Original Article Suppressing the malignant phenotypes of glioma cells by lentiviral delivery of small hairpin RNA targeting hypoxia-inducible factor-1α

Chang Gui Dong^{1*}, William K K Wu^{2*}, Su Yin Feng^{3*}, Jun Yu², Jun Fei Shao⁴, Gui Mei He¹

¹Institute of Molecular Ecology and Evolution, IAIR, East China Normal University, Shanghai 200062, PR China; ²Institute of Digestive Disease, Department of Medicine & Therapeutics and LKS Institute of Health, The Chinese University of Hong Kong, Shatin, NT, Hong Kong, China; ³Department of Neurosurgery, Wuxi NO.4 People's Hospital, Affiliated to Jiangnan University, Wuxi 214062, PR China; ⁴Department of Neurosurgery, Wuxi People's Hospital of Nanjing Medical University, Wuxi 214023, China. *Equal contributors.

Received September 1, 2013; Accepted October 3, 2013; Epub October 15, 2013; Published November 1, 2013

Abstract: Hypoxic microenvironment of solid tumors is known to shape malignant phenotypes of cancer cells through the dimeric transcription factor hypoxia-inducible factor (HIF)-1. In the present study, the therapeutic effect of targeting α subunit of HIF-1 in glioma cells via lentiviral delivery of small hairpin RNA (shRNA) was evaluated. Data from quantitative real-time PCR and immunohistochemistry demonstrated that HIF-1 α was progressively upregulated during the development of gliomas. Lentiviral shRNA targeting HIF-1 α led to substantial loss of cell viability, G_{o}/G_{1} -phase cell cycle arrest, apoptosis, and impairment of cell motility and invasiveness in human glioma U87MG cells. Xenograft experiments in nude mice further showed that HIF-1 α -shRNA inhibited tumor growth and caused persistent repression of HIF-1 α and its target genes, including VEGF, GLUT1 and MMP2, up to 25 days post-inoculation. Taken together, lentiviral delivery of shRNA is a promising therapeutic approach for targeting HIF-1 α in glioma.

Keywords: Hypoxia-inducible factor, glioma, short hairpin RNA, lentivirus

Introduction

Many solid tumors contain regions of hypoxia where the supply of oxygen fails to meet the metabolic need of actively proliferating tumor cells, leading to the formation of central necrotic zone surrounded by a rim of viable cells. Such hypoxic intratumoral microenvironment has been shown to modulate the intracellular signaling of tumor cells and promote the acquisition of aggressive phenotypes, such as unchecked cell proliferation, evasion of apoptosis, increased cell motility and invasiveness, stemness, and enhanced ability to recruit new blood vessels [1]. Emerging evidence suggests that tumor hypoxia is assoicated with poor responses to chemotherapy and radiotherapy as well as lower overall patient survival. The mechanism by which hypoxic conditions contribute to tumor aggressiveness is manifold and may include induction of endoplasmic reticulum stress [2], increased DNA damage

and impaired DNA mismatch repair [3], activation of mitogen-activated protein kinase (MAPK) signaling [4], and induction of transcription factors, such as activator protein (AP)-1 [5], nuclear factor (NF)- κ B [6] and hypoxia-inducible factor (HIF)-1 [7].

HIF-1 is a heterodimeric transcription factor composed of an oxygen-regulated α subunit and a constitutively expressed β subunit. Under normoxic conditions, proline residues 402 and 564 of HIF-1 α are hydroxylated, allowing interaction with the E3 ubiquitin ligase von Hippel-Lindau protein that tags HIF-1 α with polyubiquitin for proteasomal degradation. In hypoxic conditions, proline hydroxylation of HIF-1 α is impaired and thereby stabilizing the protein [8]. In addition to hypoxia, HIF-1 α is known to be upregulated by transforming viruses (e.g. Epstein-Barr virus, hepatitis B virus, and human papilloma virus) and bioactive lipid mediators (e.g. prostaglandin E₂ and leukotrienes) [7].



Figure 1. Successful cloning of HIF-1α-shRNA-encoding sequences into lentiviral vector was confirmed by Sanger sequencing.

Upon activation, HIF-1 translocates into the nucleus where it induces expression of genes (e.g. *VEGF*, *GLUT1*, *MMP2*) related to angiogenesis, de-differentiation, glycolysis, and metastasis, through binding to the consensus sequence 5'-RCGTG-3' [9-12].

Malignant gliomas are the most common primary brain tumors, which are associated with high mortality and morbidity. Despite recent advancements in chemotherapy, radiotherapy and neurosurgery, the prognosis of patients with malignant gliomas remains dismal, highlighting an urgent need to develop novel and effective therapeutics [13]. Hypoxia is a predominant feature of gliomas and has been found to maintain glioma stem cells and confer resistance to therapy [14]. Thus, targeting dysregulated signals associated with tumor hypoxia has been promulgated as a therapeutic approach for glioma. To this end, accumulating evidence suggest that HIF-1 α is an attractive molecular target. A small hairpin RNA (shRNA) is a sequence of RNA that self-anneals to form a hairpin structure that can be employed to silence target gene expression via RNA interference [15]. The development of lentiviral vectors for ectopic expression of shRNA has been a recent advance in the field of gene therapy. The therapeutic application of lentiviral delivery of shRNA to target HIF-1 α in gliomas, however, has not yet been explored. In the present study, we first show that HIF-1 α was increasingly upregulated during the progression of gliomas. We also demonstrate that targeting HIF-1 α with shRNA delivered by a lentiviral vector could cause sustained reversal of malignant phenotypes and prolonged repression of HIF-1 α and its target genes in glioma cells.

Materials and methods

Cell line and reagents

The human glioblastoma cell line U87MG and U251 were obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Science (Shanghai, China). Primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other chemicals were purchased from Sigma unless otherwise specified.

Patient samples and immunohistochemistry

Paraffin-embedded tissue samples consisting of a total of 5 decompression brain tissues as control and 55 glioma tissues (20 cases of glioblastoma multiforme, 13 cases of astrocytoma, 8 cases of oligodendroglioma and 4 cases of ependymoma) were used. The average age of the glioma patients was 47 years and the male to female ratio was 1.2:1. Immunohistochemical staining of HIF-1 α was performed using polyclonal anti-HIF-1α antibody (sc-13515, Santa Cruz) with standard avidin-biotin method. A proportion score based on staining intensity and percentage of positively stained cells was used to assess the expression of HIF-1α. Agematched normal and cancer tissues were obtained from anonymised excess tissues that



Figure 2. Upregulation of HIF-1 α in glioma tissues. A: prominent diffuse expression of HIF-1 α protein was detected in high-grade glioma tissues as revealed by immunohistochemistry. HIF-1 α protein expression was negligible in non-cancerous intradecompression brain (IDB) tissues. B: HIF-1 α mRNA was upregulated in glioma tissues in a tumor grade-dependent manner as measured by real-time PCR. ***P* < 0.01, significantly different from IDB group.

Table 1. Scoring of HIF-1 α immunoreactivity in intra decompression brain and glioma tissues

	Sample	HIF-1α expression				Positivity
	no.	-	+	++	+++	(%)
IDB	5	5	0	0	0	0
Grade I~II	20	17	3	0	0	15
Grade III	16	1	3	10	2	93.78
Grade IV	19	0	2	6	11	100

were no longer needed for diagnostic or clinical purposes. Use of these tissues was approved by the Ethics Committee of Nanjing Medical University (reference number: 2013-2).

Construction of HIF-1α-shRNA-expressing Ientiviral vector

The shRNAs targeting HIF-1 α mRNA were designed online (www.invitrogen.com/rnai): homo-LV-shRNA-HIF-1 α -1168, 5'-GTGATGAA-AGAATTACCGAAT-3'; homo-LV-shRNA-HIF-1 α -1577, 5'-GAAGGAACCTGATGCTTTA-3'; homo-LV-shRNA-HIF-1 α -2264, 5'-CCAGTTATGATTG-TGAAGTTA-3'; A scrambled sequence that shared no homology with the mammalian genome was used as control. Successful clon-

ing of these sequences into the pLV-U6-Puro-GFP lentivector was confirmed by Sanger sequencing (**Figure 1**). The expression of shRNA was driven by U6 promoter. This vector also encoded a green fluorescence protein (GFP). The pPACK-H1 Lentivector Packaging System (SBI) and the 293TN cell line (SBI) were used for the production of pseudoviral particles according to the manufacturer's instructions. U87MG cells were then transduced at a multiplicity of infection (MOI) of 2.

Cell culture and assays for cell proliferation, cell cycle and apoptosis

The U87MG human glioma cells were maintained in DMEM (Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (Thermo Scientific), 100 U/ml penicillin and 100 μ g/ml streptomycin (Invitrogen) at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. Cell proliferation was measured by the colorimetric Cell Counting Kit-8 (CCK-8) assay (Dojindo). The transfected cells were plated at a density of 5,000 cells/

well in 96-well plates. After incubation for 2 h to allow cell attachment to the bottom of the well. 10 µl CCK-8 solution were added to each well at the indicated time points, and the plates were incubated for another 2 h. The optical density was then determined at 450 nm using a microplate reader. For cell cycle analysis, U87MG cells were fixed with ice-cold 70% ethanol in phosphate-buffered saline, followed by incubation with 50 μ g/ml propidium iodide, 3.8 mmol/I sodium citrate and 0.5 µg/ml RNase A at 4°C for 3 h and analyzed by flow cytometry. To quantify apoptosis, annexin V/7-aminoactinomycin (7-AAD) staining was performed, and apoptosis was evaluated by flow cytometry analysis. Briefly, after transduction, both floating and attached cells were collected and subject to annexin V/7-AAD staining using an annexin V-FITC Apoptosis Detection kit (BioVision, Palo Alto, CA), according to the manufacturer's protocol. The resulting fluorescence was measured by flow cytometry using a FACS flow cytometer (BD Biosciences, San Jose, CA).

Cell migration and invasion assays

A monolayer cell migration assay and BD BioCoat Matrigel invasion chambers (8-µm

Lentiviral shRNA silencing of HIF-1 α in glioma



Figure 3. Validation of transduction efficiencies and knockdown efficacies of HIF-1 α -shRNA-encoding lentiviral vector in U87MG cells. A: transduction efficiencies of lentivirus were confirmed to be over 99% by fluorescence microscopic detection of lentiviral expression of GFP. B: All three HIF-1 α -shRNA-encoding constructs reduced HIF-1 α mRNA expression as determined by real-time PCR. C: The knockdown efficacies were further confirmed by Western blots for HIF-1 α . The results are the representative of three independent experiments. ***P* < 0.01, significantly different from LV-control group.

pores) (BD Biosciences) were used to measure the effects of HIF-1 α knockdown on the lateral motility and invasiveness of U87MG cells, respectively. For cell migration assay, U87MG cells were seeded onto 24-well plates and cultured in complete medium until confluent. The confluent monolayers were washed twice with PBS and incubated in a serum-free medium. Small linear wounds were created by gently striking a pipette tip across the monolayers. The healing of the wounds through cell migration was assessed by measuring the wound distance. For cell invasion assay, the transduced cells were seeded on the top chamber of each insert with complete medium added to the bottom chamber. After 48 h, cells on the membrane were wiped off with a cotton swab. Fixed and stained with H&E, cells on the underside of the membrane were counted from 4 microscope fields (magnification, x 200). The mean number of invading cells was expressed as a percentage relative to the control.

RNA extraction and real-time reverse transcription-PCR

Total RNA was isolated after transduction with Trizol reagent (Invitrogen, USA). A nanodrop spectrophotometer (Gene, USA) was used to measure the concentration of total RNA. Relative levels of mRNA were examined using SYBR green real-time quantitative reverse transcription-PCR (qRT-PCR) (Applied Biosystems) and normalized with GAPDH. The amplification reaction was performed using MJ real-time PCR (Bio-Rad, Hercules, CA, USA) for 40 cycles. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Western blots

Total proteins were extracted after solubilization in lysis buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 1 mM sodium vanadate, and 1 mM sodium fluoride. Protein



Figure 4. Reversal of *in vitro* malignant phenotypes of U87MG cells by lentiviral delivery of HIF-1 α -shRNA. A: LV-HIF-1A-1168 significantly reduced cell viability as determined by CCK-8 colorimetric assay. B: The same lentiviral construct induced U87MG cell cycle arrest in the G_0/G_1 -phase. C: LV-HIF-1A-1168 prominently induced apoptotic cell death as shown by increased proportion of annexin V-positive cells. D: Lateral cell motility was impaired by LV-HIF-1A-1168 as measured by wound healing assay. The number on the lower right corner of each micrograph indicates the wound size. E: Cellular invasion through Matrigel-coated transwells was reduced by LV-HIF-1A-1168. The number on the lower right corner of each micrograph indicates the number of cells invading through the membrane. The results are the representative of three independent experiments. *P < 0.05; **P < 0.01, significantly different from LV-control group.

extracts were resolved on Tris-HCl gels and Western blotting was performed using standard methodologies. Blots were developed using the enhanced chemiluminescence (ECL) reagents (Amersham Pharmacia, Buckinghamshire, UK) and visualized using the Gene-Genius Imaging System (Syngene, Frederick, MD, USA).

Nude mice xenograft model

Transduced U87MG cells stably expressing firefly luciferase were trypsinized, collected and resuspended in PBS. Then 1×10^7 U87MG cells in 0.2 ml PBS were injected subcutaneously into the flank region of 6-week-old female BALB/c nu/nu mice. After inoculation, the mice were maintained under sterile condition and the size of tumor formed was measured by bioluminescence detection using the Xenogen IVIS system. Tumor volume (V) was also estimated according to the following formula: $V = L \times W^2/2$, where L is the mid-axis length and W is the mid-axis width. At the end of the experiment, the mice were sacrificed and the tumors were excised. All experimental procedures were conducted in conformity with institutional guidelines for the care and use of laboratory animals, and protocols were approved by the Institutional Animal Care and Use Committee of Nanjing Medical University. Undertaken or supervised by trained animal technicians and performed so as to minimize any discomfort to the mice.

Results

Progressive upregulation of HIF-1 α during the progression of gliomas

Intermediate-to-high levels of cytoplasmic and nuclear expression of HIF-1 α were observed in



Figure 5. Modulation of HIF-1 α target genes in U87MG cells by LV-HIF-1A-1168. A: Downregulation of GLUT-1, VEGF, Bcl-2 and MMP2 and upregulation of Bax mRNAs were confirmed by real-time PCR. B: Western blots showed a concordant deregulation of protein expression of HIF-1 α target genes by LV-HIF-1A-1168. The results are the representative of three independent experiments. ***P* < 0.01, significantly different from LV-control group.

glioma tissues as revealed by immunohistochemical staining. By contrast, there was only negligible expression of HIF-1 α in non-cancerous intradecompression brain tissues (**Figure 2A**). The upregulation of HIF-1 α was assoicated with tumor grade (**Table 1**) but not histological subtypes. Concordant with immunohistochemical staining, real-time PCR showed the stagedependent upregulation of HIF-1 α mRNA in glioma tissues, with more than 15-fold increase of HIF-1 α mRNA in grade IV glioma samples as compared with intradecompression brain tissues (**Figure 2B**).

Effective knockdown of HIF-1 α and repression of HIF-1 α target genes by shRNA in U87MG glioma cells

Three shRNA-encoding constructs, namely HIF-1A-1168, HIF-1A-1577 and HIF-1A-2264, targeting different sites of HIF-1 α transcript were packaged into pseudotyped lentiviral particles. The transduction efficiencies of all three lentiviral vectors as well as the control vector were confirmed to be more than 99% in U87MG glioma cells by fluorescence microscopy (Figure 3A). Transduction of U87MG cells with HIF-1αshRNA-encoding lentiviral vectors also significantly reduced the mRNA levels of HIF-1a (Figure 3B). Among these three shRNA-encoding vectors, HIF-1A-1168 showed the strongest inhibition, with more than 80% repression of HIF-1 α mRNA expression. The reduction of HIF- 1α mRNA levels was paralleled by decreased

HIF-1 α protein levels as measured by Western blots (**Figure 3C**), in which HIF-1A-1168 also exhibited the strongest inhibitory effect. The HIF-1A-1168-encoding lentiviral vector was therefore used in subsequent experiments.

Induction of cell cycle arrest and apoptosis and impairment of cell motility and invasiveness by HIF-1 α -shRNA in U87MG

Functional assays were performed to characterize phenotypic changes after knockdown of HIF-1 α . The viability of U87MG cells transduced with HIF-1A-1168-encoding or control vector was measured by CCK-8 assay. Knockdown of HIF-1 α significantly reduced the viability of U87MG cells from day 3 to day 5 post-transduction (Figure 4A). In line with this finding, cell cycle analysis revealed that knockdown of HIF- 1α induced G_{α}/G_{1} -phase cell cycle arrest with concomitant reduction of proportion of cells in S and G₂/M phases (Figure 4B). In addition to the effect on cell cycle, HIF-1α knockdown induced apoptosis in U87MG where phosphotidylserine externalization was increased by ~9fold as measured by Annexin V staining (Figure 4C). As enhanced cell motility and invasiveness have been implicated in the oncogenic action of HIF-1 α , these malignant phenotypes were determined by monolayer wound healing and transwell invasion assays, respectively. Results showed that HIF-1 α knockdown substantially reduced the lateral motility and invasiveness of U87MG cells (Figure 4D and 4E).



Figure 6. In vivo tumor-suppressing and gene-modulating effects of LV-HIF-1A-1168 on U87MG xenograft in nude mice. A: Bioluminescence emitted by LV-HIF-1A-1168-transduced luciferase-expressing U87MG xenograft (right) was prominently reduced when compared with control (left) on day 25 post-inoculation. B: measurement of tumor size after xenograft removal from nude mice on day 25 post-inoculation confirmed the tumor-suppressing effect of LV-HIF-1A-1168. C: LV-HIF-1A-1168 prominently reduced mRNA level of HIF-1 α and modulated the mRNA expression of HIF-1 α -target genes in xenograft tissues. D: Western blots showed a concordant deregulation of protein expression of HIF-1 α and its target genes in xenograft tissues. The results are the representative of three independent experiments. ***P* < 0.01, significantly different from LV-control group.

Modulation of HIF-1 α target genes by HIF-1 α -shRNA

HIF-1 α has been shown to induce GLUT-1, VEGF, Bcl-2 and MMP2 but repress the expression of Bax. To confirm if HIF-1 α -shRNA could inhibit the transcriptional activity of HIF-1 α , the mRNA and protein levels of these important cancerrelated genes were determined. Results showed that HIF-1A-1168-encoding vector substantially reduced both the mRNA and protein expression of GLUT-1, VEGF, Bcl-2 and MMP2 but induced Bax in U87MG cells (**Figure 5**).

Sustained repression of glioma growth and target gene HIF-1 α expression in vivo by shRNA

To determine whether lentiviral delivery of shRNA could elicit sustained repression of HIF- 1α and persistent inhibition of malignant phenotypes of glioma cells *in vivo*, firefly luciferase-

expressing U87MG cells transduced with HIF-1A-1168-encoding or control lentivirus were inoculated subcutaneously into immunodeficient mice in which tumor growth was monitored by bioluminescence. At 25 days postinoculation, the bioluminescence and tumor volume of U87MG xenograft transduced with HIF-1A-1168-encoding vector were significantly lower that those transduced with the control vector (**Figure 6A**). Importantly, HIF-1 α -shRNA persistently reduced the mRNA and protein levels of GLUT-1, VEGF, Bcl-2 and MMP2 but induced Bax in the xenograft tissues (**Figure 6B**).

Discussion

Hypoxia is a common feature of glioma in which extensive hypoxic areas predict poor clinical outcomes. Increased tumor expression of HIF-1 α , a transcription factor whose proline hydroxylation and proteasomal degradation are reduced in hypoxic conditions, has been reported in glioma patients and is positively correlated with tumor progression and poor prognosis. In line with these findings, immunohistochemical staining revealed that HIF-1α protein expression was upregulated in glioma tissues in a tumor grade-dependent manner. In addition to post-translational regulation, sustained hypoxia has been shown to induce the transcription of HIF-1 α [16]. To address if transcriptional activation is involved in HIF-1 α upregulation in glioma tissues, HIF-1a mRNA level was determined by real-time PCR and found to be elevated. This finding indicates that transcriptional mechanism at least in part accounts for HIF-1a upregulation in glioma. In this respect, upregulation of HIF-1α mRNA has been sporadically reported in other types of solid tumors, such as gastric and prostate cancer [17, 18]. However, it is the first time to observe such a phenomenon in glioma. The mechanism by which hypoxia induces the mRNA expression of HIF-1α remains unclear but reactive oxygen species-mediated overexpression and activation of Rho GTPase has been reported to induce HIF-1α mRNA in hypoxic renal cell carcinoma [19]. Our finding also corroborated the idea that targeting HIF-1a mRNA by shRNA-mediated RNA interference is a sound approach of therapeutic intervention.

In the present study, lentiviral delivery of HIF-1α-shRNA triggered substantial phenotypic changes in glioma cells, including lowered cell proliferation, G_0/G_1 -phase cell cycle arrest, apoptosis and reduced cell motility and invasiveness. These observations are concordant with previous findings that HIF-1 α plays a central and pleotropic role in shaping oncogenic phenotypes of cancer cells through its extensive signaling network. In this regard, HIF-1αmediated expression of GLUT-1, a glucose transporter, has been implicated in the metabolic adaptation of cancer cells to hypoglycemic microenvironment [20]. Aberrant activation of HIF-1 α also represses apoptosis through deregulating the balance between pro- (e.g. Bax) and anti-apoptotic (e.g. Bcl-2) members of the Bcl-2 family [21]. In addition, HIF-1 α may promote angiogenesis and cellular invasiveness through induction of VEGF and MMP-2, respectively [9, 22-24]. Importantly, all these HIF-1α-associated molecular abnormalities could be abrogated by lentiviral delivery of HIF-1a-shRNA in glioma cells. Our findings substantiated the therapeutic value of targeting HIF-1 α in the treatment of glioma.

HIF-1 α is a promising therapeutic target for cancer treatment. Research effort has been put forth to identify novel inhibitors for inhibiting this oncogenic transcription factor. Existing small-molecule inhibitors mainly mediate their actions through decreasing HIF-1a protein level, DNA binding or transactivation. Nevertheless, their off-target actions may undermine the clinical utility [7]. Novel methods are therefore urgently needed for specific targeting of HIF-1 α . In this regard, gene-specific silencing of shRNA together with the high efficiency of gene transfer and long-term expression of transgenes by lentivirus open a window of cancer gene therapy. Lentivirus also has relatively low immunogenicity when compared with other viral vectors [25]. Nevertheless, insertional mutagenesis caused by transgene integration may still pose some clinical concerns. The use of integrase-defective lentivirus may circumvent this issue but the stability of transgene expression remains questionable [26].

In summary, our results demonstrate that lentiviral delivery of shRNA to target HIF-1 α significantly reduces the malignant phenotypes of glioma cells and restores the deregulation of multiple target genes of HIF-1 α . Such anticancer effects persist after xenograft implantation into nude mice 25-day post-inoculation. Our findings support that the delivery of HIF-1 α -shRNA through lentivirus may be a clinically useful treatment approach for glioma patients.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (NSFC, 81272791) and the Natural Science Foundation of Jiangsu Province (BK, 2009078).

Disclosure of conflict of interest

None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Address correspondence to: Jun F Shao, Department of Neurosurgery, Wuxi People's Hospital of Nanjing Medical University, Wuxi, 214023, PR China. Tel: 86-0510-82700778; E-mail: wxbrain1@163.com; Gui M He, Institute of Molecular Ecology and Evolution, IAIR, East China Normal University, Shanghai 200062, China. Tel: 86-021-62235786; E-mail: gmhe@bio.ecnu.edu.cn

References

- Wilson WR and Hay MP. Targeting hypoxia in cancer therapy. Nat Rev Cancer 2011; 11: 393-410.
- [2] Koumenis C. ER stress, hypoxia tolerance and tumor progression. Curr Mol Med 2006; 6: 55-69.
- [3] Keysar SB, Trncic N, Larue SM and Fox MH. Hypoxia/reoxygenation-induced mutations in mammalian cells detected by the flow cytometry mutation assay and characterized by mutant spectrum. Radiat Res 2010; 173: 21-6.
- [4] Zeng M, Kikuchi H, Pino MS and Chung DC. Hypoxia activates the K-ras proto-oncogene to stimulate angiogenesis and inhibit apoptosis in colon cancer cells. PLoS One 2010; 5: e10966.
- [5] Yao KS, Xanthoudakis S, Curran T and O'Dwyer PJ. Activation of AP-1 and of a nuclear redox factor, Ref-1, in the response of HT29 colon cancer cells to hypoxia. Mol Cell Biol 1994; 14: 5997-6003.
- [6] Koong AC, Chen EY and Giaccia AJ. Hypoxia causes the activation of nuclear factor kappa B through the phosphorylation of I kappa B alpha on tyrosine residues. Cancer Res 1994; 54: 1425-30.
- [7] Tang CM and Yu J. Hypoxia-inducible factor-1 as a therapeutic target in cancer. J Gastroenterol Hepatol 2012; 28: 401-405.
- [8] Maxwell PH, Wiesener MS, Chang GW, Clifford SC, Vaux EC, Cockman ME, Wykoff CC, Pugh CW, Maher ER and Ratcliffe PJ. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature 1999; 399: 271-275.
- [9] Shyu KG, Hsu FL, Wang MJ, Wang BW and Lin S. Hypoxia-inducible factor 1alpha regulates lung adenocarcinoma cell invasion. Exp Cell Res 2007; 313: 1181-1191.
- [10] Semenza GL, Jiang BH, Leung SW, Passantino R, Concordet JP, Maire P and Giallongo A. Hypoxia response elements in the aldolase A, enolase 1, and lactate dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor 1. J Biol Chem 1996; 271: 32529-32537.
- [11] Forsythe JA, Jiang BH, Iyer NV, Agani F, Leung SW, Koos RD and Semenza GL. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol Cell Biol 1996; 16: 4604-4613.
- [12] Ebert BL, Firth JD and Ratcliffe PJ. Hypoxia and mitochondrial inhibitors regulate expression of

glucose transporter-1 via distinct Cis-acting sequences. J Biol Chem 1995; 270: 29083-29089.

- [13] Dahlrot RH, Kristensen BW, Hjelmborg J, Herrstedt J and Hansen S. A population-based study of high-grade gliomas and mutated isocitrate dehydrogenase 1. Int J Clin Exp Pathol 2013; 6: 31-40.
- [14] Yang L, Lin C, Wang L, Guo H and Wang X. Hypoxia and hypoxia-inducible factors in glioblastoma multiforme progression and therapeutic implications. Exp Cell Res 2012; 318: 2417-2426.
- [15] Castanotto D and Rossi JJ. The promises and pitfalls of RNA-interference-based therapeutics. Nature 2009; 457: 426-433.
- [16] Pialoux V, Mounier R, Brown AD, Steinback CD, Rawling JM and Poulin MJ. Relationship between oxidative stress and HIF-1 alpha mRNA during sustained hypoxia in humans. Free Radic Biol Med 2009; 46: 321-326.
- [17] Pipinikas CP, Carter ND, Corbishley CM and Fenske CD. HIF-1alpha mRNA gene expression levels in improved diagnosis of early stages of prostate cancer. Biomarkers 2008; 13: 680-691.
- [18] Yoshikawa T, Tsuburaya A, Miyagi Y, Sekiguchi H, Kimura M, Cho H and Kobayashi O. Up-regulation of hypoxia-inducible factor-1 alpha and VEGF mRNAs in peritoneal dissemination of patients with gastric cancer. Anticancer Res 2006; 26: 3849-3853.
- [19] Turcotte S, Desrosiers RR and Beliveau R. HIF-1alpha mRNA and protein upregulation involves Rho GTPase expression during hypoxia in renal cell carcinoma. J Cell Sci 2003; 116: 2247-2260.
- [20] Williams KJ, Telfer BA, Airley RE, Peters HP, Sheridan MR, van der Kogel AJ, Harris AL and Stratford IJ. A protective role for HIF-1 in response to redox manipulation and glucose deprivation: implications for tumorigenesis. Oncogene 2002; 21: 282-290.
- [21] Sasabe E, Tatemoto Y, Li D, Yamamoto T and Osaki T. Mechanism of HIF-1alpha-dependent suppression of hypoxia-induced apoptosis in squamous cell carcinoma cells. Cancer Sci 2005; 96: 394-402.
- [22] Zhou J, Li K, Gu Y, Feng B, Ren G, Zhang L, Wang Y, Nie Y and Fan D. Transcriptional upregulation of RhoE by hypoxia-inducible factor (HIF)-1 promotes epithelial to mesenchymal transition of gastric cancer cells during hypoxia. Biochem Biophys Res Commun 2011; 415: 348-354.
- [23] Luo Y, He DL, Ning L, Shen SL, Li L, Li X, Zhau HE and Chung LW. Over-expression of hypoxiainducible factor-1alpha increases the invasive potency of LNCaP cells in vitro. BJU Int 2006; 98: 1315-1319.

- [24] Damert A, Ikeda E and Risau W. Activator-protein-1 binding potentiates the hypoxia-induciblefactor-1-mediated hypoxia-induced transcriptional activation of vascular-endothelial growth factor expression in C6 glioma cells. Biochem J 1997; 327: 419-423.
- [25] Nayak S and Herzog RW. Progress and prospects: immune responses to viral vectors. Gene Ther 2010; 17: 295-304.
- [26] Banasik MB and McCray PB Jr. Integrase-defective lentiviral vectors: progress and applications. Gene Ther 2010; 17: 150-157.