

Insulin-mediated signaling promotes proliferation and survival of glioblastoma through Akt activation

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Background. Metabolic complications such as obesity, hyperglycemia, and type 2 diabetes are associated with poor outcomes in patients with glioblastoma. To control peritumoral edema, use of chronic high-dose steroids in glioblastoma patients is common, which can result in de novo diabetic symptoms. These metabolic complications may affect tumors via profound mechanisms, including activation of insulin receptor (InsR) and the related insulin-like growth factor 1 receptor (IGF1R) in malignant cells.

Methods. In the present study, we assessed expression of InsR in glioblastoma surgical specimens and glioblastoma response to insulin at physiologically relevant concentrations. We further determined whether genetic or pharmacological targeting of InsR affected oncogenic functions of glioblastoma in vitro and in vivo.

Results. We showed that InsR was commonly expressed in glioblastoma surgical specimens and xenograft tumor lines, with mitogenic isoform-A predominating. Insulin at physiologically relevant concentrations promoted glioblastoma cell growth and survival, potentially via Akt activation. Depletion of InsR impaired cellular functions and repressed orthotopic tumor growth. The absence of InsR compromised downstream Akt activity, but yet stimulated IGF1R expression. Targeting both InsR and IGF1R with dual kinase inhibitors resulted in effective blockade of downstream signaling, loss of cell viability, and repression of xenograft tumor growth.

Conclusions. Taken together, our work suggests that glioblastoma is sensitive to the mitogenic functions of insulin, thus significant insulin exposure imposes risks to glioblastoma patients. Additionally, dual inhibition of InsR and IGF1R exhibits promise for treating glioblastoma.

Keywords: Akt, glioblastoma, IGF1R, insulin, insulin receptor.

It has been extensively documented that metabolic complications, such as obesity, hyperglycemia, and type 2 diabetes, are associated with increased cancer risk and poor prognosis in cancer patients.¹ Several recent studies suggest that glioblastoma patients affected by these metabolic complications have worse prognosis compared with those with normal metabolic conditions.^{2–5} However, the exact mechanisms through which compromised glucose metabolism affects tumors remain unsolved. One potentially important mechanism is

mediated by the increased insulin levels that promote tumor cell growth and survival via activation of the signaling network of insulin receptor (InsR) and insulin-like growth factor 1 receptor (IGF1R). Patients with glioblastoma are commonly treated with glucocorticoids for the management of peritumoral edema.⁶ Chronic use of glucocorticoid in glioblastoma patients is known to induce insulin resistance, which upregulates gluconeogenesis and leads to hyperglycemia.⁶ In these patients, acute hyperglycemia is often managed with insulin

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and insulin analogs, raising concerns over the oncogenic activity of insulin.

Insulin and the related IGF1 and IGF2 have mitogenic activities in addition to their roles in the regulation of metabolic homeostasis. The receptors of these ligands, InsR and IGF1R, are widely expressed and aberrantly activated in human cancers.⁷ InsR and IGF1R form homodimers or heterodimers that are differentially recognized by their ligands. The InsR homodimer is primarily activated by insulin. However, the fetal isoform InsR-A, which is commonly expressed in cancers, can also be activated by IGF2. The IGF1R homodimer and the InsR/IGF1R heterodimer receptors are preferentially activated by IGF1.⁷ IGF1R is overexpressed in many cancers and promotes malignant phenotypes and therapeutic resistance. However, trials involving monotherapy with IGF1R neutralizing antibodies have shown limited therapeutic potential.^{8,9} The ineffectiveness of IGF1R-specific antibodies can be attributed, at least in part, to compensatory activation of InsR.¹⁰ The roles of insulin and InsR in neoplasms have not been studied as extensively as IGF1R-mediated signaling. Epidemiological data indicate that obesity and diabetic conditions not only are associated with increased risks of certain cancers, but also predict poorer outcomes in cancer patients.^{11–14} Even in nondiabetic patients, elevated plasma insulin levels are associated with higher rates of recurrence and mortality in some cancer types, such as prostate and breast cancer.^{13,14} Diabetic patients who are treated with insulin, insulin analogs, or insulin-inducing sulfonylurea experience higher cancer-related mortality rates compared with those treated with non-insulin therapies, such as metformin.^{15,16} The mitogenic activity of insulin may partially explain the poor outcome seen in cancer patients treated with insulin. This is supported by observations in breast cancer where tumor cells were responsive to insulin at physiologically relevant concentrations yet exhibited repressed growth after insulin depletion.^{17,18} Similar results were obtained in prostate cancer where elevated insulin levels induced by a high-carbohydrate diet activated Akt in xenograft tumors and promoted tumor growth.¹⁹ These observations collectively suggest that insulin and InsR-mediated signaling have important implications in tumor progression and therapeutic response.

Insulin has important functions in the central nervous system, and impaired response to insulin plays critical roles in development of neurodegenerative diseases.²⁰ For example, the brains of patients with Alzheimer disease appear to be insulin resistant.²¹ Interestingly, Paris et al.²² reported that orthotopic glioblastoma tumor development was repressed in genetic mouse models of Alzheimer disease. This suggests that insulin resistance in the brain leads to an unfavorable environment for glioblastoma. IGF1R-specific inhibitors exhibit preclinical activities in glioblastoma xenograft models.^{23,24} A small subset of advanced glioma has been shown to overexpress IGF2.²⁵ Furthermore, a rare pediatric CNS tumor, atypical teratoid/rhabdoid tumor, produces insulin via an autocrine mechanism.²⁶ In the present study, we demonstrated that glioblastoma cells were responsive to insulin at physiologically relevant concentrations and that dual inhibition of InsR and IGF1R repressed glioblastoma xenograft growth. These findings highlight the need to revisit current management of impaired glucose metabolism in patients with glioblastoma and suggest the InsR/IGF1R pathway as a therapeutic target in this disease.

Materials and Methods

Tumor Samples and Cell Culture

Glioma surgical specimens were provided by the Vanderbilt Molecular Neurosurgical Tissue Bank in accordance with protocols approved by the institutional review board. Two normal neural tissue samples, VU10006 and VU10367, were acquired from patients undergoing epilepsy surgery. VU10369 and VU10827 are established as primary xenograft lines. VU11044 is a primary culture. Additional patient-derived glioblastoma xenograft lines have been described in our previous publications.^{27–29} Normal astrocytes were purchased from Lonza. All glioblastoma ex vivo cultures were derived from enzymatically dissociated subcutaneous xenograft tumors and maintained for less than 8 passages in neurobasal medium supplemented with 2% B-27 serum substitute (Life Technologies), 20 ng/mL epidermal growth factor, and 20 ng/mL basic fibroblast growth factor (Peprotech). This medium also has high concentration of insulin contained in B-27. The method to enrich CD133+ glioblastoma cells has been described in our previous publications.²⁸

Antibodies and Chemicals

The antibodies used in this study, including anti-p-IGF1R β /InsR β (#3024), IGF1R β (#3018), phospho-S473 Akt (#4060), phospho-T308 Akt (#2965), Akt (#2920), phospho-extracellular signal-regulated kinase (ERK)1/2 (#4370), and ERK1/2 (#4696), were purchased from Cell Signaling. Monoclonal antibody against InsR β (sc-57342) was purchased from Santa Cruz Biotechnology. Mouse monoclonal antibody against actin (#MAB1501) was purchased from Millipore. OSI-906 (L-5814) was purchased from LC Laboratories; and BMS-754807 (CT-BMS75) was purchased from Chemtek. Antibodies used for immunohistochemical staining were rabbit anti-IGF1R (1:75 dilution; #14534, Cell Signaling) and rabbit anti-InsR (1:100; sc-57342, Santa Cruz Biotechnology).

Plasmids and Lentivirus Production

The pLKO.1 lentiviral vectors directing expression of short hairpin (sh)RNA, shIGF1R-1 (TRCN0000039675), shIGF1R-2 (TRCN000000426), shInsR-1 (TRCN0000000380), and shInsR-2 (TRCN0000121123) were purchased from Thermo Scientific. The human InsR-A and InsR-B plasmids were kindly provided by Ingo Leibiger at Karolinska Institute, Sweden. The pCDH-puro-myr-Akt1 was described in our previous publication.³⁰ The lentiviral vectors were cotransfected with the packaging vectors psPAX2 and pCI-VSVG (Addgene) into 293FT cells by Lipofectamine 2000 (Invitrogen) to produce lentivirus. Cells were infected by viral supernatants at an approximate multiplicity of infection of 5. After infections, cells were selected with 1 μ g/mL puromycin for at least 48 h before experiments.

PCR and Quantitative Real-time PCR

Total RNA was isolated using the Illustra RNAspin kit (GE Healthcare) and reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad). Expression of InsR isoforms was assessed by PCR for 33 cycles of 95°C for 30 s and 60°C for 45 s. Gene expression was measured by quantitative real-time (qRT) PCR

using SYBR Green Mastermix (Bio-Rad). The reaction comprises 40 cycles of 95°C for 20 s and 60°C for 45 s. Primers are listed in Supplementary Table 1.

In Vivo Assay

All animal experiments were performed in accordance with a protocol approved by the Vanderbilt University Institutional Animal Care and Use Committee. Female athymic nude mice were used for all in vivo assays. For the intracranial model, 5000 T4105 cells were suspended in 10 μ L phosphate buffered saline and implanted into the right cerebrum of mice to establish tumors. Following tumor implantation, mice were maintained until development of overt neurological symptoms, such as significant weight loss, lethargy, or hunched posture. Median survival was determined by the Kaplan–Meier estimator using GraphPad Prism 5.0 software. For the subcutaneous model, 500 000 T4121 cells were suspended in 50 μ L phosphate buffered saline, mixed with an equal volume of Matrigel (BD Biosciences), and implanted into both flanks. When tumors became palpable, mice were treated with 50 mg/kg OSI-906 suspended in 25 mM tartaric acid once daily by oral gavage following a 5-days-on/3-days-off schedule. Tumor size was measured every 3 days by calipers.

Statistical Analysis

Statistical difference was assessed using GraphPad Prism 5.0 software. Data are presented as mean \pm SD unless otherwise indicated. $P < .05$ was considered significant.

Results

InsR and IGF1R Are Commonly Expressed in Glioblastoma Tumors

Although mounting evidence suggests that obesity and type 2 diabetes are associated with increased cancer risk and mortality, it remains unclear whether and how these observations are attributable to elevated insulin levels. In particular, glioma patients are often subjected to insulin therapy due to glucocorticoid-induced hyperglycemia. In order to interrogate this question, we first assessed expression of InsR and IGF1R in high-grade glioma and normal brain tissues obtained from epilepsy surgeries. Both receptors were found expressed across normal and malignant tissues (Fig. 1A). Expression of these 2 receptors was also readily detected in ex vivo cultures from a panel of patient-derived glioblastoma xenograft (PDX) lines (Fig. 1B). Immunohistochemical staining of patient specimens further confirmed expression of both receptors in glioblastoma tumors with apparent patterns of membrane staining (Fig. 1C and Supplementary Fig. 1A). InsR is expressed in 2 isoforms by alternative splicing.⁷ The longer isoform B (InsR-B) is predominantly expressed in metabolic organs, such as muscle, fat, and liver. The isoform A (InsR-A), which lacks exon 11, is expressed during development and the primary isoform is found in neoplasms. Using a primer set that flanks exon 11, we showed that isoform A was predominantly expressed in glioblastoma (Supplementary Fig. 1B). Because InsR-A has important functions in fetal cells, we asked whether its expression differed between glioblastoma stem cells and differentiated

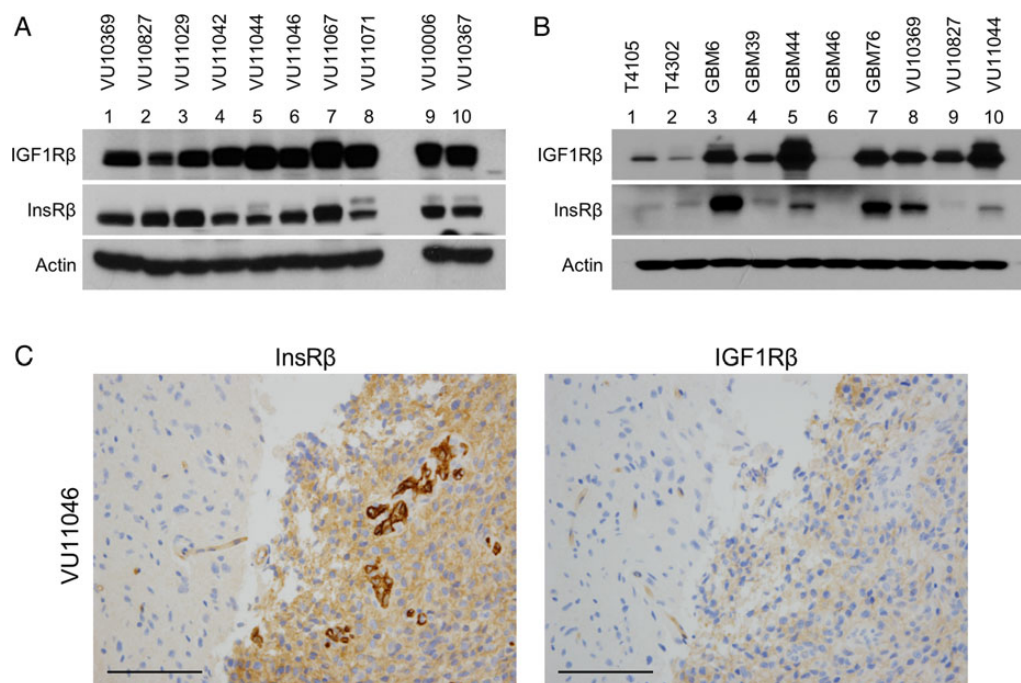


Fig. 1. Characterization of InsR and IGF1R expression in glioblastoma. (A) Expression of InsR and IGF1R in high-grade glioma surgical specimens (lane 1–8) compared with normal brain tissues (lane 9–10). (B) Immunoblotting of InsR and IGF1R in glioblastoma ex vivo cultures. (C) Representative images (400 \times) of immunohistochemical staining of InsR and IGF1R in paraffin-embedded sections of a glioblastoma patient specimen, VU11046. The scale bars represent 100 μ m.

progenies. We separated these 2 cell subpopulations by selection for the CD133 cell surface marker as described in our previous publications²⁸ and employed the same PCR primer set to show that isoform A predominated in both CD133+ and CD133- cells (Supplementary Fig. 1C).

Glioblastoma May Produce IGF1 and IGF2 but Not Insulin

The ligands of InsR and IGF1R—ie, insulin, IGF1, and IGF2—do not passively penetrate through the blood-brain barrier.³¹ Brain insulin is primarily derived from circulation via an active transportation mechanism, although local production of insulin has been reported.^{31,32} The exact concentration of insulin in normal brain remains an issue of debate. Some earlier studies demonstrated that insulin levels in rodent brain were over 10 times higher than plasma levels,^{33,34} while results from other groups suggested much lower concentrations.^{35,36} In addition, there is a lack of knowledge of how much insulin is available in the tumor microenvironment in brain. We assessed production of insulin, IGF1, and IGF2 in a group of glioblastoma ex vivo cultures by qRT-PCR using previously published primers. IGF2 mRNA was measurable in GBM6, GBM39, and VU11044, while IGF1 mRNA was only detected in GBM46 (Supplementary Fig. 2A and B). We further detected IGF2 in conditioned medium derived from the aforementioned 3 lines using a growth factor array, but IGF1 in GBM46-derived conditioned medium was marginally detectable (Supplementary Fig. 2C). Insulin expression was not detected by either qRT-PCR or enzyme-linked immunosorbent assay in any lines we tested (negative data not shown). These results suggest that insulin and IGF1 in glioblastoma is likely regulated via endocrine mechanisms, whereas autocrine IGF2 may play a role in a subset of tumors.

Insulin at Physiologically Relevant Concentrations Stimulates Glioblastoma Cell Growth

Although the mitogenic activities of insulin are well recognized, it has not been well documented whether glioblastoma significantly responds to insulin at physiologically relevant concentrations. As such, the direct tumor-promoting activities of insulin remain obscure. Our glioblastoma ex vivo cultures were grown in chemically defined neurobasal medium supplemented with epidermal growth factor, basic fibroblast growth factor, and B-27, which has been widely used to maintain the parental tumor genotypes and phenotypes.³⁷ An important component of the B-27 supplement is insulin (final concentration >1 µg/mL). To avoid the interference of insulin in medium, regular B-27 supplement was replaced by insulin-free B-27 in this assay. Cells were then treated by each ligand following a serial dilution method. Our results demonstrated that insulin, IGF1, and IGF2 each dose-dependently promoted cell growth, but responsiveness to individual ligands varied among different tumors. Some tumors, such as T4105 and T4302, appeared to be more sensitive to insulin than IGF1 and IGF2 (Fig. 2A and Supplementary Fig. 3A). In T4105, insulin at 0.5 ng/mL (normal fasting serum level) exhibited significant growth-promoting activities, while insulin at 5 ng/mL was almost as effective to promote T4105 cell growth as complete medium (Fig. 2B). In contrast, IGF1 and IGF2 exhibited only moderate growth-promoting

activities at 5 ng/mL but not 0.5 ng/mL (Supplementary Fig. 3D), suggesting a primary role of the InsR homodimer in these tumors. GBM44 was similarly responsive to insulin and IGF1, whereas IGF2 was less active (Fig. 2B and C and Supplementary Fig. 3E). This tumor is likely sustained by a mixture of InsR and IGF1R homodimeric and heterodimeric receptors. VU11044 was preferentially stimulated by IGF1 and IGF2, suggesting a composition of mainly IGF1R homodimer and/or the InsR/IGF1R heterodimer (Supplementary Fig. 3B). In contrast, the InsR/IGF1R-negative line, GBM46, did not respond to all 3 ligands (Supplementary Fig. 3C). Additionally, insulin at these physiologically relevant concentrations induced apparent activation of downstream signal pathways in T4105 cells, such as Akt and ERK (Fig. 2E). Consistent with the observations in cell growth assays, IGF1 was less potent than insulin in T4105, while IGF2 at 0.5 or 5 ng/mL did not exhibit significant impact on signal transduction (Fig. 2E). In GBM44, activation of the InsR/IGF1R pathway selectively activated Akt signaling; and IGF1 appeared to be more potent than insulin, whereas IGF2 was not active at indicated concentrations (Fig. 2E). Of note, GBM44 harbors a BRAF^{V600E} mutation, whereas T4105 carries an H1047Y mutation in phosphatidylinositol-3-kinase catalytic alpha polypeptide. Collectively, these results suggest that insulin at physiologically relevant levels may have significant growth-stimulatory activities in glioblastoma cells and important influence on Akt signaling.

Depletion of InsR Impairs Proliferation and Survival of Glioblastoma Cells

To specifically interrogate functions of InsR in glioblastoma cells, we depleted InsR expression using lentivirus-mediated expression of 2 distinct shRNA sequences (Fig. 3A). T4105 cells with reduced InsR expression exhibited significantly compromised growth and capacity for neurosphere formation (Fig. 3B and C). Similar observations were made in additional ex vivo cultures derived from glioblastoma PDX lines or primary cultures, such as GBM44 and VU11044 (Supplementary Fig. 4A and B). Deletion of InsR induced a G1 cell cycle arrest in T4105 cells associated with increased expression of cyclin-dependent kinase inhibitor 1A (p21^{WAF1/CIP1}) (Fig. 3D and Supplementary Fig. 4C). Additionally, activation of caspase-3/7 and increased staining for annexin V suggested a moderate activation of apoptotic cell death following knockdown of InsR (Fig. 3E and Supplementary Fig. 4D). These results collectively indicate that InsR-mediated signaling has important functions to maintain proliferation and survival of glioblastoma cells.

Loss of InsR Induces Upregulation of IGF1R

The functions of InsR and IGF1R are tightly intertwined. While knockdown of InsR in T4105 cells reduced activities of downstream signaling molecules, such as Akt and ERK, we found that it induced a significant upregulation of IGF1R expression (Fig. 4A). IGF1R mRNA levels were also increased in cells depleted of InsR, suggesting that the feedback mechanism involved transcriptional regulation (Fig. 4B). Selective depletion of IGF1R also impaired growth of T4105 cells and attenuated phosphorylation of Akt and ERK (Fig. 4A and C). However, induction of InsR expression in the absence of IGF1R was limited

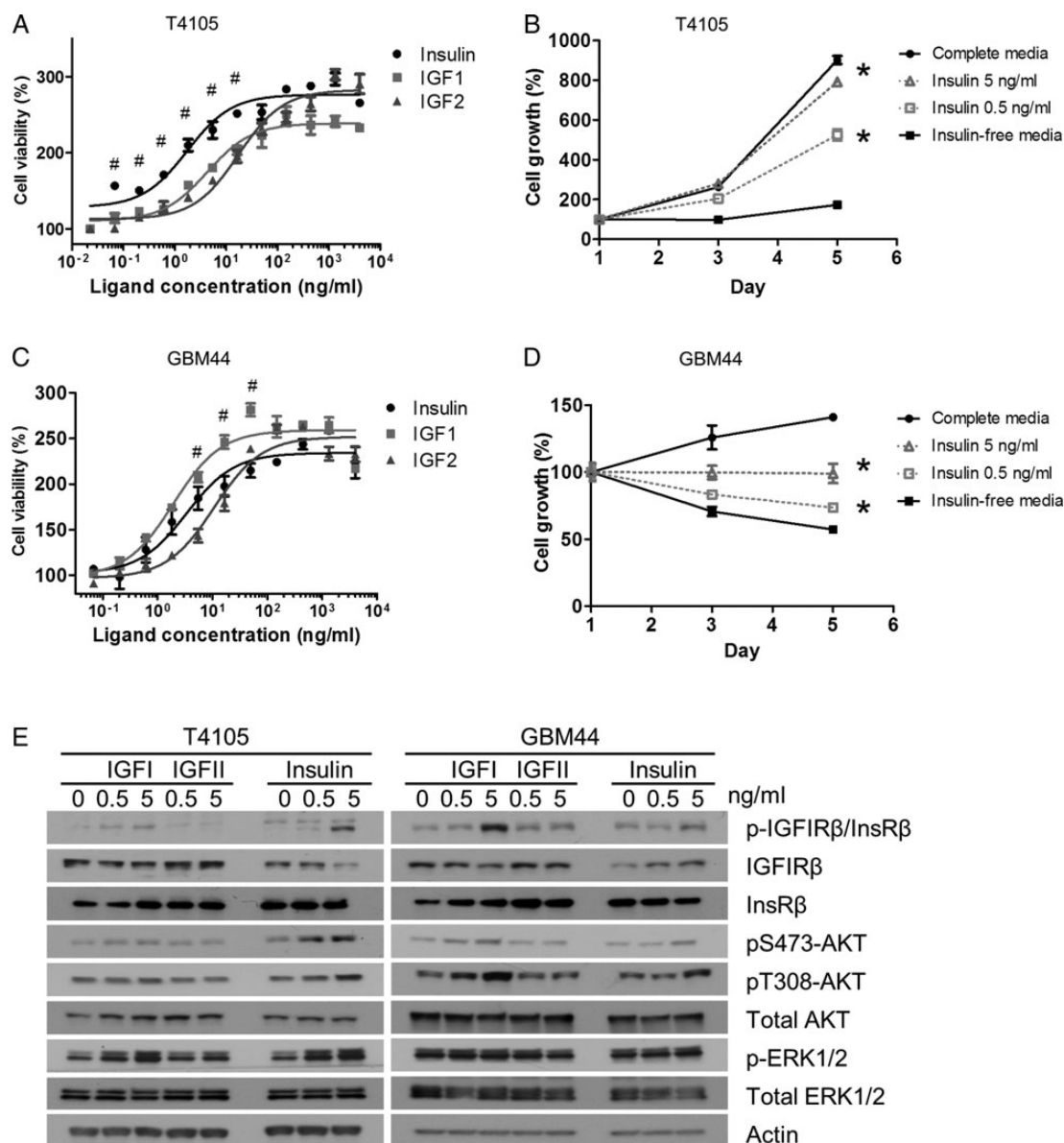


Fig. 2. Insulin, IGF1, and IGF2 stimulate glioblastoma cell growth and downstream signaling. (A and C) T4105 and GBM44 cells were cultured in insulin-free medium and treated with insulin, IGF1, or IGF2 with a 3-fold serial dilution for 5 days. Dose response curves were fitted by GraphPad Prism 5 using a 3-parameter nonlinear regression algorithm. Viability readings of cells cultured in insulin-free medium were assigned as 100%. $^{\#}P < .05$ by Student's *t*-test for insulin vs IGF1. (B and D) Following insulin starvation, growth rates of T4105 or GBM44 cells in the presence of 0.5 or 5 ng/mL insulin were determined as described in methods. $^*P < .01$ by Student's *t*-test for insulin vs insulin-free medium. (E) T4105 and GBM44 cells were starved in insulin-free medium overnight. Cells were stimulated by indicated ligands for 30 min and harvested for immunoblotting with actin as the loading control.

(Fig. 4A and B). Additional glioblastoma lines, such as GBM44 and VU11044, also required both receptors to adequately sustain cell growth (Supplementary Fig. 4A and B). Finally, knockdown of either InsR or IGF1R significantly prolonged survival of animals bearing orthotopic T4105 tumors (Fig. 4D). Taken together, these results suggest that both InsR and IGF1R are implicated in regulation of proliferation and survival in glioblastoma.

Dual Inhibition of InsR and IGF1R Represses Glioblastoma Tumors

We next assessed the anti-glioblastoma potential of dual InsR/IGF1R inhibitors, OSI-906 and BMS-754807. Cells derived from primary xenograft lines, such as T4105, T4121, and GBM44, or the primary culture VU11044, all exhibited dose-dependent loss of cell viability in the presence of OSI-906 or BMS-754807 (Fig. 5A, Supplementary Fig. 5A–C). Neurosphere formation by

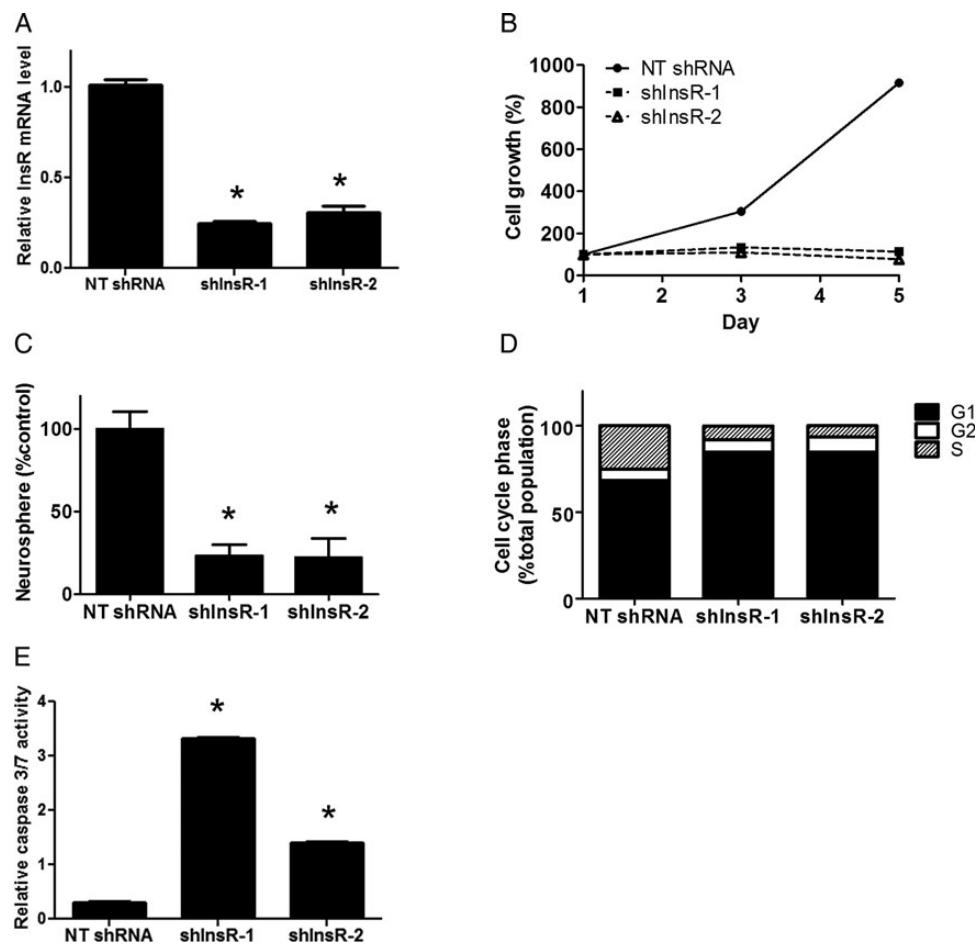


Fig. 3. Knockdown of InsR impairs glioblastoma proliferation and survival. (A) T4105 cells were infected with lentivirus directing expression of nontargeting shRNA (NT) or shRNA sequences specific to InsR. After selection with 1 μ g/mL puromycin for 2 days, relative InsR mRNA levels were determined by qRT-PCR. (B) Cells were then used to determine the growth rate and (C) neurosphere formation. (D) Representative cell cycle distribution and (E) caspase-3/7 activation were assessed 3 days after puromycin selection. Caspase activation was measured by the Caspase-Glo 3/7 kit (Promega) and normalized to the corresponding cell viability. * $P < .001$ by Student's *t*-test.

T4105 cells was also significantly decreased by OSI-906 (Fig. 5B). We have identified one PDX line, GBM46, that did not show measurable InsR or IGF1R expression. This line was resistant to both InsR/IGF1R inhibitors (Supplementary Fig. 5D). Administration of either OSI-906 or BMS-754807 in both T4105 and GBM44 potently inhibited Akt activity as shown by decreased phosphorylation levels at the 2 key activation residues, threonine 308 and serine 473 (Fig. 5C and Supplementary Fig. 5E). ERK was also significantly suppressed in T4105 cells but not in GBM44, suggesting that regulation of the mitogen-activated protein kinase kinase/ERK pathway by InsR/IGF1R is context dependent. Activation of Akt by lentivirus-directed expression of constitutively active myristoylated Akt1 protected T4105 cells against OSI-906, suggesting that phosphatidylinositol-3 kinase/Akt signaling is a key downstream target of InsR and IGF1R in glioblastoma (Fig. 5D). Of note, expression of InsR and IGF1R was significantly increased in the presence of dual kinase inhibitors (Fig. 5C and Supplementary material, Fig. 5E), suggesting a negative feedback mechanism that crucially regulates expression of these 2 receptors in glioblastoma.

Currently available inhibitors for InsR and IGF1R do not effectively penetrate the blood-brain barrier. Therefore, we evaluated the *in vivo* therapeutic efficacy of OSI-906 using a subcutaneous xenograft model. T4121 tumors were established at both flanks in athymic nude mice. Treatment was initiated when most tumors were palpable. OSI-906 was orally administered by a 5-days-on/3-days-off schedule, which did not induce significant weight loss (Supplementary Fig. 6A). Both protein and mRNA levels of InsR and IGF1R were upregulated in xenograft tumors 4 h after OSI-906 administration, while phosphorylation of the receptors was decreased (Supplementary Fig. 6B and C), which confirmed significant blockade of the pathway in tumors. Treatment with OSI-906 repressed tumor growth and significantly decreased tumor weight at the endpoint of the experiment (Fig. 5E and F). The results were consistent with our *in vitro* observations that depletion of insulin in medium significantly impaired cell growth but did not completely eradicate all tumor cells. Our findings show therapeutic potential of dual InsR/IGF1R inhibitors in glioblastoma but suggest that more potent drug combinations or

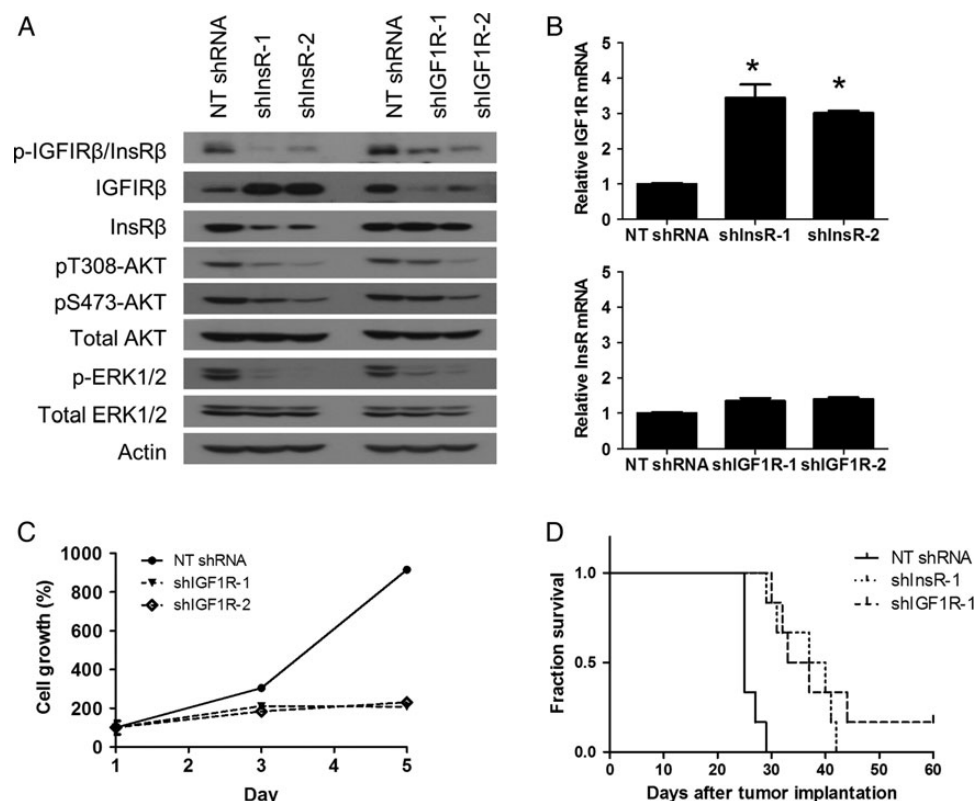


Fig. 4. IGF1R is also implicated in glioblastoma proliferation and survival. (A) Following completion of puromycin selection, T4105 cells were subject to immunoblotting for indicated proteins and (B) qRT-PCR for expression of InsR and IGF1R. The mRNA levels of InsR and IGF1R were normalized to actin mRNA. * $P < .001$ by Student's *t*-test. (C) Growth curves after IGF1R knockdown were determined as described in Fig. 3B. (D) Following lentiviral infection and puromycin selection, 5000 T4105 cells were injected intracranially into athymic nude mice ($n = 6$). Animals were sacrificed upon development of neurologic signs. Median survival for the NT group was 28 days, and for the InsR-knockdown group and IGF1R-knockdown group 38 days (log-rank $P = .0011$) and 36 days (log-rank $P = .0004$), respectively.

combinations with conventional chemoradiotherapy are required to effectively treat glioblastoma.

Discussion

It has been increasingly recognized that metabolic abnormalities have an adverse impact on outcomes of cancer patients. However, the exact role that elevated glucose and insulin exposure play in cancer progression remains unclear. The influence of metabolic complications in patients with glioblastoma is particularly important due to the common use of corticosteroids (eg, dexamethasone) and their frequent deteriorating effects on glucose metabolism.⁶ Insulin therapy is widely used to manage these complications when needed. However, the response of glioblastoma cells to insulin remains largely unexplored. We identified expression of both InsR and IGF1R in the majority of glioblastoma surgical specimens and PDX lines. Levels of these 2 receptors appear to be higher in VU10369 and VU11046 tumors than in adjacent normal tissues. Whether this represents a general observation needs to be further explored in a wider range of samples. Our results showed dose-dependent mitogenic activities of insulin, IGF1, and IGF2 across concentrations of multiple orders of magnitude. We also identified an inverse

correlation between the activation of the InsR/IGF1R pathway and the expression of both receptors in glioblastoma. Suppression of the pathway by either RNA interference or dual kinase inhibitors significantly increased InsR and/or IGF1R expression. These observations suggest that glioblastoma tumors are highly sensitive to the changes in the activity of this pathway and develop a feedback mechanism for effective utilization of the available ligands in the tumor microenvironment. Therefore, it is reasonable to speculate that the availability of ligands plays important roles in regulating the InsR/IGF1R pathway in glioblastoma.

Administration of insulin has been shown to rapidly affect brain functions and ameliorate dementia symptoms in patients with Alzheimer disease, indicating that exogenous insulin is biologically active in the CNS.³⁸ In the current study, we demonstrated that a subset of glioblastoma tumors were sensitive to insulin at normal fasting plasma levels. While others were more sensitive to IGF1 and IGF2, they could also be stimulated by insulin at higher concentrations. Additionally, blockade of the InsR/IGF1R pathway by small molecule inhibitors or knockdown of the receptors compromised glioblastoma xenograft tumor growth. These observations collectively suggest that insulin therapy may have a direct impact on glioblastoma progression by activating the InsR/IGF1R pathway and downstream

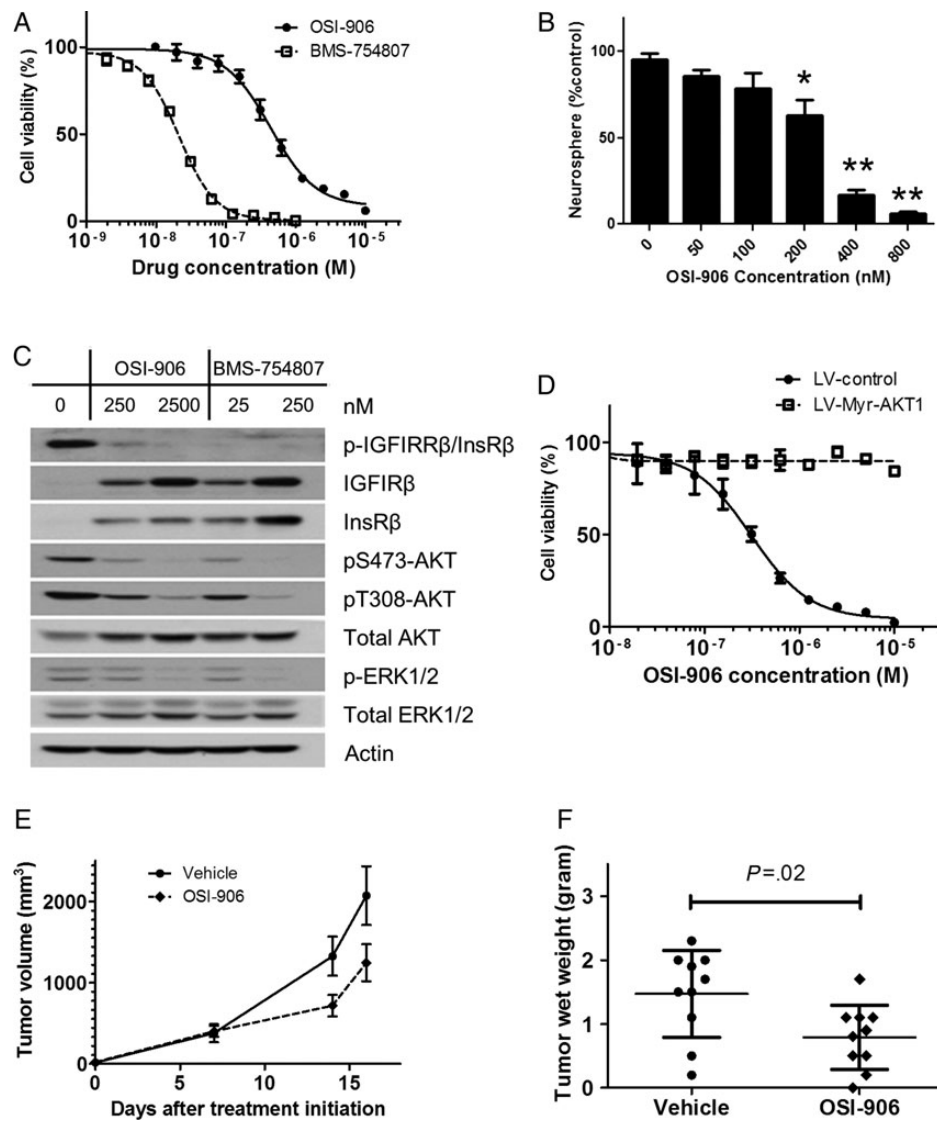


Fig. 5. Dual inhibition of InsR and IGF1R reduces glioblastoma cell viability and represses subcutaneous xenograft growth. (A) InsR/IGF1R dual inhibitors, OSI-906 and BMS-754807, dose-dependently inhibited T4105 viability and (B) neurosphere formation. * $P < .01$, ** $P < .001$ by Student's *t*-test. (C) T4105 cells were treated with OSI-906 or BMS-754807 at indicated concentrations for 24 hours, lysed, and subjected to immunoblotting. (D) T4105 cells were infected with a lentivirus directing expression of myristoylated Akt1. Dose-response curves to OSI-906 were assessed for control and Akt-activated cells. (E) T4105 subcutaneous tumors were established in both flanks of athymic nude mice ($n = 5$) and treated with 50 mg/kg OSI-906 or vehicle once daily, 5 days on, 3 days off. Data are mean \pm SE. (F) At the end of the experiment, tumors were dissected and the wet weight was measured. $P = .02$ by Student's *t*-test.

Akt signaling in tumor cells. Alternatives to insulin therapy have just emerged. Newly approved sodium glucose co-transporter 2 inhibitors, Invokana and Farxiga, reduce blood glucose levels by promoting glucose excretion into urine.³⁹ It is anticipated that these drugs may decrease levels of both blood glucose and insulin and thus represent a promising alternative approach for management of blood glucose levels in patients with glioblastoma and other insulin-responsive cancers.

Accumulating epidemiological data suggest that diabetic patients are at significantly higher risks for multiple cancer types compared with the general population.⁴⁰ Strikingly, the risk of glioma is 42% lower in patients with a history of diabetes

according to a recent meta-analysis.⁴¹ This inverse correlation may be explained by 2 hypotheses. One possibility is that brain insulin levels are actually downregulated in diabetic patients due to saturation of the insulin transporter and decreased uptake of peripheral insulin. This hypothesis is supported by findings that obese rats have reduced brain insulin levels⁴² and that a high-fat diet induces weight gain in dogs with reduced insulin levels in brain.⁴³ Alternatively, brain tissue of diabetic patients may become insulin resistant through similar mechanisms identified in peripheral tissues and therefore refractory to cellular transformation.^{21,44} Indeed, diabetic patients are at higher risks of Alzheimer disease, and patients with Alzheimer disease

show symptoms of brain insulin resistance and harbor an inversely regulated signaling network in neuronal tissues compared with glioblastoma.^{45,46} Altogether, these observations suggest that impaired brain insulin response, which is often associated with diabetes, may induce dementia, whereas aberrantly activated insulin response may promote glioma progression.

We investigated the therapeutic potential of dual InsR/IGF1R inhibitors against glioblastoma, as both receptors appeared to be implicated in regulation of malignant phenotypes and downstream signal transduction. It remains an open question why glioblastoma tumors prefer coexpression of both receptors, given the substantial overlap of their known downstream targets. One possibility is that the hybrid receptor has tumorigenic functions distinct from either homodimer. Of note, even in glioblastoma lines that are more sensitive to insulin than to IGF1, such as T4105, depletion of IGF1R had significant impact on cell proliferation and survival. It is also possible that the crosstalk between unique downstream targets of InsR and IGF1R homodimers generates important benefits to glioblastoma. Therefore, dual InsR/IGF1R inhibitors hold promise for treating glioblastoma. Repression of this pathway strongly leads to upregulated receptor expression. A better understanding of this feedback mechanism may help to improve the efficacy of InsR/IGF1R inhibitors. Taken together, our findings demonstrate an InsR/IGF1R-regulated dynamic signaling pathway that mediates an important tumor crosstalk with the host endocrine system. However, there is still a lack of small molecule InsR/IGF1R inhibitors that effectively penetrate the blood-brain barrier. Prolonged use of InsR/IGF1R inhibitors may also have an adverse impact on overall metabolism. A therapeutic window needs to be carefully defined to target this pathway in glioblastoma patients. Rationally designed drug combinations, such as cotargeting of the feedback mechanisms that control receptor expression, may generate significant therapeutic effects without excessive inhibition of this pathway. Although biomarkers that predict tumor sensitivity to InsR/IGF1R inhibitors are not yet available, the absence of InsR and IGF1R may indicate resistance to this therapy.

Supplementary Material

Supplementary material is available at Neuro-Oncology Journal online (<http://neuro-oncology.oxfordjournals.org/>).

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Conflict of interest statement. The authors have no potential conflicts of interest to disclose.

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