The HIF1α-PDGFD-PDGFRα axis controls glioblastoma growth at normoxia/mild-hypoxia and confers sensitivity to targeted therapy by echinomycin

Gong Peng†, Yin Wang†, Pengfei Ge, Christopher Bailey, Peng Zhang, Di Zhang, Zhaoli Meng, Chong Qi, Qian Chen, Jingtao Chen, Junqi Niu, Pan Zheng, Yang Liu, and Yan Liu

Abstract

Background: Glioblastoma multiforme (GBM), a lethal brain tumor, remains the most daunting challenge in cancer therapy. Overexpression and constitutive activation of PDGFs and PDGFRα are observed in most GBM; however, available inhibitors targeting isolated signaling pathways are minimally effective. Therefore, better understanding of crucial mechanisms underlying GBM is needed for developing more effective targeted therapies.

Methods: Target genes controlled by HIF1α in GBM were identified by analysis of TCGA database and by RNA-sequencing of GBM cells with HIF1α knockout by sgRNA-Cas9 method. Functional roles of HIF1α, PDGFs and PDGFRs were elucidated by loss- or gain-of-function assays or chemical inhibitors, and compared in response to oxygen tension. Pharmacological efficacy and gene expression in mice with intracranial xenografts of primary GBM were analyzed by bioluminescence imaging and immunofluorescence.

Results: HIF1α binds the PDGFD proximal promoter and PDGFRα intron enhancers in GBM cells under normoxia or mild-hypoxia to induce their expression and maintain constitutive activation of AKT signaling, which in turn increases HIF1α protein level and activity. Paradoxically, severe hypoxia abrogates PDGFRα expression despite enhancing HIF1α accumulation and corresponding PDGF-D expression. Knockout of HIF1A, PDGFD or PDGFRα in U251 cells inhibits cell growth and invasion in vitro and eradicates tumor growth in vivo. HIF1A knockout in primary GBM extends survival of xenograft mice, whereas PDGFD overexpression in GL261 shortens survival. HIF1α inhibitor Echinomycin induces GBM cell apoptosis and effectively inhibits growth of GBM in vivo by simultaneously targeting HIF1α-PDGFD/PDGFRα-AKT feedforward pathway.

Conclusions: HIF1α orchestrates expression of PDGF-D and PDGFRα for constitutive activation of AKT pathway and is crucial for GBM malignancy. Therefore, therapies targeting HIF1α should provide an effective treatment for GBM.

Keywords: HIF1α, PDGFRα, PDGF-D, Glioblastoma, Echinomycin

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Background
Glioblastoma multiforme (GBM) is the most common primary aggressive brain tumor, causing death within two years after diagnosis despite current therapies [1]. Malignant GBM is referred to as grade IV astrocytic glioma, based on WHO classification of four histology grades comprised of pilocytic, diffuse, anaplastic astrocytomas and glioblastoma, and incorporated with molecular genetic features for diagnosis [2—4]. Based on gene mutations and molecular profiling, GBM is divided into four subtypes including proneural, neural, classical and mesenchymal GBMs [4, 5]. Most proneural GBM, the most resistant subtype, have mutations in TP53 and molecular profiling, GBM is divided into subtypes including proneural, neural, classical and mesenchymal GBMs [4, 5]. Most proneural GBM, the most resistant subtype, have mutations in TP53 and 57% of GBM is caused by constitutive activation of the receptor tyrosine kinase (RTK)/RAS/PI3K signaling pathways and defective RB and/or ARF-p53 signaling pathways [6—8]. Genes encoding epithelial growth factor receptor (EGFR) and PDGFRα were altered by amplification, rearrangements and mutations, resulting in increased receptor tyrosine phosphorylation in GBM [9]. In adult GBM, EGFR amplification is the most frequent alteration (45—57% of cases), while PDGFRα amplification is the second most frequent (10 to 20% cases). The incidence of PDGFRα amplification increases to 23% in pediatric GBM [10] and 30% in high-grade pediatric gliomas [9, 11, 12]. Other genetic lesions, including PDGFRα activating mutations and gene rearrangements, as well as EGFR amplification, often occur concurrently in tumors with PDGFRα amplification [11—15]. However, overexpression of PDGFRα was detected in majority of proneural subtype GBM, which was substantially more frequent than PDGFRα genetic alterations [5]. Meanwhile, genetic alterations of components of the PDGFRα-PI3K-AKT signaling pathway occur in up to 70% of GBM [16]. Moreover, co-overexpression and co-activation of PDGFRα with EGFR often occur in GBM tumors without amplification of either gene [17—19] but with a typical feature of high angiogenesis such as the most common EGFRVIII mutant-overexpressing GBM [20, 21].

HIF1α is stabilized under hypoxic conditions and responsible for directing tumor angiogenesis. Hypoxia inactivates the prolyl-hydroxylases in cytosol and the arginine hydroxylase factor inhibiting HIFα in nucleus, leading to the prevention of recognition and degradation of HIFα by the E3 ligase Von Hippel-Lindau, and to the inhibition of HIFα transcriptional activity, respectively [22—24]. In contrast, we demonstrated an essential role of HIF1α under normoxia in leukemia/lymphoma stem cells, which is efficiently targeted by echinomycin, an inhibitor of HIF1α transcriptional activity [25, 26]. Glioblastoma typically features three-layers including a necrotic core, intermediate/hypoxic layer, and a well-oxygenated and -vascularized, highly-proliferative outer layer comprising the invasive tumor frontier [27, 28]. HIF2α is required for the growth of glioma stem cells at hypoxia [29]. Although HIF1α is highly expressed in both glioma stem and bulk tumor cells [29], its role has not been thoroughly evaluated.

The family of PDGFs and PDGFRs is comprised of PDGF-A, B, C and D, and PDGFRα and β. Overexpression of PDGF-A, B, and C has demonstrated that PDGF-PDGFR signaling plays an important role in both normal development and tumorigenesis of the central nervous system (CNS) [30, 31]. PDGFRα is expressed predominantly in glial progenitors and has a reduced expression in mature astrocytes [32]. In mice, PDGFRα overexpression, together with the loss of ARF, was reported to induce GBM via PDGFRα-PI3K-AKT activation [33]. PDGFRβ is mostly restricted in the glioma-associated stroma, but can be induced in glioma cells by microglia to enhance the migration of glioma cells [34, 35]. Its ligands, PDGF-B and PDGF-D, were both shown to be more potent mitogens for the growth and transformation of fibroblast cells than PDGF-A and PDGF-C [36, 37]. Overexpression of PDGF-B in glial progenitors of transgenic mice induced gliomas in a longer latency, and high-grade gliomas at a shorter latency once combined with Arf or Trp53 deficiency [38, 39]. However, the expression, function, and regulation of PDGF-D in CNS cells remains less known.

Here, we demonstrate that HIF1α plays a critical role in favoring the growth of GBM cells via directly inducing the expression of both PDGF-D and PDGFRα for constitutive AKT activation, which primarily occurs at normoxia or mild-hypoxia. The induced PDGF-D is essential for GBM growth in vivo via an autocrine and/or paracrine manner, to increase tumor invasion and angiogenesis in mouse models of GBM.

Materials and methods
Mice, cells and reagents
Mice
*Nod.Scid.IL2rg<sup>0</sup>* (NSG) mice were purchased from the Jackson lab. Mice at 6—8 weeks were used for the intracranial implantation of glioblastoma cells and for the treatment. All experiments were performed using mycoplasma-free cells.

Cell lines and primary GBM cells
The human GBM cell lines U251 and U87MG and mouse GBM line GL261 were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). These GBM cell lines were cultured in high-glucose DMEM supplemented with 10% FBS, 2 mM glutamine and penicillin/streptomycin (100 U/mL, each), and maintained at 37°C, 5% CO2 incubator. Unless stated
otherwise in the figures or figure legends, GBM cells were cultured at normoxia (21% oxygen).

Primary LGG and GBM samples were obtained from surgical-resections of adult gliomas at the First Hospital of Jilin University between Jan 2013 and Jun 2014 (Supplemental Table 1). The Ethics Committee of the Jilin University approved and patient consent was provided for this study. Two primary GBM tissues obtained from surgical resections were immediately placed in RPMI 1640 medium. The tissues were chopped into a fine paste with scalpels and digested in 0.25% trypsin/EDTA solution containing 100 U/mL DNase I (Sigma-Aldrich) for 30 min at 37 °C with 125 rpm shaking, and terminated by adding 10% FBS DMEM medium. Digested cells were pelleted by centrifugation, gently suspended with 1 mL of 1x lysing buffer solution (BD Bioscience) for a total of 2 min, neutralized with 10 mL DMEM medium, passed through a 70 µm filter, and pelleted by centrifugation. The isolated cells were cultured in NeuroCult basal medium containing 1x differentiation supplement (Stem Cell Technology), 40 ng/mL EGF (Invitrogen), 20 ng/mL b-FGF (Invitrogen), 1 mM glutamine, and 1x antibiotics. The primary cells at least than three passages were used for in vitro assays and the freshly separated tumor cells from mice were used for in vivo assays and tumor implantation.

Reagents

Rabbit HIF1α antibody (GTX127309, GeneTex), Phospho-Y754-PDGFRα (Ab5460, Abcam), Phospho-AKT(S473) and AKT, Phospho-ERK(T202/Y204) and Phospho-Y754-PDGFRα (Ab-1020, BD Bioscience) were purchased.

Plasmids

The full-length coding cDNAs of HIF1A from human bone marrow cells and of PDGFRA and PDGFD, both from U251 cells, were cloned into a pcDNA vector and identified by DNA sequencing. The triple HIF1A mutant form (P402A/P564A/N802A, named as HIF1α-PPN) was made by site-directed mutagenesis using HIF1A as template and a kit from Clontech. The PDGFD-dCUB construct was made by deletion of the CUB domain of PDGFD using Platinum SuperFill DNA polymerase (Invitrogen) and ligated with T4 ligase (Promega).

Cas9/CRISPR gene knockout in glioblastoma cells

Design of small Cas9-guided RNA (sgRNA) sequence was based on the website tool of Zhang’s lab (http://crispr.mit.edu/). Knockout of HIF1A or PDGFD or PDGFRα genes was performed in U251 or U87MG cells by transfecting the sgRNA-expressing plasmid of HIF1A or PDGFD or PDGFRα or control scramble sg (Sr-sg).

The plasmid is constructed in a Lenti-Crisp-V2 vector (addgene, Cambridge, MA) and expresses both Cas9 protein and sgRNA of the DNA sequence of HIF1A (5′-ccatcagattttgcgtagttg-3′) or PDGFD (5′-ttttgccacaggc-caacctc-3′) or PDGFRα (5′-cgcttttggaggctgtct-3′) or Sr-sg (5′-ggaactggctgtgtcatactc-3′). U251 or U87MG cells cultured in a 100 mm dish at about 80% confluence were transfected with the individual sgRNA-Cas9 plasmid using lipofectamine 3000 reagent. About 76 puromycin-resistant clones were picked up for each gene knockout. Positive clones were selected by immunofluorescent staining and Western blot before directly sequencing of their DNA PCR products to confirm their knockout. The Sr-sgRNA-transfected cells were selected with puromycin and drug-resistant cell pool was used as knockout control, hereafter referred to as wild type (WT).

Gene knockdown by shRNA lentivirus

Overnight culture of freshly isolated GBM cells from NSG recipients were transduced with high-titer lentiviral mixture of HIF1α-sh1 and HIF1α-sh2 or with scrambled-sh (Sr-sh) controls for 30 h before checking the expression of GFP-reporter under microscope and the 1 × 10^7 transduced/mouse were injected intracranially into NSG mice. The two lentiviral shRNA plasmids were constructed by cloning DNA oligo sequences of HIF-1α (sh1, 5′-ggagtcataagtctgga; sh2, 5′-gaaatcagctgtaa) or Sr-sh (sh1, 5′-tgtgtatctgctgtaa; sh2, 5′-gataatcgagcactgtaa) into a lentiviral shRNA vector with GFP as reporter [25].
the PCR fragment was cloned into lentiviral vector upstream of the GFP reporter to allow promoter-driven expression of GFP. For the PDGFRα promoter construct, DNA oligos of the PDGFRα enhancers (located within the first intron containing consensus HRE sites) were synthesized and inserted downstream of the PDGFRα proximal promoter. The DNA sequences of the enhancers are as follows: E1 (5′-tacccagccccgctggtctgctcgaactcctgacct-3′), E2 (5′-caaccccggagcagtctgccacactg-3′).

ChIP assay
U251 cells were starved of serum for 8 h and re-flashed with medium containing 10% FBS for 2 h before 1% formalin fixation. The rabbit HIF1α antibody was used for immunoprecipitation of sonicated SDS lysate diluted at 1:10 ratio with dilution buffer. The following steps were performed according to the guidelines for ChIP Assay Kit (17–295, Millipore). Control IgG was used as a parallel control. HIF1α-bound regions in the promoters of PDGFD and PDGFRα pulled down by ChIP were amplified by PCR using primer pairs as follows: PDGFD, forward (5′-taccaagagagtattggacacc-3′) and reverse (5′-tcctaatggtctccgcgaag-3′); PDGFRα, proximal promoter P1 forward (5′-ctgcttggtctgctcgaactcctgacct-3′) and reverse (5′-tcctaatggtctccgcgaag-3′); enhancer E1 forward (5′-ctgcttggtctgctcgaactcctgacct-3′) and reverse (5′-tcctaatggtctccgcgaag-3′); enhancer E2 forward (5′-caaccccggagcagtctgccacactg-3′) and reverse (5′-tcctaatggtctccgcgaag-3′).

Promoter activity
HEK293T cells were plated onto a 24-well plate (2 x 10^5 cells per well) 12 h before transfection. The cells were transiently transfected with various promoter plasmids and with/without HIF1α-PPN (P402A/P564A/N803A mutant) plasmid (total DNA 500 ng/per well) using lipofectamine 3000. Twenty-four or 36 h after transfection, the GFP signals of the cells were photographed under fluorescence microscope and the mean fluorescence intensity (MFI) of the cells was determined by flow cytometry. Red fluorescent control plasmid was co-transfected with these promoter plasmids as internal control to read out transfection efficiency of each sample. One of quadruplicate wells was fixed with cold methanol to stain the Flag-tagged HIF1α-PPN to confirm its expression.

In vivo tumor models
GBM cells, 1 x 10^5 in 3 µl DMEM medium, were stereotactically injected into the cerebral cortex of each anesthetized recipient NSG (for primary GBM) or C57BL/6 (for GL261) mouse at a depth of 2 mm. Mice were randomly grouped (n = 5–6/group), and treated with 250 µg/kg Liposomal Echinomycin (LEM) or vehicle via tail vein injection every other day for a total of 4 injections, beginning on day 10 (xenograft mice) or day 7 (GL261). Survival was determined based on the removal criteria to estimate the Kaplan-Meier survival curves. The WT and KO U251 cells were transduced with lentivirus of GFP-Luciferase reporter and the sorted cultured GFP-positive cells were injected in the cerebral cortex of NSG mice as with the GBM primary cells.

Clinical samples for gene expression analysis
We analyzed the gene transcript expression of HIF1α and its targets, PDGFs and PDGFRs in GBM samples (N = 174) versus low-grade glioma (LGG) samples (N = 529) from TCGA (The Cancer Genome Atlas). The Mann-Whitney test was used to determine statistical significance.

Results
High HIF1α activity and PDGFs/PDGFRs expression in GBM
To identify key molecules or pathways in driving GBM malignancy, we analyzed 174 GBM and 529 low-grade glioma (LGG) patient samples from the Cancer Genome Atlas (TCGA). This analysis revealed significantly higher expression of HIF1α and its targets in GBM vs LGG, particularly those involved in glycolysis (PDK1, PKG1, HK2, SLC2A1/GLUT1, SLC16A3/MCT4, LDHA), angiogenesis (VEGF-A), and tyrosine receptor signaling pathway (IGFBP2) (Fig. 1A). Growth factors PDGFA, PDGFB and PDGFD were also more highly expressed in GBM (Fig. 1A). Interestingly, expression of PDGFRα, a major growth factor receptor in glioma cells, was significantly reduced in GBM, whereas PDGFRβ was increased (Fig. 1A). To identify which pathways may be controlled by HIF1α in GBM, and to validate our findings from TCGA, we performed exome RNA-sequencing (RNA-Seq) on U251 cells after using Cas9-guided RNA (sgRNA) method to knockout HIF1α. Analysis of growth factors, receptors, and substrates in AKT signaling pathway, and HIF1α target genes involved in glycolysis, de novo lipogenesis, and angiogenesis, revealed that PDGF D, PDGFRα, IGFBP2, PDK1,3, SLC16A1,3/MCT1,4, and lipogenesis genes SCD and FASN were all consistently downregulated in U251 cells following genetic ablation of HIF1α, in comparison to scrambled sgRNA control (Fig. 1B,C, Table S2, S3). These data indicate that the expression of PDGFRα and PDGFD in GBM cells depends more on HIF1α compared with the expression of other receptors and growth factors, such as PDGFB. Moreover, surgically resected primary LGG (n = 3) and GBM (n = 6) tissue samples were compared for HIF1α, PDGFRα and PDGFD-D levels by Western blot. The data revealed high protein levels of HIF1α (5/6), PDGFRα (4/6) and PDGFD-D (5/6) in most of the 6 GBM cases (Fig. 1D). These results suggest a positive relationship between HIF1α and PDGF-D and PDGFRα in GBM.
HIF1α is required for the growth and invasion of GBM cells

Whether HIF1α plays a critical role in GBM growth remains undefined, although HIF1α knockdown was shown to inhibit growth and invasion of glioma cell lines in vitro [40]. We used sgRNA to target HIF1A in GBM cell lines, U251 and U87MG. The two clones with HIF1A knockout (KO) in U251 cells were confirmed by Western blot and by directly sequencing their PCR products (Fig. 2A and Fig. S1). Both HIF1A KO U251 clones exhibited reduced growth (Fig. 2B) and invasion (Fig. 2C,D). The size of tumor spheres was also reduced (Fig. 2E). In addition, HIF1A KO U251 cells cultured at low cell density exhibited increased levels of apoptotic proteins, cleaved caspase 3 (cCasp3) and cleaved PARP (bottom bands). The trend was more pronounced when

the cells were treated with the hypoxia mimic CoCl2. More strikingly, HIF1A KO U251 clones were unable to either form tumors or cause mortality in xenograft recipient NSG mice (Fig. 2G, H). Similarly, the HIF1A mutated clone of U87MG cells also exhibited greatly reduced colony formation and delayed tumor growth in xenograft recipients (Fig. S2B, C).

To test if HIF1A is critical for tumor growth in primary GBM cells, we performed similar experiments in xenograft mice using Glio-1 and Glio-2 cells. To maintain the heterogeneity of primary GBM, we used high-titer lentiviral HIF1A silencers to infect the cells prior to intracranial transplantation. As shown in Fig. S3A, infection efficiency was similar in HIF1A shRNA- or scrambled shRNA- infected cells, based on expression of the GFP reporter. Co-transfection of HEK293FT cells with

Fig. 1 High expression of HIF1α, HIF1α targets, PDGFs and PDGFRα in GBM. A Analysis of TCGA database for the mRNA levels of indicated genes in GBM samples (N = 174) versus low-grade glioma (LGG) samples (N = 529). B C HIF1α governs expression of growth factors and receptors, and specific targets and substrates associated with AKT activation. Cas9-guided RNA method was used to generate HIF1A knockout (KO) or scrambled sgRNA control (WT) U251 cells for exome RNA-sequencing. Heat-maps depict the differential gene expression profiles among two HIF1A KO U251 clones (KO1 and KO5), and WT polyclone control U251 cells, performed in triplicate for each sample. Analysis of growth factors and receptors (B) or HIF1α targets and substrates associated with AKT activation (C) are shown. D Immunoblot showing protein levels of indicated genes in freshly frozen tumor tissues from primary surgically-resected adult gliomas
HIF1A-sh-GFP and HIF1α-P2A-RFP plasmids validated the successful shRNA knockdown of HIF1A mRNA by the expression of RFP and GFP reporters, as the HIF1α-P2A-RFP plasmid allows for HIF1α and RFP to be expressed as a single mRNA transcript which is then translated as two proteins, separated by the self-cleaving peptide P2A (Fig. S3B). As shown in Fig. 2I and J, silencing HIF1A in the Glio-1 or Glio-2 primary cells also restrained tumor growth and significantly extended survival of recipient mice compared to scrambled-sh (Sr-sh) control cells (Fig. 2I, J). Taken together, these data indicate that HIF1α plays a critical role in the growth and in vitro invasion of GBM cells.

**HIF1A regulates expression of PDGFRα and PDGFD in GBM cells**

As oxygen availability is unevenly distributed throughout GBM tumors, we examined the impact of differential oxygenation on protein levels of HIF1α, PDGFRα, PDGFD, and phospho-AKT in WT or HIF1α-KO U251 cells exposed to a range of oxygen tensions. As shown in Fig. 3A, in wild type cells, mild hypoxia (5% O2, 8 h)
increased protein levels of both HIF1α and PDGFRα with minimal effect on PDGF-D and PDGFB-B, whereas moderate (5% O2, 48 h) or severe (1% O2, 48 h) hypoxia increased PDGF-D and PDGFB protein levels but dramatically reduced that of PDGFRα (Fig. 3A, S3C). However, neither mild nor moderate hypoxia increased the activation of AKT and, like PDGFRα, severe hypoxia actually reduced AKT activation despite maintaining the accumulation of HIF1α (Fig. 3A). HIF1A knockout dramatically reduced the expression of both PDGFRα and PDGFD, moderately reduced PDGFB, but increased PDGFRβ at normoxia (Fig. S3C). Combined with the growth reduction and hypoxia-induced apoptosis in HIF1A-KO cells (Fig. 2), these results indicate that HIF1α favors GBM cell growth in normoxic and mild to moderate hypoxic conditions in which the growth factors and the receptor PDGFRα and the AKT activation are all maintained persistently.

To ascertain the correlation between HIF1α and PDGFRα, we tested expression of HIF1α and PDGFRα among 35 cases of GBM tissues. We observed high expression of both proteins in 21/35 GBM cases, depicted in the representative images (Fig. 3B, upper row), and intensity of HIF-1α staining was highly correlated with that of PDGFRα, with rare instances of single-positive staining (Fig. S4C). In addition to GBM, co-expression
of HIF1α and PDGFRα proteins was also observed in other types of gliomas, but not in medulloblastoma or adjacent normal brain tissues (ANB) (Fig. S4 A,B). Similarly, we observed a correlation between HIF1α and PDGF-D expression with double positive stains observed in 23 of 38 cases of a GBM tissue microarray (Fig. S4C), and representative double-positive staining shown in Fig. 3B, bottom row.

To see if HIF1α directly regulates the expression of PDGFRα and PDGF-D in GBM, we first identified a putative hypoxia-response element (HRE) in the PDGFRα proximal promoter, and 2 HREs within intron 1 of the PDGFRα gene (Fig. 3C). We cloned the PDGFRα proximal promoter, with or without its intronic HREs, into a GFP-reporting vector and cotransfected the constructs with mutant HIF1α-PPN (a P402A/P564A/N803A mutant resistant to degradation under normoxia) into HEK293 cells. As shown in Fig. 3D and E, HIF1α did not induce the proximal promoter activity of PDGFRα (PDGFRα-P1). Adding either intronic HRE to the basic promoter upstream of the GFP reporter (PDGFRα-P1-E1 and PDGFRα-P1-E2, respectively) resulted in the activation of the PDGFRα promoters (Fig. 3D,E). The PDGFRα-P1-E1 promoter activity was also activated by hypoxia mimetic CoCl₂ in concentrations ranging from 50 to 250 μg/ml (Fig. S3D). Chromatin immunoprecipitation (ChIP) in WT U251 cells with or without 8-h serum-starvation revealed that endogenous HIF1α can bind to the regions encompassing each of the enhancers, but not to the HRE region located in the proximal basic promoter of PDGFRα under normoxic conditions (Fig. 3F). These results demonstrated that HIF1α regulates the expression of PDGFRα through binding to the PDGFRα enhancers rather than its basic promoter.

HIF1α also activated the proximal promoter activity of PDGF-D in HEK293 cells co-transfected with plasmids of stable HIF1α-PPN and the PDGF-D promoter (PDGF-D-P-GFP) (Fig. 3G). Compared to empty vector, HIF1α enhanced the promoter activity more than 2-fold, as determined by flow-cytometry (Fig. 3H). CoCl₂ also activated the PDGF-D promoter activity at concentrations ranging from 100 to 500 μg/ml (Fig. S3D). ChIP revealed endogenous HIF1α bound to the proximal promoter region of PDGF-D in U251 cells, which was also modestly
increased by serum stimulation (Fig. 4I). No specific PCR product could be detected in control IgG ChIP assay, which confirmed the specificity of HIF1α binding to PDGFD (Fig. 4I). Therefore, HIF1α directly regulated PDGF-D expression in GBM.

**PDGFRα and PDGF-D are required for invasion and growth of GBM cells**

To test if HIF1α-regulated PDGF-D and PDGFRα are essential for GBM growth and invasion, we generated PDGFRα KO or PDGFD KO U251 cells by Crispr-Cas9 sgRNA editing method (Fig. 4A,B). Knockout of PDGFRα minimally affected HIF1α, PDGF-D and PDGF-B levels, at either normoxic or hypoxic conditions (Fig. 4 C), whereas knockout of PDGFD dramatically reduced protein levels of HIF1α, PDGFRα and PDGF-B regardless of oxygen levels (Fig. 4D). Knockout of PDGFRα or PDGFD dramatically reduced growth and invasion of U251 cells in vitro when cells were seeded at low cell density (Fig. 4E, F). More importantly, these PDGFRα KO lines did not grow or cause mortality in NSG recipient mice throughout the observation period of 120 days (Fig. 4G, H). Mice that received PDGFD KO lines had significantly longer survival than mice engrafted with PDGFD WT cells (Fig. 4H). These results demonstrated that HIF1α controls GBM growth mainly through upregulating the expression of PDGFRα and PDGF-D.

**HIF1α-PDGFD-PDGFRα pathway controls constitutive activation of AKT, leading to GBM cell growth and invasion**

PDGF-D was reported to bind to and activate PDGFRβ/β homodimer and PDGFRα/β heterodimer in cells expressing both receptors [41, 42]. As PDGFRβ expression was much lower in U251 cells than PDGFRα expression, we compared PDGFR-D to other PDGFR family members for their ability to activate PDGFRα, and if so, whether such interactions result in the HIF1α accumulation in normoxia. Stimulating U251 cells with recombinant PDGF-A, PDGF-B or PDGF-D induced phosphorylation of PDGFRα to a comparable extent, which was blocked by the PDGFR-specific tyrosine kinase inhibitor AG1296 (Fig. 5A), indicating PDGFRα acts as their receptor. Although the transactivation EGFR by PDGF-B was reported in fibroblasts [43], none of these PDGFs activated the phosphorylation of EGFR in U251 cells (Fig. 5A). The activation of PDGF to PDGFRα relayed signals to its down-stream pathway activation of both AKT and ERK, as the PDGFR inhibitor blocked their phosphorylation completely. The EGFR inhibitor AG1478 at 0.5 μM concentration slightly inhibited the PDGFRα activation, as well as the activation of AKT and ERK induced by PDGF-D, whereas it completely blocked EGF-induced EGFR signaling cascades (Fig. 5A). The PDGFD-PDGFRα-AKT signaling cascade required HIF1α for its constitutive activation because knockout of HIF1α abolished the expression of both the ligand and receptor, and then the phosphorylation of AKT (Fig. 5B). This pathway activation is also required for HIF1α accumulation in normoxia (Fig. 5B) because knockout of either PDGFD or PDGFRα abolished or reduced HIF1α protein levels. Compared to AKT activation, transfection of PDGFRα to HIF1α KO U251 cells not only moderately increased AKT activation and cells growth, but also greatly enhanced the cell invasive ability in vitro (Fig. 5 C, E). Overexpression of HIF1α in HIF1α KO U251 cells completely restored the PDGFRα expression, AKT activation, and thus the colony growth and cell invasion in vitro (Fig. 6 D, E).

It is worth noting that ERK activation either in the absence of exogenous PDGF or in the presence of exogenous PDGF-D was moderately affected by ablation of HIF1α, although its activation by PDGF-A was dramatically affected by the HIF1α ablation (Fig. 5B). Since ERK activation is moderately affected by deletion of PDGFRα, and since PDGFRβ levels increased in the HIF1α knockout cells (Fig. 5C), the basal and PDGFD-induced activation of ERK may relate to PDGFD interaction with PDGFRβ or other receptors that are independent of HIF1α and PDGFRα. Nevertheless, ERK activation by PDGFRβ signaling pathway cannot compensate the loss of HIF1α-PDGFD-PDGFRα-AKT network for GBM tumor growth because knockout of either HIF1α or PDGFD or PDGFRα in U251 cells eradicated the tumor growth (Fig. 2G, H and Fig. 4G, H). As HIF1α knockout also abolished PDGF-D release (Fig. 5A, S5A), and PDGFD knockout largely reduced HIF1α protein levels, their reciprocal regulation is both autocrine and feedforward.

**Echinomycin inhibits HIF1α-PDGFD-PDGFRα-AKT signaling and induces apoptosis of GBM cells**

Having established the novel feedforward mechanism of HIF1α-PDGFR- AKT pathway, we tested the effect of HIF1α inhibitor Echinomycin in regulating this new pathway. Echinomycin inhibited PDGFRα expression and AKT activation in a dose-dependent manner under normoxic or mild hypoxic conditions (Fig. 6A). The inhibition was also time-dependent, as shown in Fig. 6B. We observed a similar dose-response when echinomycin was used to treat primary Glio-1 cells (Fig. 6C). Figures 6D and E showed that echinomycin also inhibited PDGF-D secretion in a dose-dependent manner from U251 cells. Correspondingly, Echinomycin induced apoptosis of U251 cells in a dose-dependent manner (Fig. 6F and S5B), although it had minimal effects on the viability of HIF1α KO cells at low doses when compared with WT cells, confirming its on-target effect (Fig. S5C).
Similar to HIF1A KO cells, the PDGFRα KO cells also exhibited resistance to Echinomycin treatment (Fig. S5D), which suggests that the two proteins work on the same pathway for cell viability.

**Targeting HIF1α by liposomal echinomycin inhibits tumor growth and prolongs survival of GBM-xenografted NSG mice**

To test the impact of pharmacologically targeting the HIF1α-PDGFD-PDGFRα axis in GBM in vivo, we took advantage of an improved formulation of Echinomycin which we developed recently using liposomes (LEM) to treat solid tumors [44, 45]. LEM prolonged survival in mice xenografted with primary Glio-1 or Glio-2 tumors by about 20 days (Fig. 6G,H). By sequencing the PCR products of the genomic DNA encompassing exons 2–9 of the TP53 gene, we found that Glio-1 cells were surprisingly contained the same R273H hot-spot mutation as U251 cells, albeit heterozygous, whereas no TP53 mutation was observed in Glio-2 (Fig. S5E). However, regardless of TP53 mutation status, LEM conferred similar survival advantages to mice engrafted with either Glio-1 or Glio-2, indicating that HIF1α and HIF1α-controlled PDGFD-PDGFRα-AKT signaling contributes more to the sensitivity to Echinomycin treatment. Indeed, in comparison to vehicle controls, Echinomycin inhibited the Glio-1 tumor growth as seen in the reduced tumor size of representative sections (Fig. 6I, top), and the proliferation as judged by the reduced proliferative marker Ki67 in contrast to the increased apoptotic marker cleaved-caspase3 (Fig. 6I, bottom). Echinomycin also reduced immunofluorescent staining of HIF1α and PDGF-D in GBM tissues (Fig. 6J).

These results demonstrate that Echinomycin effectively targets the HIF1α-PDGFD-D axis to inhibit GBM growth.

**Overexpression of PDGF-D stimulates tumor growth and angiogenesis in immunocompetent mice, and renders sensitivity to echinomycin treatment**

Unlike PDGF-B, which is expressed and released as an active homodimer, PDGF-D is expressed and released as an inactive homodimer that is activated by extracellular...
serine proteinases [46]. Thus, it has been unclear whether PDGF-D played a significant role in GBM pathogenesis. In murine GL261 GBM cells, ectopic overexpression of PDGF-D (Fig. 7A) significantly accelerated tumor growth and mortality of recipient mice (Fig. 7B,C). Moreover, overexpression of the active form of PDGF-D (i.e. PDGFD-dCUB), resulting from deletion of the inhibitory CUB domain (Fig. 7A) [46] had an even more pronounced effect (Fig. 7B,C). The data indicates that GBM tumors are capable of proteolytically activating the potent growth factor PDGF-D. In response to a cycle of treatment (Fig. 7D), Echinomycin effectively neutralized the growth advantage of PDGFD-transduced GL261 tumors and conferred equivalent therapeutic effects for both transduced and un-transduced GL261 tumors (Fig. 7E,F). Immunofluorescence of PDGFRα and angiogenic marker CD31 in tumor tissues revealed an increase in GL261-PDGFD vs GL261-vector tissues (Fig. 7G). LEM treatment of the GL261 brain tumor greatly reduced the expression of Pdgfd, Pdgfra and Igfbp2, as detected by quantitative RT-PCR, compared to vehicle control tumors (Fig. S6).

Taken together, we demonstrate that HIF1α is a crucial effector on the constitutive activation of AKT...
through controlling the expression of PDGF-D and PDGFRα in an autocrine and feedforward manner for GBM growth and malignancy, which could be therapeutically targeted by LEM (Fig. 7H).

Discussion
GBM is characterized by cellular heterogeneity, integrated oncogenic signaling pathways, intratumorally intricate microenvironments, and distinct regions including a necrotic/hypoxic core surrounded by intermediate/hypoxic layer and by highly proliferative, well-oxygenated and -vascularized outer layer/frontier [27, 28]. Collectively, these complex features of GBM underpin therapeutic strategies targeting single pathways in isolation [47, 48]. HIF1α expression pattern was heterogenous seen in the hypoxic zone and the normoxic frontier of GBM [49]. Our studies revealed that, to different extents, HIF1α ablation reduced constitutive activation of both AKT and ERK signaling pathways in U251 cells. Thus, HIF1α may function as a converging point between these signaling pathways via controlling the expression of PDGF-B, PDGF-D and PDGFRα for the malignancy of GBMs. In addition, based on RNA-Seq data of U251 cells, HIF1α may also control IGF-IGF1R-AKT and FGF signaling pathways for GBM growth and invasion. Thus, HIF1α is a crucial master transcriptional factor that orchestrates the expression of growth factors, receptors, signal-pathway substrates, and angiogenic factors under conditions of normoxia and physiological hypoxia. Inhibiting or deleting HIF1α greatly restrains growth advantages exploited by GBM and may disrupt the reciprocal or feedback regulation between PDGFRα-PI3K-AKT and EGFR-ERK pathways once targeting them separately [50]. Meanwhile,
targeting HIF1α could largely block angiogenesis via inhibiting expression of VEGF, PDGF-B and PDGF-D; the latter two of which may promote angiogenesis through binding VEGFR2 and coreceptor neuropilin 1, respectively [51, 52]. Taken together, these features provide a rationale for supporting development of HIF1α-targeting therapies, such as echinomycin, for the treatment of GBM.

HIF1α is normally degraded under conditions of normoxia, although noncanonical mechanisms are known to promote HIF1α stability regardless of oxygen tension. Our studies provide additional evidence for noncanonical HIF1α stabilization, as PDGFD and PDGFRα are both required for high HIF1α accumulation in GBM cells under normoxia. While PDGFD was found to be a hypoxia-induced gene in our study, expression of PDGFRα was only induced by mild hypoxia or normoxia, and AKT activation was only induced under normoxia in GBM cells. Therefore, the paracrine activity of PDGFD and PDGF-D originating from intermediate/hypoxic middle-layer of GBM via canonical HIF1α stabilization may trigger the HIF1α stabilization in the GBM cells located in the oxygen-rich leading edge of the tumor. More importantly, as PDGFD, PDGFRα, and HIF1α were all required for GBM cell growth and invasion under normoxia and in xenografted mice, our work implies that the PDGFD-PDGFRα-HIF1α axis could be an essential event for GBM malignancy, which is predominantly functional under normoxic or mildly hypoxic conditions. The HIF1α driven PDGFD and PDGFRα transcription in these conditions suggest that HIF1α promotes a feedforward PDGFD-PDGFRα-AKT signaling in the GBM leading edge. Thus, our findings provide mechanistic insight as to how GBM invades to surrounding tissues.

PDGF-A stimulation or overexpression and PDGFB overexpression in glial or neural progenitors of mice have been demonstrated each acting a driver for glioma-like neoplasms or glioma-genesis in those mouse models [53–55]. However, their roles in the growth and malignancy of human glioma including GBM cell lines have not been revealed. We showed here that PDGF-D is a potent growth factor for both human and mouse GBM cells. Whether it is able to initiate glioma-like brain tumors in mice remains to be defined.

PDGF and PDGFR are frequently co-expressed in human glioma cell lines as well as high-grade gliomas [28, 31]. Previous study showed that PDGFB promoter was activated in breast cancer cells by HIF1α under hypoxia [56]. Consistently, we showed here that HIF1α induced PDGFB, as well as PDGF-D, in U251 cells under hypoxia. In addition, our data showed that PDGF-D was unexpectedly as potent as PDGF-A and PDGFB in U251 cells which predominantly expressed PDGFRα, suggesting that PDGF-D may activate PDGFRα via another receptor rather than PDGFRβ. These findings provide important new insights on how PDGF-D promotes GBM pathogenesis.

TP53 mutation is observed in up to 54% of proneural GBM, a subtype which also displays frequent overexpression of PDGFRα [5]. Mutant TP53 often drives chemotherapy resistance to the first-line drug temozolomide and is associated with poor prognosis [57, 58]. Targeting HIF1α by liposomal Echinomycin could exert significant therapeutic effects in mouse models of GBM regardless of TP53 mutations. This effectiveness of Echinomycin is consistent with our previous observation that Echinomycin is effective against TP53 mutated AML [59].

Conclusions
This report is the first to reveal PDGF-D as a potent growth factor for human GBM cells. HIF1α plays a critical role in constitutive activation of the AKT signaling pathway for GBM growth by controlling expression of PDGF-D and PDGFRα under normoxia and physiological hypoxia. The HIF1α inhibitor Echinomycin abolishes the HIF1α-PDGFD-PDGFRα feedforward axis for constitutive activation of AKT pathway and therefore provides a novel and potentially effective therapeutic approach for GBM.

Statistical analysis
All statistical analysis was performed using the SPSS 21.0 statistical software program. Quantitative data were expressed as mean ± SD. Comparison of two groups was analyzed with student’s T test. Survival between groups was compared with the log-rank test. A value of p < 0.05 was considered statistically significant.

Abbreviations
ABN: Adjacent normal brain tissues; cCasp3: Cleaved caspase 3; ChIP: Chromatin immunoprecipitation assay; CNS: Central nervous system; EGFR: Epithelial growth factor receptor; ERK: Extracellular regulated protein kinase; FASN: Fatty acid synthase; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; GBM: Glioblastoma multiforme; GLUT1: Glucose transporter subunit alpha; HK2: Hexokinase 2; HIF: Hypoxia-inducible factor; IGFBP2: Insulin like growth factor binding protein 2; LDHA: Lactate dehydrogenase A; LEM: Liposomal Echinomycin; LGG: Low-grade glioma; MCT4: Monocarboxylate transporter 4; NSG: NOD-SCID IL-2receptor gamma null; PARP: Poly(ADP-Ribose) polymerase; PDGF: Platelet-derived growth factor; PDGFD: Platelet-derived growth factor; PDGFRα: Platelet-derived growth factor receptor subunit alpha; PDGFRβ: Platelet-derived growth factor receptor subunit beta; PDK1: Pyruvate dehydrogenase kinase 1; PGK1: Phosphoglycerate kinase 1; PPK1: Phosphoglycerate kinase 1; RTK: Receptor tyrosine kinase; SCD: Stearoyl-CoA desaturase; sRNA: Small guide RNA; TGCA: The cancer genome atlas; VEGF-A: Vascular endothelial growth factor A

Supplementary Information
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Primary Glio-1 cells have an R273H mutation of TP53 which is also carried by Echinomycin for 72 h prior to determining cell viability by MTT assay. E. PDGFRA or PDGFRB treated for 48 h with different concentrations of Echinomycin. C, D. Western blots of α-stains of 23/38 cases with low, moderate, high, or very high scores were used to visualize nuclei (blue). The correlation of staining intensity for α in GBM tissue microarray. Sections of brain tissue was performed with PDGF-D and HIF1α antibodies as described in D. The double positive staining for HIF1α and PDGF-D in GBM. Microarray of brain tumor with adjacent normal tissues was co-stained with PDGFRα and HIF1α double positive staining for HIF1α and PDGF-D, which was presented as percentage of total tumor subtype cases. ANB, adjacent normal brain tissue; OD, oligodendrocytoma; OA, oligoastrocytoma; AA, anaplastic astrocytoma; MB, medulloblastoma; EP, ependymoma. Data shown are representative of two independent experiments. C. Correlation of staining intensity for HIF1α with PDGF-Rα in GBM. Microarray of brain tumor with adjacent normal tissues was co-stained with primary rabbit HIF1α antibody and mouse PDGF-Rα antibody, and with secondary antibodies goat anti-rabbit Alexa-Fluor 594 and goat anti-mouse Alexa-Fluor 488 after washing away primary antibodies. DAPI was used to visualize nuclei (blue). B. Summary of microarray cases with double positive staining for HIF1α and PDGF-Rα, which was presented as percentage of total tumor subtype cases. ANB, adjacent normal brain tissue; OD, oligodendrocytoma; OA, oligoastrocytoma; AA, anaplastic astrocytoma; MB, medulloblastoma; EP, ependymoma. Data shown are representative of two independent experiments. C. Correlation of staining intensity for HIF1α with PDGF-Rα in GBM. Microarray of brain tumor with adjacent normal tissues was co-stained with primary rabbit HIF1α antibody and mouse PDGF-D antibody, and with secondary antibodies donkey anti-rabbit Alexa-Fluor 488 and donkey anti-goat Alexa-Fluor 594 after washing away primary antibodies. DAPI was used to visualize nuclei (blue). The correlation of staining intensity for HIF1α and PDGF-D are shown. E. Correlation of staining intensity for HIF1α with PDGF-D in GBM tissue microarray. IF staining of a 33 cases tissue microarray of GBM and 5 cancer adjacent normal cerebral tissue was performed with PDGF-Rα and HIF1α antibodies together as described in A. The double positive stains of cases with low, moderate, high, or very high scores were analyzed for the correlation of HIF1α with PDGF-D. C. Co-expression of HIF1α and PDGF-D in GBM. Sections of brain tumor with adjacent normal brain tissue microarray were co-stained with primary rabbit HIF1α antibody and goat PDGF-D antibody, and with secondary antibodies donkey anti-rabbit Alexa-Fluor 488 and donkey anti-goat Alexa-Fluor 594 after washing away primary antibodies. DAPI was used to visualize nuclei (blue). The correlation of staining intensity for HIF1α and PDGF-D were shown. E. Correlation of staining intensity for HIF1α with PDGF-D in GBM tissue microarray. IF staining of a 33 cases tissue microarray of GBM and 5 cancer adjacent normal cerebral tissue was performed with PDGF-Rα and HIF1α antibodies as described in D. The double positive stains of 23/38 cases with low, moderate, high, or very high scores were analyzed for the correlation of HIF1α with PDGF-D. Figure S5. TP53 mutation did not affect GBM response to Echinomycin. A. PDGF-DD levels in the medium of HIF1α KO or WT U251 cells. Levels of released PDGF-D protein were measured by ELISA. B. Echinomycin induced apoptosis of U251 cells. Annexin V staining was performed on U251 cells that were treated for 48 h with different concentrations of Echinomycin. C, D. HIF1α-KO or PDGFRα-KO cells are resistant to Echinomycin. WT, HIF1α-KO or PDGFRα-KO U251 cells were treated with different concentrations of Echinomycin for 72 h prior to determining cell viability by MTT assay. E. Primary Glio-1 cells have an R273H mutation of TP53 which is also carried by U251 cells. Sequence chromatograms are shown with arrows indicating R273H mutation. Figure S6. Echinomycin reduced the expression of HIF1α target genes. Empty vector-transfected GL261 brain tumor cells were orthotopically transplanted to recipient mice, and the mice were treated with vehicle or LEM as detailed in methods. Twenty-four hours after the final dose, the tumor cells were isolated and the cDNA was used to perform qRT-PCR. Table S1. Characteristics of Clinical Glioma Samples. Table S2. RNA-Seq data of growth factor related genes expressed in WT and HIF1α KO in U251 cells. Table S3. RNA-Seq data of metabolism related genes expressed in WT and HIF1α KO in U251 cells.

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Availability of data and materials
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Declarations
Ethics approval and consent to participate All procedures involving experimental animals were approved by Institutional Animal Care and Use Committees of the University of Maryland School of Medicine and the First Hospital of Jinlin University.

Consent for publication
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Competing interests
Authors disclosed no potential conflicts of interest.

Author details
1Institute of Translational Medicine, the First Hospital of Jinlin University, Changchun, Jilin, China. 2Division of Immunotherapy, Department of Surgery and Comprehensive Cancer Center, Institute of Human Virology, University of Maryland School of Medicine, Baltimore, USA. 3Department of Neurosurgery, Neuroscience Research Center, The First Hospital of Jinlin University, Changchun, Jilin, China. 4Beijing Pediatric Research Institute, Beijing Children’s Hospital, Capital Medical University, National Cancer for Children’s Health, Beijing, China. 5Department of Neurosurgery, Beijing Children’s Hospital, Capital Medical University, National Cancer for Children’s Health, Beijing, China. 6OncoC4, Inc., Rodsville, MD, USA.

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