

Geranylgeranyltransferase I regulates HIF-1 α promoting glioblastoma cell migration and invasion

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Abstract Glioblastoma multiforme is a highly migratory and invasive brain tumor in which hypoxia inducible factor-1 α (HIF-1 α) plays important roles. However, the underlying mechanisms regulating the action of HIF-1 α in glioma cell migration and invasion ability remain unclear. We reported here that HIF-1 α was regulated by geranylgeranyltransferase I (GGTI), a protein prenylation transferase, and then promoted glioma cell migration and invasion. The migratory and invasive ability of glioma cells were enhanced by hypoxia treatment but inhibited by down-regulation of HIF-1 α . GGTI activity inhibition or GGTI specific β subunit (GGTI β) knocking-down decreased HIF-1 α protein level.

In addition, down-regulation of GGTI β inhibited migration and invasion of glioma cells under hypoxia, while GGTI β over-expression promoted it. Furthermore, the effect of GGTI β over-expression on cell migration and invasion was abolished by HIF-1 α down-regulation. In summary, our study showed, for the first time, that HIF-1 α was regulated by protein prenylation transferase GGTI and mediated the effect of GGTI on glioma cell migration and invasion.

Keywords Geranylgeranyltransferase I · Hypoxia inducible factor-1 α · Migration · Invasion · Glioma

Abbreviations

GGTI Geranylgeranyltransferase I
HIF-1 α Hypoxia inducible factor-1 α
ECM Extracellular matrix
FT Farnesyltransferase

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Introduction

Glioblastoma multiforme (GBM) is the most common and malignant primary tumor developed in the central nervous system. Despite advances in surgery and adjuvant therapy, the median overall survival time does not exceed 15 months [1–3] due to the highly invasive capacity of tumor cells. Hypoxia and its master regulator, hypoxia inducible factor-1 α (HIF-1 α) play key roles in glioma migration and invasion [4–7].

Although HIF-1 α maintains at a low level in normoxic cells due to proteasome mediated protein degradation [8], it plays a pivotal role in the cellular response to tumor hypoxia, which represents a major obstacle to the success

of radiotherapy and chemotherapy [7]. It is reported that HIF-1 α was upregulated in many common human cancers, such as prostate cancer, breast cancer and glioma [6, 9–11]. Suppression of HIF-1 α expression via antisense oligonucleotides reduces the survival of glioblastoma cells and accelerates p53-independent apoptosis [12]. In addition, knocking-down of HIF-1 α by RNA interference attenuates human glioma cell migration and invasion [7, 13, 14]. Although HIF-1 α function is extensively studied under hypoxic stress, the mechanism regulating the action of HIF-1 α is still unclear. Given its pivotal role in cancer biology, the underlying mechanisms involved in the activation of HIF-1 α in glioma cells need to be further understood.

It is reported that members of the Rho small GTPases, which play essential roles in a variety of cellular events such as cell adhesion and invasion [15, 16], are activated in response to hypoxia and required for the induction of HIF-1 α expression and transcriptional activity in hypoxic cells [17–19]. Rho GTPases recycle between GTP-bound active form and GDP-bound inactive states, and this process is regulated by GTPase activating proteins and guanine nucleotide exchange factors [20]. Importantly, before being activated by combining with GTP, Rho GTPases need to be translocated from the cytosol to the plasma membrane [21], which is achieved by prenylation, a lipid modification mainly catalyzed by geranylgeranyltransferase I (GGTI). GGTI acts to covalently couple a lipid moiety to the cystine of C-terminal “CAAX” box (Cys-aliphatic-aliphatic-X) of the GTPases [21, 22]. GGTI has two subunits: an α subunit (GGTI α) which is shared with farnesyltransferase (FT) and a distinct β subunit (GGTI β) [23]. As for FT, it has another distinct β subunit highly homologous with GGTI [23].

Since Rho small GTPases are demonstrated to be upregulated or highly mutated in many types of human tumors [15, 16], GGTI has been considered as a good cancer therapeutic target and great efforts have been made to develop GGTI inhibitors as anticancer drugs [23]. Many studies reported that GGTI specific inhibitors could induce apoptosis and inhibit cell proliferation in many tumors [23–25]. However, little is known about the migratory and invasive effect of GGTI on tumor cells.

In summary, because Rho GTPases could induce the expression of HIF-1 α in hypoxic cells [17–19] and their activation need to be prenylated by GGTI first, we deduced that GGTI might regulate HIF-1 α and play roles in tumor cell migration and invasion. In the present study, we found that HIF-1 α promoted glioma cell migration and invasion, while down-regulation of HIF-1 α inhibited it. GGTI regulated HIF-1 α induction and GGTI β down-regulation inhibited glioma cell migration and invasion, similar to that of HIF-1 α . Finally, over-expression of GGTI β induced

migration and invasion were abrogated by HIF-1 α down regulation. These results suggested that GGTI regulated HIF-1 α induction, and then promoted glioma cell migration and invasion.

Materials and methods

Materials

U251 and U87 cells were purchased from Shanghai Cell Bank, Type Culture Collection Committee, Chinese Academy of Sciences. pCAG-YFP-GGTI β was kindly gifted by professor ZG Luo at Shanghai Institute of Neuroscience of Chinese Academy of Sciences. Dulbecco's modified Eagle's medium, F-12 (DMEM/F-12) and Lipofectamine 2000 were purchased from Invitrogen (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from Sijiqing Biological Engineering Materials Co., Ltd. Transwell invasion chambers were purchased from Corning Incorporated (Corning, USA). GGTI-298 was obtained from Calbiochem (Schwallbach, Germany). Following antibodies were used in this study: rabbit polyclonal anti-GGTI β (kindly gifted by professor ZG Luo); mouse monoclonal anti-HIF-1 α (BD Transduction Laboratories, USA); mouse monoclonal anti-FT β and rabbit polyclonal anti-GGT α /FT α (Santa Cruz Biotech, USA); mouse monoclonal anti- β -actin (Millipore Co., USA).

Cell culture

U251 and U87 cells were cultured in 5 % CO₂ and 95 % humidified atmosphere air at 37 °C in Dulbecco's modified Eagle's medium, F-12 (DMEM/F-12) supplemented with 10 % FBS. For hypoxic exposure, cells were placed in a sealed Modular Incubator Chamber (150i, thermo Inc., USA) flushed with a gas mixture containing 1 % O₂, 5 % CO₂ and 94 % N₂.

Small interfering RNAs and plasmids transfection

U251 and U87 cells were transiently transfected with siRNAs by using Lipofectamine 2000 according to the manufacturer's instructions. siRNA duplexes purchased from Shanghai GenePharma Co. were listed below:

HIF-1 α si1217: (5'-GCCGCUCAAUUUAUGAAUATT-3');
 HIF-1 α si2791: (5'-CCAGUUAUGAUUGUGAAGUUA
 TT-3');
 GGTI β si217: (5'-GGUGAACAAAGAUGAUUATT-3');
 GGTI β si1015: (5'-GGAGGAAAGUGGAAUUGUT
 T-3').

Wound healing assay

A scratch was made in the middle of the well with a pipette tip 24 h after transfection and the monolayer was washed twice with phosphate-buffered saline (PBS, pH 7.4). Thereafter, the cells were maintained in serum-free media and incubated in hypoxic condition (1 % O₂) at 37 °C for 24 h. Then five randomly selected fields at the lesion border were photographed using an inverted microscope (Olympus IX71). The number of cells across the wound was normalized to that of hypoxia group.

Invasion assay

Cell invasion was assessed by matrigel precoated transwell inserts (8.0-µm pore size with polyethylene tetraphthalate membrane) according to the manufacturer’s protocol. To

assess invasion, filters were precoated with 10 µg of matrigel (BD Biosciences). A pretreated cell suspension (1 × 10⁵) in serum-free culture media was added into the inserts, and each insert was placed in the lower chamber filled with culture media containing 10 % FBS as a chemoattractant. The invasion chambers were incubated in hypoxic condition (1 % O₂) at 37 °C for 24 h. And then the invasion assay was performed as described [26].

Gelatin zymography assay

Cells (5 × 10⁵) were seeded in 6-well plates in DMEM/F-12 media containing 10 % FBS for 24 h and transfected with GGTI β siRNA or HIF-1α siRNA. Thereafter, cells were incubated in serum-free DMEM/F-12 for an additional 24 h in hypoxic condition (1 % O₂) at 37 °C. Then preformed as described previously [27].

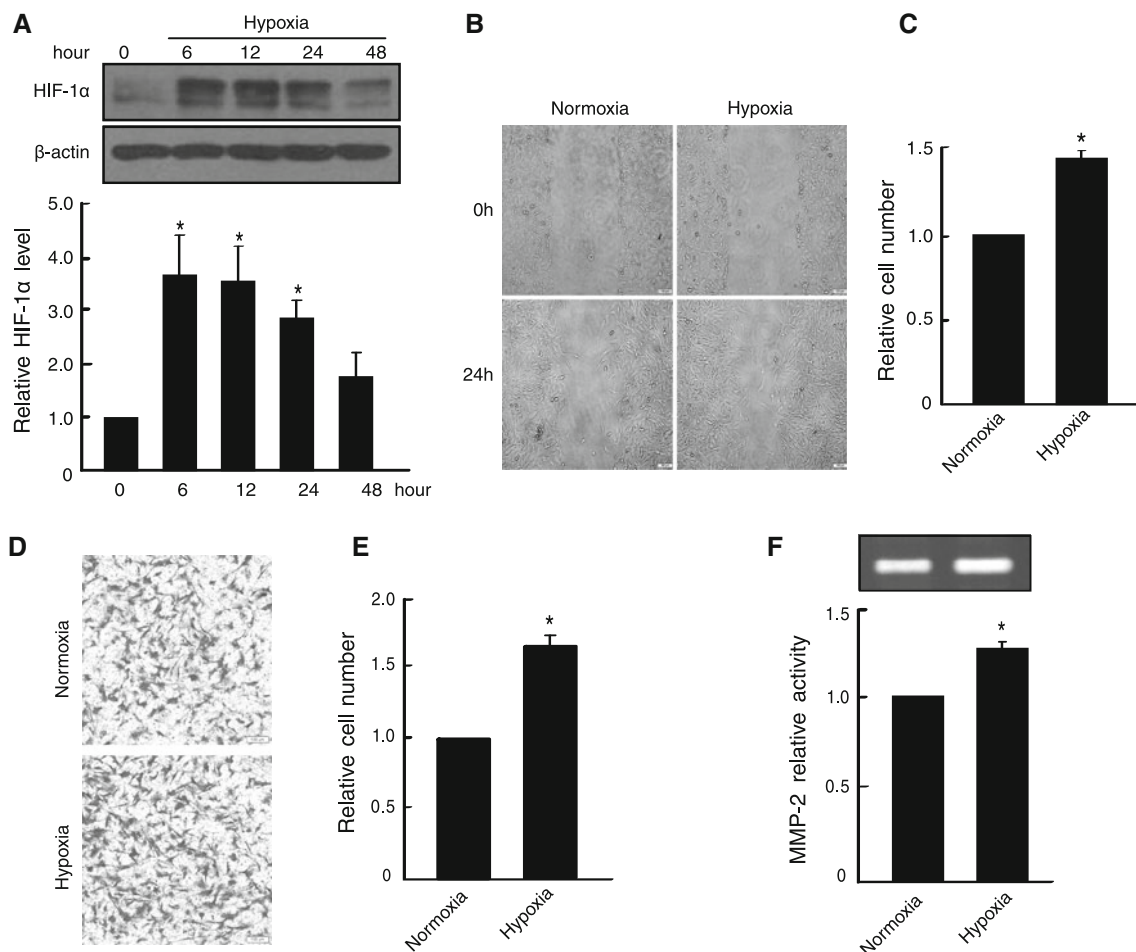


Fig. 1 Hypoxia promotes the migration and invasion of glioma cells. **a** U251 glioma cells were grown under normoxia (20 % O₂) or hypoxia (1 % O₂) conditions for the indicated time. The protein level of HIF-1α was analyzed by Western blot. β-actin served as an internal control. **b, c** Hypoxia promotes glioma cell migration examined by wound healing assay. Representative digital pictures were taken at 0

and 24 h. **d, e** Hypoxia promotes glioma cell invasion examined by matrigel precoated transwell assay. **f** Hypoxia promotes MMP2 excretion examined by gelatin zymography. Results are presented as mean ± SEM from three separate experiments. Scale bar 100 µm. **P* < 0.05

Western blot analysis

At the designated time, the cells were removed quickly from the hypoxic chamber, washed twice with PBS, and lysed using ice-cold sodium dodecyl sulphate (SDS) buffer (60 mM Tris-HCl pH 6.8, 70 mM SDS, 10 % Glycerol) supplemented with protease inhibitors, and western blot was performed as described [28]. Band densities were quantified by ImageJ software (NIH; version 1.45), and the densitometric results were shown. The relative amount of proteins was determined by normalizing the densitometry value of interest to that of the internal loading control.

Statistical analysis

The results are representative of experiments repeated at least three times and presented as the mean \pm SEM.

Fig. 3 GGTI regulates HIF-1 α induction under normoxia and hypoxia conditions in glioma cells. **a** U251 glioma cells were grown under hypoxia (1 % O₂) conditions for the indicated time. The protein level of GGTI β , Rho and the membrane level of Rho were analyzed by western blot. β -actin or Na⁺-K⁺-ATPase served as internal control respectively. **b** The effect of GGTI β si217, si1015 or siNC on GGTI β expression in glioma cells. **c** U251 cells were transfected with siNC and GGTI β si1015, and exposed to 1 % O₂ for 24 h. The level of FT α and FT β were analyzed by Western blot. **d** U251 and U87 cells were transfected with siNC and GGTI β si1015, and exposed to normoxia or hypoxia conditions for 24 h. GGTI β and HIF-1 α level was analyzed by western blot. **e, f** Quantitative analysis of the relative protein levels of HIF-1 α and GGTI β in glioma U251 and U87 cells. **g** U251 cells were treated with DMSO or GGTI-298 and exposed to normoxia or hypoxia conditions for 24 h. HIF-1 α protein expression was analyzed by Western blot. **h** U251 and U87 cells were transfected with siNC and HIF1- α si2791, and exposed to normoxia or hypoxia conditions. GGTI β and HIF-1 α level was analyzed by Western blot. **i, j** Quantitative analysis of the relative protein levels of HIF-1 α and GGTI β in glioma U251 and U87 cells. β -actin was used as the loading control. Results are presented as the mean \pm SEM from three experiments. * P < 0.05

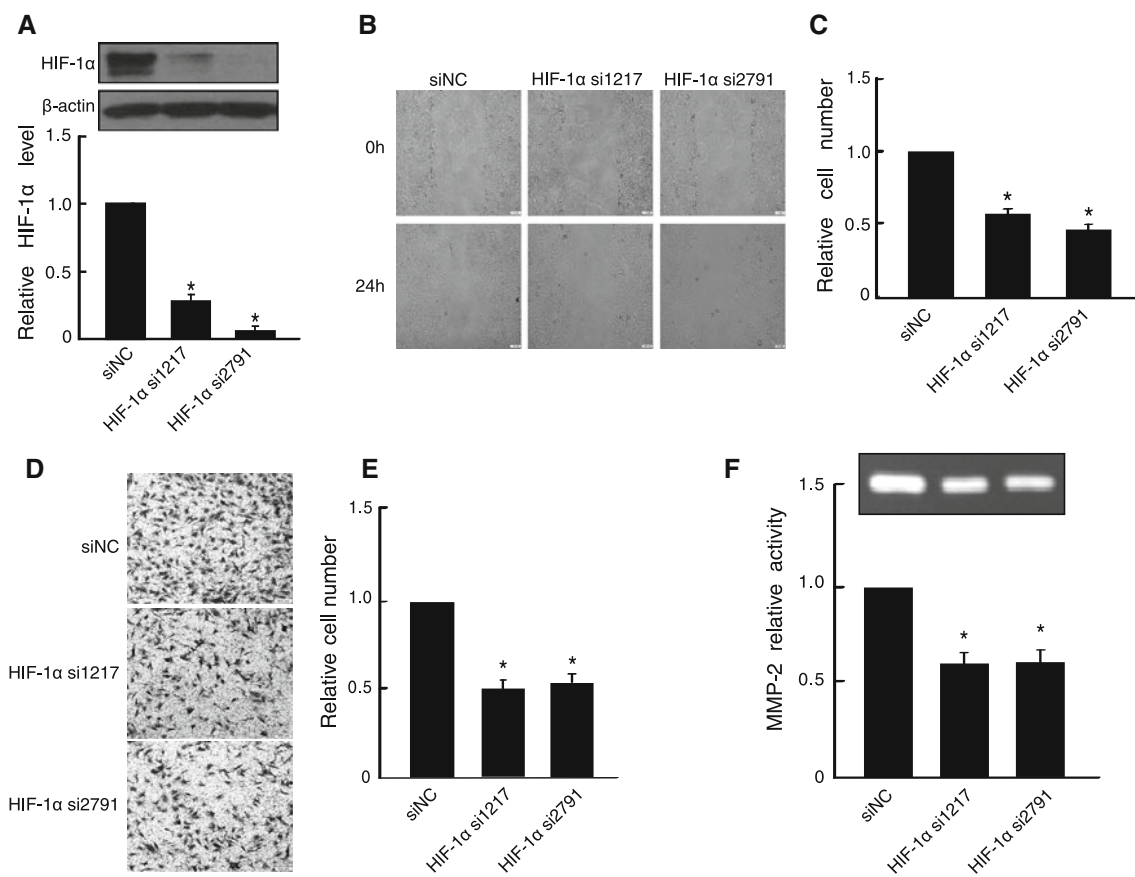
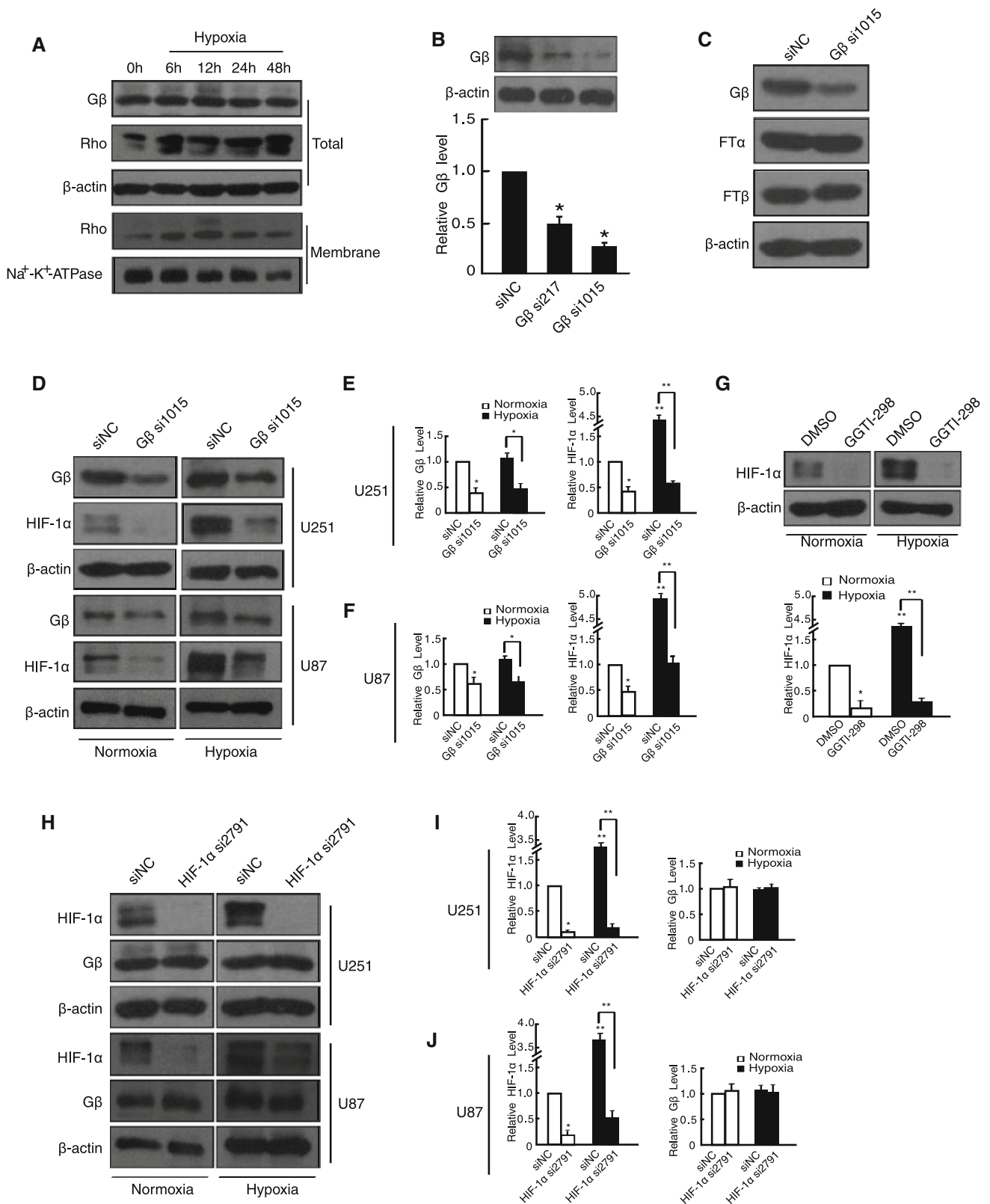


Fig. 2 Down-regulation of HIF-1 α inhibits hypoxia-induced glioma cell migration and invasion. **a** Immunoblot was applied to detect the effect of HIF-1 α si1217 and si2791 on HIF-1 α expression. β -actin was used as a loading control. **b–e** 24 h after being transfected with siNC, HIF-1 α si1217 and si2791, cells were subjected to wounding

assay and invasion assay under hypoxia condition. **f** 24 h after transfection, cells were exposed to hypoxia condition for further 24 h and the supernatant was harvested and subjected to gelatin zymography. Scale bar 100 μ m. Results are presented as the mean \pm SEM from three experiments. * P < 0.05



All statistical analyses were performed using SPSS software (SPSS; version 17.0). Statistical comparisons were performed using Student's *t* test with two tails or

one-way ANOVA for multiple comparisons. *P* values <0.05 were considered statistically significant (**P* < 0.05).

Results

Hypoxia promotes the migration and invasion of glioma cells

It is reported that HIF-1 α is activated under hypoxia and plays a key role in glioma migration and invasion [6]. But the mechanism regulating the action of HIF-1 α is still unclear. To further elucidate the role and regulating mechanism of HIF-1 α in malignant gliomas, we first investigated the effects of HIF-1 α on glioma U251 cell migration and invasion. As is shown in Fig. 1a, the protein level of HIF-1 α increased at 6, 12, 24 and 48 h after hypoxia, and had a peak at 6 and 12 h, suggesting that hypoxia model has established successfully. The result of wound healing assay revealed that the wound

healed obviously and had the tendency to fuse under hypoxia (Fig. 1b). Compared with the normoxic conditions, the migratory cell numbers increased by 46 % under hypoxic conditions (Fig. 1c). In addition, the result of matrigel pre-coated transwell assay showed that the number of invasive cells in hypoxia was higher than that under normoxic conditions by 67 % (Fig. 1d, e), suggesting that hypoxia also promoted cell invasion.

Many studies have pointed out a strong interaction between invasiveness of human gliomas and degradation of extracellular matrix (ECM) by matrix metalloproteases (MMPs), especially by MMP2 [29, 30]. Thus, the MMPs activity is considered as an index of tumor invasion ability. We therefore detected the effect of hypoxia treatment on the excretion of MMP-2 by gelatin zymography. As is

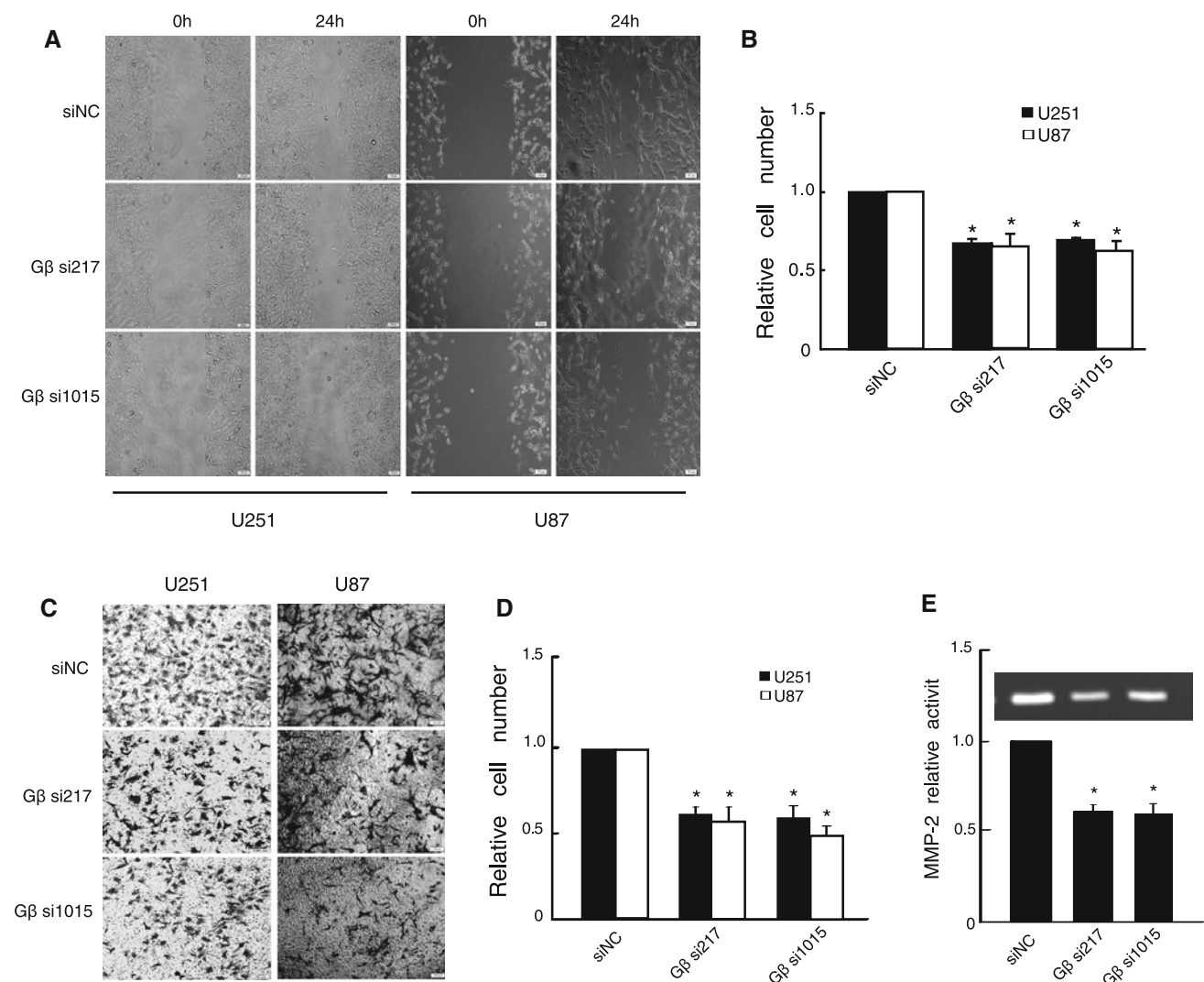


Fig. 4 Depletion of GGTI β inhibits hypoxia-induced cell migration and invasion. **a–d** U251 and U87 cells were transfected with siNC, GGTI β si217 and si1015. After 24 h of transfection, cells were subjected to wounding healing assay or invasion assay under hypoxia condition. Representative microphotographs are shown. **e** 24 h after

transfection, cells were exposed to hypoxia condition for further 24 h and the supernatant was harvested and subjected to gelatin zymography. Scale bar 100 μ m. Results are presented as the mean \pm SEM from three independent experiments. * $P < 0.05$

shown in Fig. 1f, hypoxia treatment increased the excretion of MMP-2 significantly (Fig. 1f). Collectively, all these results indicated that hypoxia treatment promoted cell migration and invasion, consistent with the previous studies [6, 7, 12–14].

Down-regulation of HIF-1 α inhibits hypoxia-induced cell migration and invasion

Next, we observed the migration and invasion behavior of cells by down-regulating the HIF-1 α using RNA interference approach. After being transiently transfected with HIF-1 α siRNAs (si-1217 and si-2791), the cells were harvested and the down-regulation efficacy was examined by western blotting. The results showed that both siRNAs knocked down HIF-1 α remarkably (Fig. 2a).

The result of wound healing assay showed that the number of migratory cells transfected with HIF-1 α si1217 and si2791 was significantly reduced to 59 and 51 % respectively, compared with siNC group under hypoxia conditions (Fig. 2b, c). Furthermore, HIF-1 α si1217 and si2791 decreased the number of invasive cells by about 47 and 45 % respectively (Fig. 2d, e), which were examined by matrigel precoated transwell assay. To further confirm the above result, we also used gelatin zymography to study the effect of HIF-1 α down-regulation on the excretion of MMP-2. The intensity of the gelatinolytic bands clearly decreased to 59 and 60 % in U251 cells transfected with HIF-1 α si1217 or si2791 respectively (Fig. 2f). These results indicated that down-regulation of HIF-1 α reduced glioma cell migration and invasion under hypoxia conditions.

GGTI regulates HIF-1 α induction under hypoxic conditions

It is reported that Rho small GTPases are upregulated in hypoxia-driven angiogenesis and are involved in HIF-1 α induction [17–19]. Turcotte et al. [31] even reported that the upregulated protein level of Cdc42, Rac1 and RhoA, the known substrates of GGT, was mainly located at membranes after hypoxia. Since Rho family members need to be prenylated by GGTI and then translocated to membranes before being activated [21], the above reports hinted us the possibility that GGTI may regulate HIF-1 α under hypoxia. Thus, we detected the protein level of GGTI β upon hypoxia treatment first. Unexpectedly, we have not found any increase in GGTI β protein level (Fig. 3a). However, we found that the total and membrane protein level of RhoA increased gradually after hypoxia treatment, similar to the change of HIF-1 α , suggesting that GGTI activity increased under hypoxia condition (Fig. 3a). Next, we used the siRNAs to downregulate GGTI β and examined the level of HIF-1 α . As the Fig. 3b, c showed, GGTI β

siRNAs (GGTI β si217 and si1015) knocked down GGTI β significantly (Fig. 3b), but have not affect the level of GGT α /FT α or FT β (Fig. 3c), suggesting the specificity of GGTI β siRNA. In addition, we found that the protein level of HIF-1 α decreased after down-regulation of GGTI β under either normoxia or hypoxia conditions in both glioma U251 and U87 cells (Fig. 3d–f). Similarly, GGTI-298, a GGTI specific inhibitor, decreased the protein level of HIF-1 α by 87 and 91 % under normoxia and hypoxic conditions, respectively (Fig. 3g). On the contrary, we tested the possibility that HIF-1 α regulates GGTI. Since HIF-1 α si2791 had a better down-regulating efficiency on HIF-1 α than that of HIF-1 α si-1217 (Fig. 2a), we selected HIF-1 α si2791 to do this experiment. The result showed that in glioma U251 and U87 cells, down-regulation of HIF-1 α has not change the protein level of GGTI β (Fig. 3h–j) under either normoxia or hypoxic conditions, suggesting that GGTI was located at the upstream of HIF-1 α and could regulate HIF-1 α in glioma cells.

Depletion of GGTI β inhibits cell migration and invasion under hypoxia

Next, we wonder whether GGTI had a similar effect to that of HIF-1 α on cell migration and invasion. As is shown in Fig. 4a, b, compared with siNC group, the number of migratory cells transiently transfected with GGTI β si217 or si1015 was reduced by 33 and 31 % in glioma U251 cells, 34.5 and 38 % in glioma U87 cells under hypoxic conditions respectively. Similarly, the invasive ability after down-regulation of GGTI β was reduced to 61 and 59 % (U251 cells), 57 and 48 % (U87 cells) of the level of siNC group respectively (Fig. 4c, d). In addition, down-regulation of GGTI β reduced the excretion of MMP2 significantly (Fig. 4e). These results indicated that, similar to the effect of HIF-1 α , depletion of GGTI β reduced cell migration and invasion under hypoxia conditions.

GGTI regulates cell migration and invasion via HIF-1 α

The above data indicated that GGTI could not only regulate HIF-1 α , but also affect the migratory and invasive behavior of glioma cells. Therefore, we examined whether HIF-1 α could mediate the function of GGTI in cell migration and invasion by co-transfecting GGTI β and HIF-1 α si2791. U251 cells were transfected with pCAG–YFP–GGTI β plasmid, which expressed YFP at the same time to indicate the positive cells, and the GGTI β overexpression was verified by the YFP fluorescence and western blot (Fig. 5a, b). In addition, western blot result showed that the GGTI β and HIF-1 α si2791 were co-transfected into glioma U251 cells successfully (Fig. 5b) [32]. In line with the above result (Fig. 2b, c), the number of migratory cells decreased after

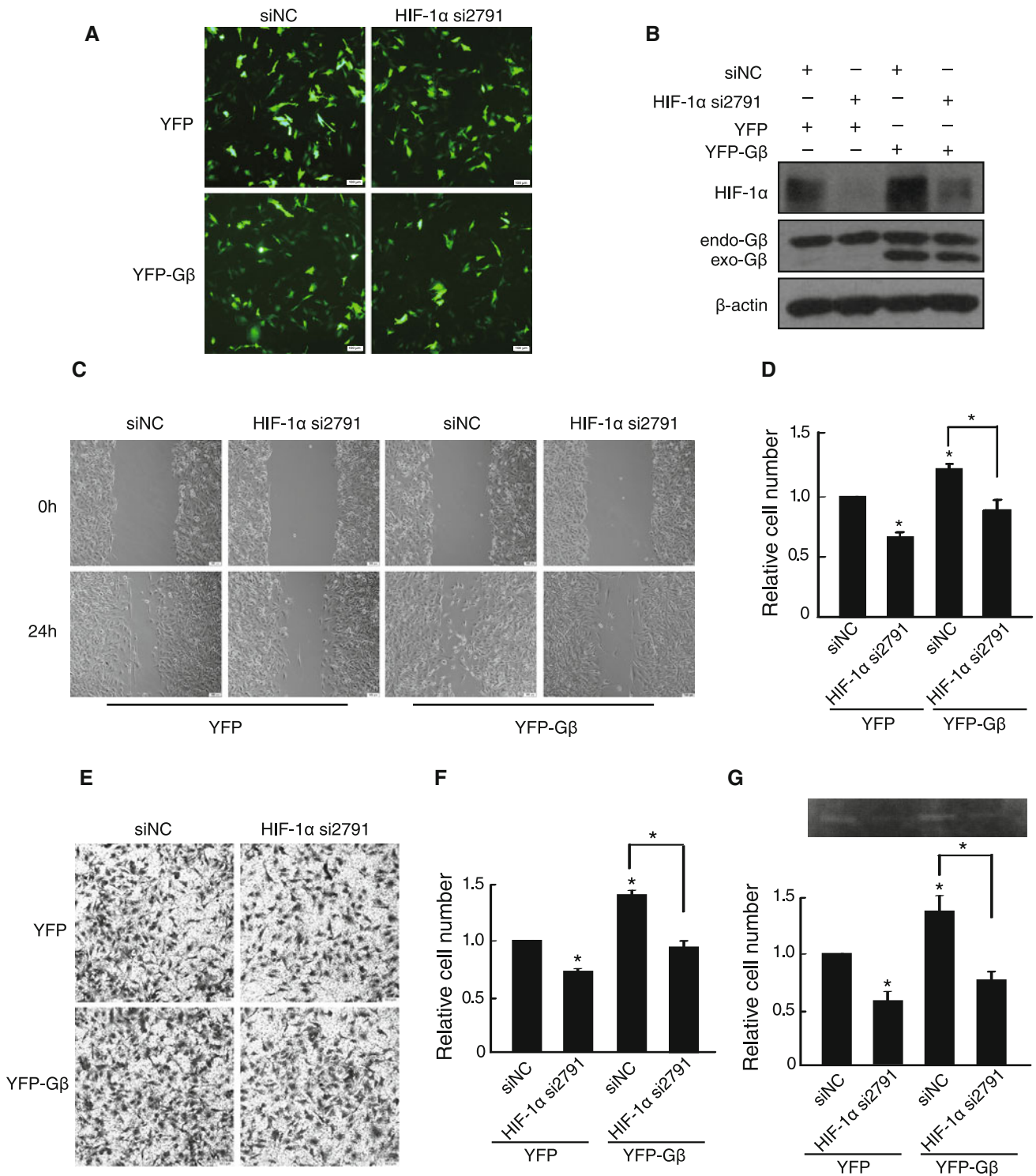


Fig. 5 GGTI β regulates cell migration and invasion via HIF-1α in glioma cells. **a, b** 24 h after being transfected with indicated plasmid and/or siRNA, cells were exposed to hypoxia conditions for further 24 h. YFP fluorescence and immunoblot was applied to detect the transient co-transfection efficiency. **c–f** Cell invasion and migration

were examined by using cell invasion assay and wound healing assay. **g** 24 h after transfection, cells were exposed to hypoxia condition for further 24 h and the supernatant was harvested and subjected to gelatin zymography. Results are presented as the mean ± SEM from three independent experiments. *Bar* indicates 100 μm. **P* < 0.05

down-regulation of HIF-1 α in hypoxia and increased after GGTI β over-expression (Fig. 5c, d). Furthermore, the effect of GGTI β over-expression on cell migration and invasion was partially abolished by depletion of HIF-1 α (Fig. 5c–f). In addition, GGTI β over-expression also increased the excretion of MMP2, which was blocked by down-regulation of HIF-1 α at the same time. Above results suggested that GGTI regulates cell migration and invasion partially via HIF-1 α in glioma cells under hypoxia.

Discussion

Hypoxia is one feature of glioblastoma, which is associated with poor prognosis, increased angiogenesis, tumor growth and resistance to radio- and chemotherapy [13]. HIF-1 α , the key hypoxia regulatory factor, has been shown to be important in promoting both angiogenesis and invasion. Upregulation of HIF-1 α induced by hypoxic stress is an essential event in the activation of glioma cell motility through alteration of invasion-related molecules, while knocking down of HIF-1 α in glioma cells reduces migration and invasion [6, 7, 12–14]. Our data also showed that hypoxia promotes the migration and invasion of glioma cells, and down-regulation of HIF-1 α inhibits them (Figs. 1, 2). These data supported that HIF-1 α and hypoxia play important roles in glioma cell migration and invasion [4–6, 33] and that inhibition of HIF-1 α is an attractive therapeutic strategy to target the tumor microenvironment.

Although the role of HIF-1 α in glioma cell progression is well elucidated [6, 7, 12–14], the regulatory mechanism is not clear so far. Some studies demonstrated that mTOR and Myc could regulate HIF-1 α induction [34–37]. Some reports suggested that the Rho small GTPases are upregulated under hypoxia and also involved in HIF-1 α induction [17–19]. Rho small GTPases are often upregulated or mutated in tumors and play important roles in cell migration and invasion [16, 38–41]. The biological function of Rho GTPases depends on post-translational isoprenylation by GGTI, which in turn leads to their attachment to plasma membranes and then activation [21]. In our study, although the protein level of GGTI β was not increased under hypoxia, the total and membrane level of RhoA was upregulated after hypoxia treatment, consistent with the previous studies [31, 42], suggesting that GGTI was activated under hypoxia. And we also found that specific down-regulation of GGTI β can reduce the expression of HIF-1 α while the level of GGTI β has not changed after HIF-1 α down-regulation, suggesting the regulation of GGTI on HIF-1 α expression (Fig. 3).

It has been shown that a number of geranylgeranylated proteins play important roles in tumorigenesis and metastasis [23, 43, 44]. Many studies have reported that GGT

inhibitors could induce apoptosis and inhibit cell proliferation in many tumors [43–46]. Similarly, we found that down-regulation of GGTI β also inhibited cell migration and invasion while GGTI β over-expression promoted them under hypoxia (Fig. 4, 5). Furthermore, the effect of GGTI on cell migration and invasion under hypoxia was partially abolished by knocking down of HIF-1 α (Fig. 5). These results suggested HIF-1 α mediated the effect of GGTI on cell migration and invasion.

Taken together, the results presented here indicated that GGTI regulated cell migration and invasion partially via HIF-1 α . For the first time, our data provided the molecular mechanism of GGTI–HIF-1 α signal pathway involved in glioma cell migration and invasion under hypoxia. As for the roles of Rho GTPases, the bridge between GGTI and HIF-1 α , will be further studied in GGTI–HIF-1 α signal pathway, which might play an important role in glioma progression.

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