GRIM-19 opposes reprogramming of glioblastoma cell metabolism via HIF1α destabilization

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The metabolism that sustains cancer cells is adapted preferentially to glycolysis, even under aerobic conditions (Warburg effect). This effect was one of the first alterations in cancer cells recognized as conferring a survival advantage. In this study, we show that gene associated with retinoid-interferon-induced mortality-19 (GRIM-19), which was previously identified as a tumor suppressor protein associated with growth inhibition and cell apoptosis, contributes to the switch between oxidative and glycolytic pathways. In parallel to this, vascular endothelial growth factor, which promotes neovascularization, is also regulated. We have identified hypoxia-inducible factor 1α (HIF1α) as the downstream factor of GRIM-19 in human glioblastoma cell lines. Downregulation of GRIM-19 promotes HIF1α synthesis in a STAT3-dependent manner, which acts as a potential competitive inhibitor for von Hippel-Lindau (pVHL)–HIF1α interaction, and thereby prevents HIF1α from pVHL-mediated ubiquitination and proteasomal degradation. Taken together, it is concluded that GRIM-19, a potential tumor suppressor gene, performs its function in part via regulating glioblastoma metabolic reprogramming through STAT3-HIF1α signaling axis, and this has added new perspective to its role in tumorigenesis, thus providing potential strategies for tumor metabolic therapy.

Introduction

Cellular energy metabolism is one of the major processes affected during transition from normal to cancer cells. As a support for rapid proliferation, cancer cells choose to use glycolysis even in the presence of oxygen (Warburg effect) to fuel macromolecules for the synthesis of essential cellular components, rather than inclining on mitochondrial oxygenic respiration (1,2). Moreover, pre-adaptation to glycolytic metabolism provides several advantages for tumor cells, such as lower production of reactive oxygen species, protection from apoptosis and exertion of tumor drug resistance (3–5). It has already been proposed that aerobic glycolysis might be the Achilles’ heel for cancer cells (6).

In glioblastoma, the most frequent and malignant adult brain tumor, glycolytic metabolism is elevated to an average of 3-fold than that in normal brain, with regulation by well-known oncogenes such as phosphoinositide 3-kinase, Akt and hypoxia-inducible factor 1 (HIF1α) (7,8). The expression profile of a set of genes coding for glycolysis and the tricarboxylic acid cycle in clinical cases confirms this metabolic switch (9). However, there is only little evidence on how cancer cells in glioblastomas can modify metabolic pathways. A better understanding of the interactions between oncogenes and tumor suppressor genes with these pathways may shed light on new therapeutic strategies for these tumors.

Gene associated with retinoid-interferon-induced mortality-19 (GRIM-19) was originally identified by a research group interested in unveiling the molecular basis of cell death associated with exposure to interferon-β and retinoic acid (10). Further experiments to characterize GRIM-19 showed that its overexpression induced apoptotic cell death in response to interferon-β/retinoic acid treatment, whereas GRIM-19 knockdown seemed to confer cell resistance to these factors (10,11). In this context, GRIM-19 inhibition confers some growth advantage. Studies performed by different groups demonstrated that GRIM-19 is also a component of complex I of the mitochondrial respiratory chain (MRC), which is responsible for adenosine triphosphate (ATP) production (12–14). Indeed, GRIM-19 is essential for early embryonic development in mice as demonstrated by genetic ablation experiments. Homologous deletion of the GRIM-19 gene resulted in embryonic lethality by day 9.5, with the retarded growth and abnormal mitochondrial structure, morphology and distribution exhibited in the blastocysts (15).

The initial discovery of GRIM-19 as a growth suppressor in a genetic screen hinted that it could be a target for genetic inactivation events. This hint was supported by the finding of mutations in the coding region of the GRIM-19 gene in Hürthle cell thyroid carcinomas (16). More recently, studies reported that GRIM-19 protein expression was lost or severely repressed in numerous human carcinomas such as renal, colorectal, prostate, cervical and glioblastomas (17–21). These observations were further corroborated by an upregulation of STAT3, a multifaceted growth mediator in the cells, specifically revealed in the carcinoma tissues. Overexpression of GRIM-19 in multiple human tumor cell lines induces apoptotic cell death (22,23), and inhibition of its pro-apoptotic activity by viral oncoproteins occurs (13), indicating a potential tumor suppressor like function for this protein.

In 2003, two groups simultaneously reported that GRIM-19 directly interacted with STAT3, inhibiting its gene stimulatory function (11,24). It counteracted constitutive STAT3-induced cellular transformation and suppressed src-induced cell motility and growth (24,25). A protein related to cell cycle transition—GW112—was subsequently found to interact with GRIM-19, reducing its ability to mediate apoptotic cell death (26). GRIM-19 upregulation in human gastric cancer SGC-7901 cells decreased cell invasion via its inhibitory role in GW112 expression and the binding activity of the upstream transcriptional regulator nuclear factor-κB (27). The studies described highlight that GRIM-19 function as a tumor suppressor seems to occur at multiple levels. However, although GRIM-19 role in tumor suppression has been widely investigated, the molecular mechanisms beneath it are still far from defined. Especially, as an important component of MRC, it has remained to be determined if and how GRIM-19 is involved in the metabolic reprogramming and neovascularization, significant hallmarks of tumors besides tumor growth and migration.

In this study, we utilized glioblastoma cell lines to dissect the functional roles of GRIM-19 in tumor metabolic adaptation. Downregulation of GRIM-19 in T98G cells significantly increases cellular deoxyglucose uptake and lactate production. Activities of...
the key enzymes involved in glycolysis are elevated, all indicative of higher aerobic glycolysis. Consistently, pyruvate dehydrogenase (PDH) in these cells is intensely phosphorylated, further confirming a switch from oxidative respiration to glycolytic pathway. The effects of GRIM-19 are not limited to the above metabolic changes. Targeting GRIM-19 induces vascular endothelial growth factor (VEGF) and significantly increases tumorigenic capacity of glioblastoma cells in vivo. Additionally, we have identified HIF1α as the potential downstream mediator of the above-mentioned effects. Downregulation of GRIM-19 promotes HIF1α synthesis in a STAT3-dependent manner, which acts as a potential competitive inhibitor for von Hippel-Lindau (pVHL)–HIF1α interaction, and thereby prevents HIF1α from pVHL-mediated ubiquitination and proteasomal degradation. Taken together, the present results demonstrate that GRIM-19, a potential tumor suppressor gene, performs its function in part via regulating cellular metabolism through STAT3-pVHL-HIF1α signaling axis, which would provide a possible explanation for the Warburg effect and thus offer a new perspective to the roles of GRIM-19 in gliomagenesis.

Materials and methods

Cell lines
Glioblastoma cell lines T98G and U251 were obtained from Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT).

Plasmids, small hairpin RNA and generation of stably transfected cells
Knockdowns by small hairpin RNA (shRNA) were performed using pSuper.neo (OligoEngine, Seattle, WA) following the manufacturer’s instructions. The following sequences were used: GRIM-19, 5'-GGGACTG GAGCATAATGAA-3' and scramble, 5'-GCCGATCCCTAGATGTG-3'. The plasmids were then transfected into T98G and U251 cells by Nucleofection 2000 (Invitrogen, New York, NY). After 2 weeks of selection using 400 μg/ml G-418, polyclonal stable cell lines were established.

Hexokinase 2, PKM2 and phosphofructokinase 1 enzymatic activities
Hexokinase (HK2), PKM2 and phosphofructokinase (PFK1) enzymatic activities were measured in the cell lysates with respective enzyme activity assays (Jiemei Genetech Corporation Ltd, Shanghai, China) according to manufacturer’s recommended protocols.

Lactate production
Accumulation of lactate in the culture medium was measured over a period of 24 h using a lactate assay kit (Nanjing Jiancheng Corporation Ltd, Shanghai, China). The lactate assay kit is based on the reduction of the tetrazolium salt 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride in an reduced form of nicotinamide adenine dinucleotide-coupled enzymatic reaction to formazan, which is water-soluble and exhibits an absorption maximum at 492 nm. Total viable cell number was used for normalization.

Cell viability assay
The transduced cells were seeded in 96-well tissue culture plates at a concentration of 1000 cells per well supplemented with or without 4500 mg/l glucose. At indicated time points, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide was added to each well at a final concentration of 150 μg/ml. After 4 h of incubation at 37°C, absorbance at 570 nm was measured using a spectrophotometer (Benchmark; Bio-Rad, Hercules, CA).

Enzyme-linked immunosorbent assay for VEGF production
The culture medium of T98G cells was collected, and 100 μl aliquots were used to determine VEGF levels. Immunoreactive VEGF was quantified using a sandwich enzyme-linked immunosorbent assay (R&D Systems) according to the manufacturer’s recommended protocol.

In vivo tumor formation and immunohistochemistry
Intracranial transplantation of glioblastoma cells into nude mice was performed as described (28) in accordance with a Shandong University Institutional Animal Care and Use Committee approved protocol concurrent with national regulatory standards. Briefly, 10^5 transduced cells were counted and implanted into the right frontol lobes of athymic BALB/c nu/nu mice. Mice were maintained up to 12 weeks. Brains of euthanized and phosphate-buffered saline perfused mice were collected, fixed in 4% paraformaldehyde and paraffin embedded. Immunohistochemistry staining was performed as described previously (29). The primary antibodies used were monoclonal antibodies to CD34 and CD31 (Santa Cruz Biotechnology). High-resolution images of the stained sections were obtained using OLYMPUS DP72 Digital Camera and DP controller software (Olympus, Essex, UK). The tumor volumes were determined as follows: maximal axial diameter of the tumor was measured on the hematoxylin and eosin−stained sections. Tumor volume was calculated from the formula: tumor volume = (maximum diameter)^2 × (minimum diameter)^2 × 0.5 (30,31).

Luciferase reporter assay
Cells were seeded onto 48-well plates, transfected with reporter plasmid pGL2-HIF1αLuc; control reporter pSV-Renilla; together with shRNA-GRIM19 or pJAX-GRIM-19 expression vector. FLuc and RLuc activities were determined using the dual-luciferase assay system (Promega, Madison, WI) 24 h later.
Cells were lysed in hypotonic buffer by a Dounce homogenizer (40 strokes). Intact cells were removed by centrifugation at 53g for 10 min. The nuclei were collected by centrifugation at 800g for 10 min, washed and lysed in isonicotic buffer by sonication. The supernatant was taken as the cytosolic fraction.

Statistical analysis
Descriptive statistics were generated for all quantitative data with presentation of mean ± standard deviation (SD). Significance was tested by one-way analysis of variance followed by independent sample two-tailed t-tests and Tukey’s test for multiple comparisons using statistical software SPSS (IBM corporation, Poughkeepsie, New York). For in vivo studies, Kaplan Meier curves and log-rank analysis were performed using MedCalc software (Mariakerke, Belgium). All statistical tests were two-sided, and P < 0.05 was considered statistically significant.

Results
Knockdown of GRIM-19 in glioblastoma cells enhances glycolytic metabolism

We have shown previously that upregulation of GRIM-19 induced apoptosis and suppressed glioma cell proliferation and motility, indicating that GRIM-19 is necessary for gliomagenesis. To determine whether GRIM-19 is involved in the energetic transformation of glioblastomas, we separately introduced a pSuper.neo vector of GRIM-19 shRNA and a pJAX vector of wild-type GRIM-19 into T98G glioblastoma cells. Immunoblotting indicated notable depletion of GRIM-19 in the pSuper.neo-GRIM19-shRNA transfected cells and significant upregulation of GRIM-19 was shown in the pJAX-GRIM-19 transfected cells compared with their separate controls (Supplementary Figure 1a, available at Carcinogenesis Online). Additionally, knockdown of GRIM-19 in T98G cells promoted cell proliferation. Conversely, upregulation of GRIM-19 led to decreased growth in these cells (Supplementary Figure 1b, available at Carcinogenesis Online).

To investigate whether GRIM-19 is involved in the metabolic reprogramming of glioblastomas, we first examined the glycolytic activity by measuring cellular retention rates of deoxyglucose. We found that the T98G cells with downregulation of GRIM-19 displayed 2-fold increase in the retention rate of intracellular deoxyglucose. Overexpression of GRIM-19 resulted in a reversed effect, indicating decreased glycolytic metabolism (Figure 1a). We subsequently detected the plasma membrane expression of GLUT1, which plays a key role in deoxyglucose uptake by facilitating assimilation of extracellular deoxyglucose. As the data shown in Figure 1b, in comparison with the non-targeting control cells, level of GLUT1 expressed on the plasma membrane was substantially enhanced in the T98G cells that were depleted for GRIM-19 (Figure 1b), accounting at least partially for the elevated uptake of deoxyglucose in these cells.

HK, pyruvate kinase and PFK are three key enzymes involved in the glycolytic pathway. We next sought to determine the expression and activity of the respective enzyme. Quantitative real-time–PCR results showed that knockdown of GRIM-19 resulted in elevated messenger RNA (mRNA) expression levels for HK2, PKM2 and PFK1 in the T98G cells (Figure 1c). Consistent with these changes at transcriptional level, enzymatic activities of HK2, PKM2 and PFK1 were also increased (Figure 1d), further supporting an opposite role of GRIM-19 in the glucose metabolism of glioblastomas.

A hallmark of Warburg effect is the reduction of pyruvate, the end product of glycolysis, to lactate acid. We, therefore, examined lactate acid levels in the medium of the cells with or without GRIM-19 depletion. As shown in Figure 1c, downregulation of GRIM-19 resulted in a marked increase in secreted lactate acid in the medium of T98G cells. A similar phenomenon was manifested in another glioblastoma cell line, namely, U251 (Figure 1c), thus further strengthening the concept of GRIM-19 role in extracellular lactate production, a characteristic of glycolytic metabolism.

Loss of GRIM-19 contributes to the switch from mitochondrial respiration to glycolysis in glioblastoma cells

As an important component of the MRC that is essential for complex I assembly and electron transfer activity, it is validated that loss of GRIM-19 could result in inhibition of oxidative phosphorylation (OXPHOS). To confirm the effect of GRIM-19 in the switch between mitochondrial respiration and glycolysis, we analyzed PDH, a key metabolic bridge linking oxidative and glycolytic pathways, and associated proteins in the transduced cells. Results showed that knockdown of GRIM-19 resulted in higher phospho-PDH level. Consistently, the expression of pyruvate dehydrogenase kinase 1 (PDK1), which phosphorylates and inactivates PDH, was upregulated, all indicating that depletion of GRIM-19 would contribute, at least partially, to the metabolic switch from mitochondrial respiration to glycolysis in the T98G cells (Figure 2a).

Increased growth of the GRIM-19-depleted cells suggested that these cells may be depending on enhanced glycolysis to support increased proliferation by directing glucose away from the tricarboxylic acid cycle. Indeed, when the transduced cells were cultured in glucose-free medium, the GRIM-19 knockdown cells began to grow at a slower pace than the scrambled controls (Figure 2b). Interestingly, there was no significant change in the total cellular ATP levels, confirming that the cells with low levels of GRIM-19 relied on glycolytic metabolism as their major energy source with no further detectable changes after inhibition of OXPHOS (Figure 2c).

In view of the dual functions of GRIM-19 in mitochondrial respiration and glycolysis, it was surmised that loss of GRIM-19 might have promoted glycolytic metabolism as part of a compensatory response due to diminished oxidative capacity. To ascertain this, we examined cellular deoxyglucose uptake and lactate secretion in the presence of a mitochondrial respiratory inhibitor, rotenone. Results showed that glycolysis was increased in the presence of rotenone in the scrambled control cells. Strikingly, deoxyglucose uptake and lactate production also remained elevated in the GRIM-19 knockdown cells even in the presence of the oxidation inhibitor (Figure 2d). These data suggested that upregulated glycolysis in the GRIM-19-depleted cells did not result solely from non-specific compensation for decreased mitochondrial oxidative function. Instead, GRIM-19 might as well regulate glycolytic metabolism via activation of a specific signaling pathway.

GRIM-19 is involved in VEGF expression and endothelial cell proliferation in glioblastoma cells

A key mechanism for promotion of angiogenesis during tumor development is elevated expression of VEGF, which mitogenically stimulates proliferation of endothelial cells and for their recruitment into new blood vessels. We, therefore, determined control of GRIM-19 in VEGF expression in glioblastoma cells. As depicted in Figure 3, stable knockdown of GRIM-19 in T98G cells significantly increased VEGF at mRNA (Figure 3a), intracellular (Figure 3b) and secreted protein (Figure 3c) levels, strongly suggesting an opposite role of GRIM-19 in glioblastoma cells for the induction of VEGF expression.

As VEGF supports brain tumor angiogenesis through regulation of endothelial cell proliferation and survival (32), we examined whether downregulation of GRIM-19 in glioblastoma cells could significantly impact endothelial cell growth. A co-culture chamber coated with a permeable membrane was utilized, in which the transduced T98G cells were cultured in the upper well, whereas human microvascular endothelial cells were planted in the lower well. By direct cell number counting on 10 microscopic fields of human microvascular endothelial cells, the T98G cells depleted of GRIM-19 significantly increased endothelial cell numbers and proliferation in comparison with the control cells (Figure 3d). These data were consistent with the effect of GRIM-19 on VEGF induction in the above study and suggested a specific role for GRIM-19 in the VEGF-mediated angiogenesis by affecting endothelial cell growth.

Targeting GRIM-19 in glioblastoma cells increases tumorigenic capacity and reduces survival of mice-bearing intracranial xenografts

Considering the in vitro involvement of GRIM-19 in glioma cell proliferation, survival, metabolic adaptation and VEGF production, we extended this study to determine the impact of GRIM-19 knockdown on tumorigenic capability of glioblastoma cells in vivo. When the T98G cells transduced with shRNA targeting GRIM-19...
or non-targeting control shRNA were intracranially implanted into immunocompromised mice, we observed a significant increase in tumor formation (Figure 4a) and a decrease in the survival of tumor-bearing mice when GRIM-19 was targeted (Figure 4b).

We next performed immunohistochemistry on paraffin-embedded mouse brain tumor samples with an antibody directed against CD34, which detects new blood vessels. In contrast to variable and sporadic CD34 immunostaining in the glioblastomas...
derived from the cells transduced with scrambled control shRNA, the glioblastomas depleted of GRIM-19 displayed a much higher micro-vessel density (Figure 4c), confirming an involvement of GRIM-19 in glioma angiogenesis. Staining with an antibody against CD31, another blood vessel marker, manifested the same micro-vessel density profiles in the xenograft tumors (data not shown).

In order to investigate the role of GRIM-19 in glioblastoma metabolic switch in vivo, we examined the mRNA expression levels of HK2, PKM2, PFK1 and PDK1 in the xenograft tumors. Quantitative real-time–PCR analysis showed that decreased expression of GRIM-19 correlated with significantly elevated levels of all tested transcripts in the xenograft tumors derived from the cells depleted of GRIM-19 in comparison to the controls (Figure 4d), suggestive of higher glycolytic metabolism in the GRIM-19 knockdown tumors. Collectively, the present data suggested that GRIM-19 was involved in the transformation of glioblastoma cells. More strikingly, its loss appeared to maintain the metabolic and vascular potential of glioblastomas.

An opposite role of GRIM-19 in HIF1α expression

Considering the broad involvement of GRIM-19 in the metabolic reprogramming and angiogenesis of glioblastomas, we next sought to determine the precise mechanisms underlying these effects. The oxygen-dependent protein HIF1α is a major oncogenic factor involved in different human cancers. It acts as a transcriptional factor to activate genes that permit metabolic adaptation, such as HK2, GLUT-1 and PDK1, and facilitate production of VEGF (33). A serial analysis of gene expression database of potential GRIM-19 target genes were examined in Hela cells, where HIF1α was induced, which correlated with GRIM-19 depletion as evidenced by a 1.8-fold increase in transcripts (H.Lu et al., unpublished data). As such, it was hypothesized that the roles of GRIM-19 in glioblastoma metabolism and angiogenesis may be linked to HIF1α.

To test this hypothesis, we first sought to determine whether HIF1α is indeed trans-activated by GRIM-19. Our result showed that a moderate increase in luciferase activity of HIF1α promoter region was coupled with knockdown of endogenous GRIM-19, whereas upregulation of GRIM-19 in T98G cells reduced HIF1α transcriptional activity (Figure 5a). A comparable result was observed in U251 glioblastoma cells (data not shown), confirming an opposite role of GRIM-19 in HIF1α trans-activation. More strikingly, knockdown of GRIM-19 resulted in significant augmentation in HIF1α protein levels (Supplementary Figure 2a, available at Carcinogenesis Online) under normoxic conditions. Nuclear/cytosol fractionation analysis detected HIF1α protein primarily from the isolated nuclei in the GRIM-19-depleted cells, suggesting functional activation of HIF1α (Figure 5b).

We additionally investigated the subcellular distribution of HIF1α by immunofluorescence analysis. As shown in Figure 5c, in comparison with the scrambled cells that exhibited a weak staining for HIF1α in both the cytoplasm and nuclei, the cells knockdown of GRIM-19 tended to present strong detection of HIF1α predominantly localized in the nuclei. When GRIM-19 was upregulated in the knockdown cells via the adenovirus-mediated gene transfer, strong nuclear localization of HIF1α merely disappeared (Figure 5c). This evidently confirmed the control over HIF1α activation by GRIM-19 depletion. To probe for evidence of increased HIF1α activation in vivo, we measured protein expression of HIF1α in xenograft tumors derived from the GRIM-19 knockdown and control cells. As predicted, HIF1α was significantly elevated when GRIM-19 was downregulated (Supplementary Figure 2b, available at Carcinogenesis Online).

GRIM-19-mediated glioblastoma metabolic adaptation requires HIF1α

To step further to address whether HIF1α represents a functional link for the glycolytic shift and neovascularization observed in the glioblastomas cells depleted of GRIM-19, we introduced HIF1α small interfering RNA into both control and GRIM-19 knockdown cell lines. As predicted, interrupting HIF1α signaling lowered PDK1 protein level (Figure 5d) as well as the enzymatic activities of HK2, PKM2 and PFK1 (Figure 5e; data not shown) to comparable levels in both control and GRIM-19-depleted cells. Likewise,
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Deoxylglucose uptake and lactate production were significantly reduced (Supplementary Figure 2a, available at Carcinogenesis Online). These data demonstrated that HIF1α was not only required for the basal level of glycolysis but also involved in the GRIM-19-mediated metabolic reprogramming.

In contrast to glycolytic metabolism, which was largely dependent on functional HIF1α, VEGF induction in the GRIM-19 knockedown cells was relatively unaffected by HIF1α silencing, although rather unexpectedly, a moderate increase in VEGF secretion was observed (Figure 5f). These data suggested that though upregulation of glycolysis and induction of VEGF were mediated by constitutively transcriptionally active HIF1α in the basal condition, both events seemed to occur via independent pathways in GRIM-19-depleted glioma cells. A possible explanation for this would be that the effect of HIF1α on VEGF production was dependent on a functional mitochondrial activity. In this regard, the possibility of existence of negative feedback loops was considered whereby loss of GRIM-19 might activate compensatory mechanisms to induce VEGF production.

Involvement of STAT3 in the regulation of HIF1α by GRIM-19

Previous studies including ours have demonstrated that GRIM-19, a potent tumor suppressor gene, restrains cell growth mainly via repressing STAT3 activity (11). It is well established that activated STAT3 induces expression of a number of cellular proto-oncogenes (34), cell cycle-regulating proteins (35) and antiapoptotic proteins (36), all of which contribute to oncogenesis. Remarkably, it became recognized that STAT3, even to a lesser extent, is involved in the metabolic abnormalities of cancer cells and energy derivation through glycolysis (37). As such, we surmise that STAT3 may be a linker connecting GRIM-19 depletion and HIF1α activation.

To test this hypothesis, we first set out to examine the expressions of STAT3 in the T98G cells transduced with shRNA targeting GRIM-19 and non-targeting control. Results showed that knockdown of GRIM-19 resulted in elevated expression of HIF1α and a concomitant hyper-phosphorylation of STAT3. Overexpression of GRIM-19, on the other hand, led to the opposite effects (Figure 6a), indicative of inverse regulation of STAT3 and HIF1α by GRIM-19. When T98G cells were transfected with constitutively activated STAT3 in a dose-dependent manner, protein levels of HIF1α ascended accordingly until it was abolished by the overexpression of GRIM-19 in these cells (Figure 6b). These results evidently suggested that phospho-STAT3 might be involved in the normoxic stabilization of HIF1α protein controlled by GRIM-19.

To confirm STAT3 as a linker bridging GRIM-19 depletion and HIF1α activation, GRIM-19 knockdown cells were transfected with a dominant-negative STAT3 plasmid. As shown in Figure 6c, the upregulation of HIF1α resulted from GRIM-19 loss was significantly reversed in the cells expressing the STAT3 mutant compared with the control, indicating that activated STAT3 was indeed required for GRIM-19 depletion–induced HIF1α expression (Figure 6c).

The most well-known mechanism for HIF1α regulation is at the post-translational phase, i.e. pVHL-mediated HIF1α ubiquitination and proteasomal degradation. To test whether STAT3 affected the association between pVHL and HIF1α, a crucial step in HIF1α...
regulation, we transfected T98G cells with vectors of both constitutively active STAT3 and wild-type pVHL in the presence of MG132, a proteasome inhibitor. As the data shown in Figure 6d, along with increasing addition of activated STAT3, the interaction between pVHL and HIF1α decreased, indicating that STAT3 might act as a competitive inhibitor of pVHL binding to HIF1α. When GRIM-19 was overexpressed into these cells, STAT3 was less phosphorylated; concomitantly, the interaction between pVHL and HIF1α was elevated (Figure 6d). Taken together, our results suggested that GRIM-19 control over HIF1α stability at least partially relied on STAT3 regulated pVHL–HIF1α interaction. As such, increased glycolysis in response to GRIM-19 loss might be largely dependent on STAT3-pVHL-HIF1α signaling axis.

Discussion

Tumor metabolism, characterized by an adaptive switch from oxidative respiration to glycolysis, even in the presence of oxygen (Warburg effect), confers a survival advantage to cancerous cells (1,2). It favors synthesis of essential cellular components required for rapid cell duplication and produces lactate to condition the microenvironment, promote tumor invasion and suppress antitumor T-cell immune responses (38,39). In addition, reduced cellular respiration may result in lower production of reactive oxygen species and protection from apoptosis (4). A rising from present results, the role of GRIM-19 in control of glioblastoma metabolic reprogramming and neovascularization is greatly amplified and this would form a new molecular basis for designing of more effective therapeutic strategies to target these tumors.

We have previously reported that the expression levels of GRIM-19 were inversely correlated with grades/aggressiveness of glioblastomas, which are determined by pathologic evaluation of the tumors (18). This finding suggests that GRIM-19 might represent a valuable marker for the prognosis of glioma patients. In this study, targeting GRIM-19 in the glioblastoma cells caused a marked decrease in the survival rates of xenograft animals, evidently supporting the association between low expression of GRIM-19 and a worse clinical outcome.

Although a GRIM-19 cancer connection has been extensively documented, the precise mechanism through which GRIM-19 suppresses tumors remains unclear. As a tumor suppressor protein initially discovered as a pro-apoptotic factor, numerous studies have reported that GRIM-19 inhibition confers growth advantage to the cells (10,18). In addition, the negative role of GRIM-19 in tumor pathogenesis seems to occur at multiple levels including cell invasion and metastasis (24). In this study, we provided further direct evidence for the function of GRIM-19 in modulating the balance between oxidative respiration and glycolytic pathway, therefore adding a new perspective to support a role of GRIM-19 in tumor suppression.

As an important component of complex I of the MRC, which is responsible for ATP production, it is speculated that reduced mitochondrial respiration resulted from GRIM-19 depletion could be an important mechanism that contributes to enhanced glycolysis in the glioblastoma cells. Indeed, PDH complex, which lies at the crossroad of aerobic respiration and glycolysis, was highly phosphorylated in response to GRIM-19 loss, a hallmark of metabolic switch from OXPHOS to glycolytic metabolism. Interestingly, levels of glycolysis remained elevated in the cells lacking GRIM-19 compared with the scrambled controls even in the presence of an oxidation inhibitor. These data evidently suggested that increased glycolysis responsive to GRIM-19 depletion was not only a non-specific compensation for reduced mitochondrial oxidative capacity but also resulted from activation of a specific signaling pathway. To this end, HIF1α, which is usually induced in low oxygen tension, was significantly elevated in the glioblastoma cells by depletion of GRIM-19 in normoxic conditions.
A number of studies have described mutations in enzymes involved in the Krebs cycle and OXPHOS associated with various cancers through HIF1α activation resulted from a switch to glycolytic metabolism (40,41). As an essential MRC component, GRIM-19 may share a similar mechanism as GRIM-19 depletion reduced cellular respiratory flux as well; however, it seems to play some unique roles in HIF1α activation. It has been established that GRIM-19 exerts its tumor suppressor function mainly through restraining cell growth by direct inhibition on STAT3 trans-activation (11,42). STAT3, a major oncogenic factor, was also reported to be involved in metabolic abnormalities of cancers and closely correlated to accumulation of HIF1α in several cell types (37). It is thus speculated that HIF1α induced by GRIM-19 depletion might be dependent on STAT3 activation. Indeed, enhanced STAT3 activity was required for GRIM-19-regulated HIF1α protein stability. An in-depth research further demonstrated that high phosphorylated STAT3 acted as a potential competitive inhibitor for pVHL–HIF1α interaction, and thereby prevented HIF1α from pVHL-mediated ubiquitination and proteasomal degradation. An estimated model of GRIM-19 role in regulating cell glycolytic metabolism is summarized in Figure 6e.

Another major finding in this study is that through regulation of glycosylation and induction of VEGF are mediated by constitutively transcriptionally active HIF1α in the basal conditions (33), both processes appear to be regulated by different pathways in the GRIM-19-depleted glioblastoma cells. It is generally accepted that of the full spectrum of regulatory pathways on tumor angiogenesis, Ras, phosphoinositide 3-kinase/Akt and SP1 pathways have been conserved to be alternative controls in VEGF induction independent of HIF1α (43,44). Compensatory activation of these pathways might have accounted for the phenomena manifested in the HIF1α and VEGF double-knockdown cells.

In summary, we have provided unequivocal evidence demonstrating that GRIM-19, a potential tumor suppressor gene, performs its function in part via regulating cellular metabolism through STAT3-HIF1α signaling axis. The novel findings would provide a new perspective to roles of GRIM-19 in tumorigenesis and progression. As a corollary, regulation of tumor metabolism and angiogenesis by GRIM-19 may provide an important area for cancer diagnosis and therapeutic intervention.

Supplementary material

Supplementary Figures 1 and 2 can be found at [http://carcin.oxfordjournals.org/](http://carcin.oxfordjournals.org/)
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Conflict of Interest Statement: None declared.

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