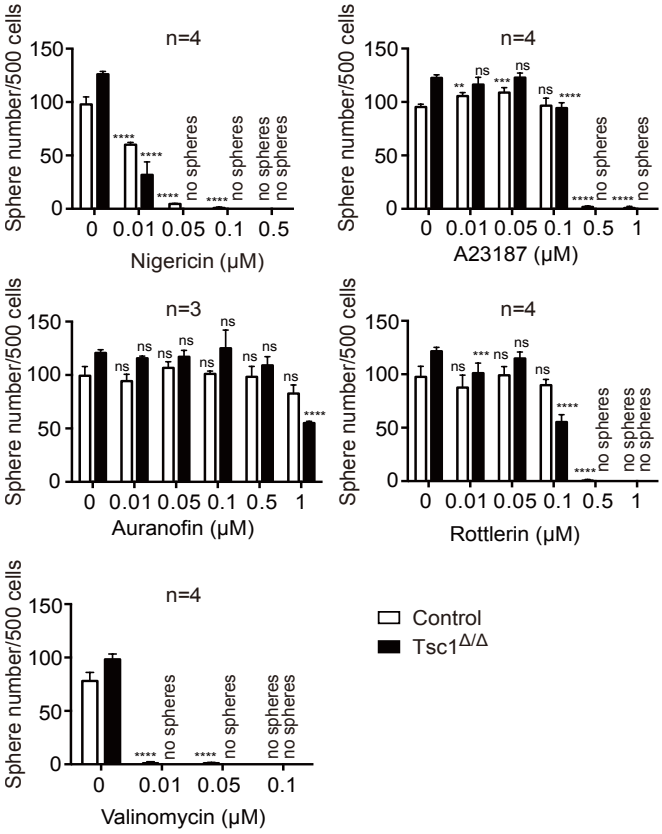

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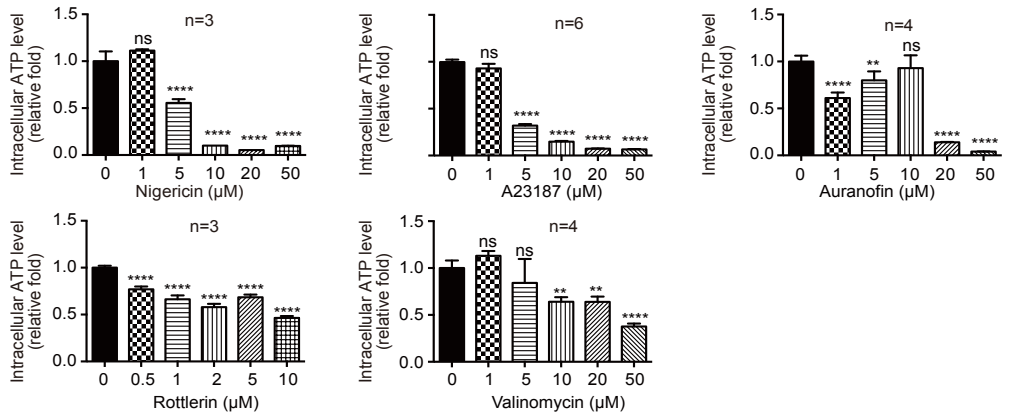
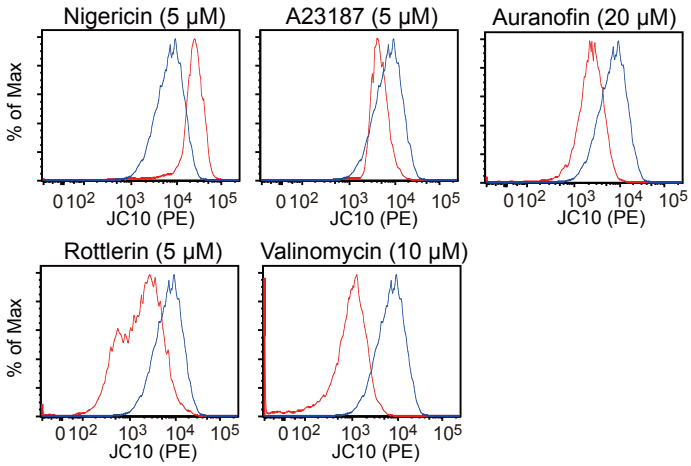


Fig. 16



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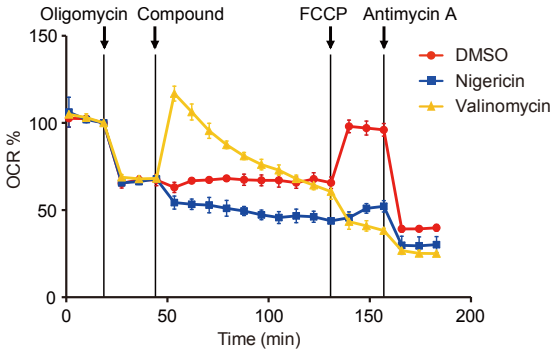

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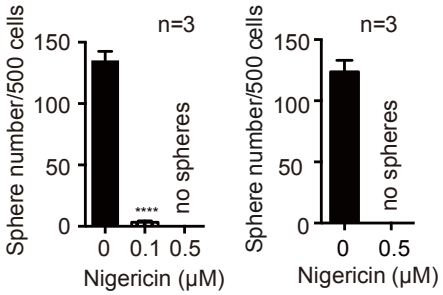

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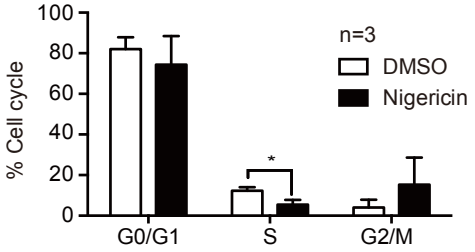

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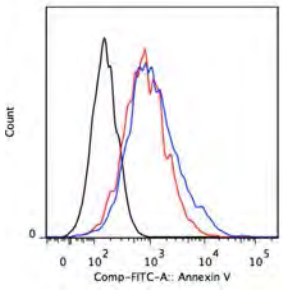

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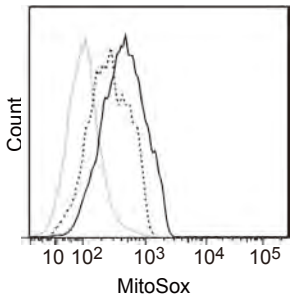

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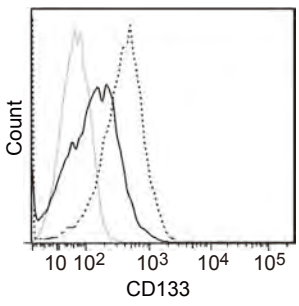

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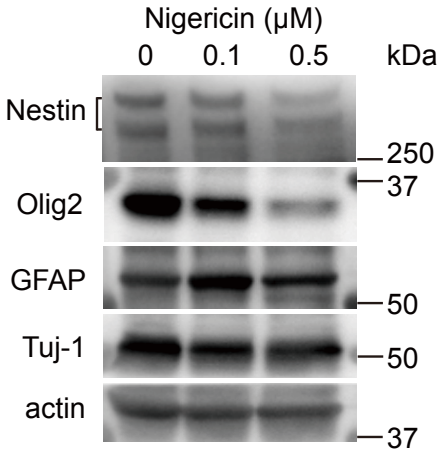

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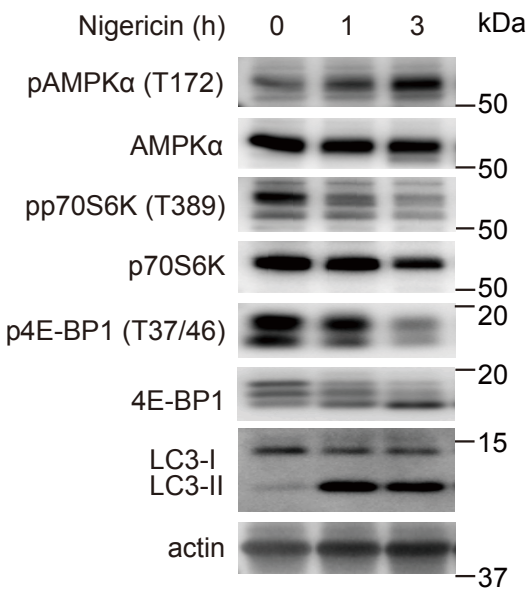

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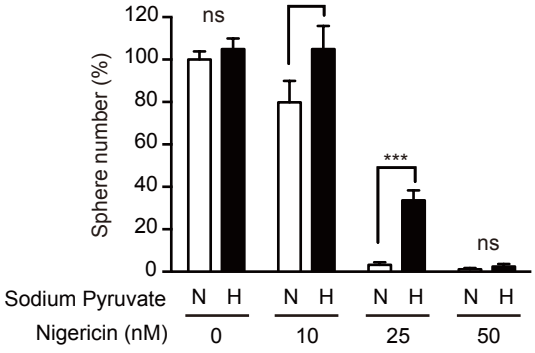

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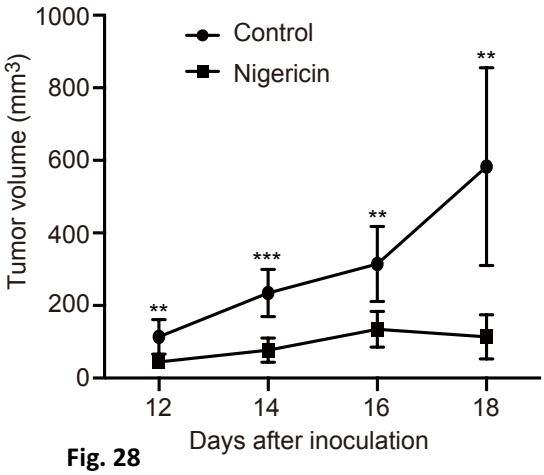

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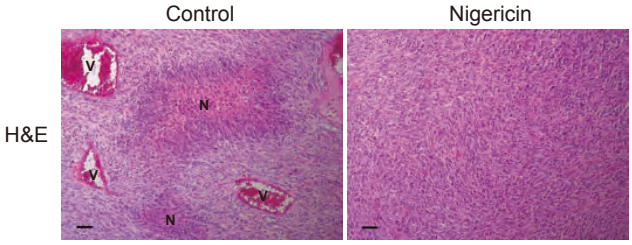

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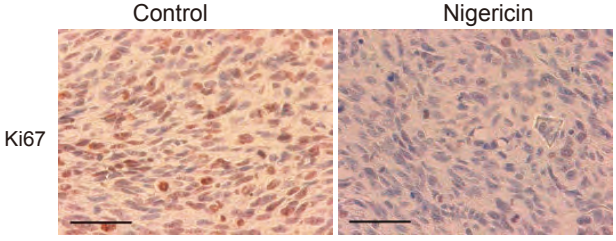

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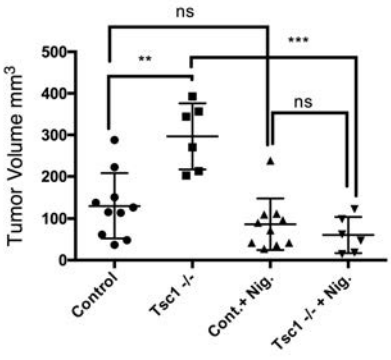
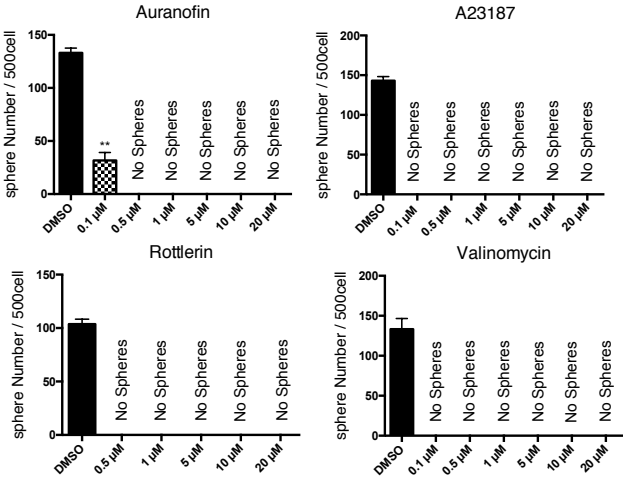


Fig. 30



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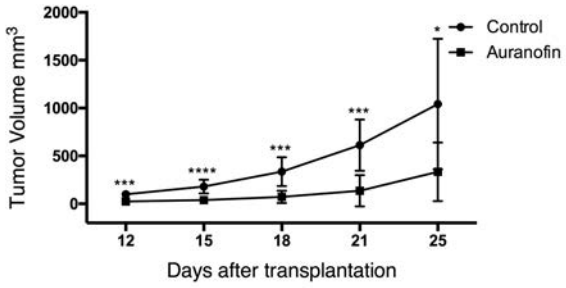

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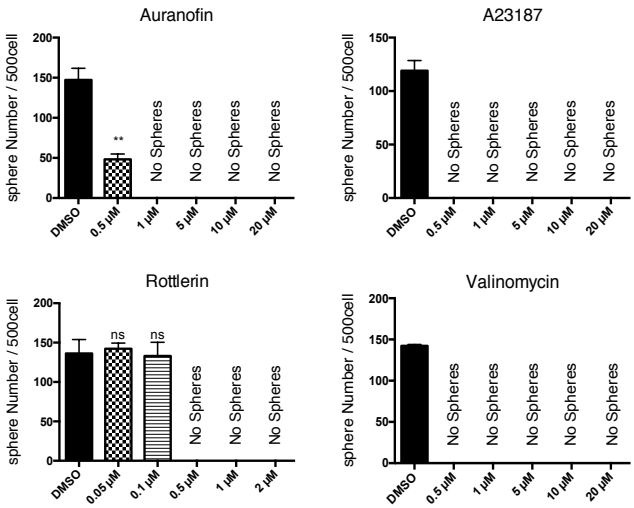

平成２８年　８月　２日

学位論文審査報告書（甲）

１．学位論文題目（外国語の場合は和訳を付けること。）

Molecular mechanisms of metabolic regulation of glioma stem cells by mTORC1 activation

（mTOR 複合体１活性化による神経膠腫幹細胞の代謝調節機構）

２．論文提出者　(1) 所　　属　　生命科学　専攻

(2) 氏（ふり）　名（がな）　モハメド ヒガージ モハメド アーメド

３．審査結果の要旨（600～650 字）

神経膠芽腫（グリオーマ）は、極めて悪性度の高い脳腫瘍である。グリオーマにおいて顕著に活性化し、患者の生命予後とも相関するシグナルが mTOR である。mTOR 複合体 1 (mTORC1)は、セリンスレオニンキナーゼ複合体であり、蛋白合成や代謝調節に寄与する。本研究では、mTORC1 によるエネルギー代謝とがんの悪性化に焦点を当て研究を進めた。マウスグリオーマモデルにおいて、mTORC1 活性を過剰に亢進させる系を用いて解析した結果、mTORC1 の異常活性化は、グリオーマ形成に寄与する glioma-initiating cell (GIC)を増加させることを観察した。さらに、mTORC1 活性型グリオーマ細胞では、ATP 産生亢進が顕著であることから、mTOR 依存的なエネルギー産生亢進が、GIC の増幅に寄与していると考えられた。そこで、これらの細胞を用いて、化合物スクリーニングを行ったところ、ミトコンドリア活性阻害を示す複数の化合物が、mTORC1 活性型グリオーマ細胞に対し強い増殖抑制効果を示すことが判明した。これらの中には、ヒトグリオーマ細胞に対しても、顕著な ATP レベルの低下、AMPK の活性化、オートファジーの亢進を誘導し、さらには生体内での増殖抑制効果を示すものも存在した。以上の結果より、グリオーマの悪性進展には、エネルギー代謝調節が極めて重要な役割を果たしていると考えられた。本研究結果は、グリオーマの悪性化制御機構における新知見であり、本論文が学位に値すると評価された。

４．審査結果　(1) 判　　定（いずれかに○印）　（合　格）・　不合格

(2) 授与学位　　博　士（　理学　）