

AGR2 Expression is Regulated by HIF-1 and Contributes to Growth and Angiogenesis of Glioblastoma

Xing-Yu Hong · Jing Wang · Zhe Li

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Abstract Glioblastoma multiforme (GBM) tumors are the most common type of brain tumors characterized by extensive angiogenesis that is mostly orchestrated by tumor hypoxia. The hypoxia induced factor-1 (HIF-1) transcriptional complex is the “master control switch” for hypoxia. Dysregulation of anterior gradient protein 2 (AGR2) expression is associated with tumor growth and metastasis. Whether AGR2 is a hypoxia-responsive factor and affects tumor progression via angiogenesis remains unknown. Here, we show that GBM cell lines, U87 and LN18, exhibited enhanced hypoxic responses compared with control normal human astrocytes, and a corresponding HIF-1-dependent increase in AGR2 mRNA and protein. Recombinant AGR2 and conditioned medium from GBM cells induced human umbilical vein endothelial cell (HUVEC) migration and tube formation, which were abrogated by anti-AGR2 neutralizing antibodies. Expression of the HIF-1 α oxygen-dependent degradation domain mutant in cells resulted in elevated AGR2 levels and an increased ability to induce HUVEC migration and tube formation in vitro and enhanced growth and vascularity of tumor xenografts in vivo, which were prevented by AGR2 knockdown. Taken together, these results indicate that

AGR2 expression is regulated by HIF-1 and plays an important role in control of glioblastoma growth and vascularity. Our findings suggest that inhibiting AGR2 may represent a new therapeutic target for anti-angiogenic cancer treatment.

Keywords Glioblastoma Multiforme · Anterior gradient protein 2 · Hypoxia · Hypoxia induced factor-1 · Tumor angiogenesis

Introduction

Cells that are under hypoxic stress can either develop an adaptive response that includes increasing the rate of angiogenesis or undergo cell death by promoting apoptosis and/or necrosis. The ability of tumor cells to maintain a balance between an adaptation to hypoxia and cell death is regulated by a family of transcription factors called hypoxia-inducing factors, which are essential for the regulation of the expression of a large number of hypoxia-responsive cytokines including vascular endothelial growth factor (VEGF). Tumor hypoxia facilitates the likelihood of angiogenesis, metastases, tumor recurrence, and the invasive potential [1–3]. The HIF-1 transcriptional complex is the “master control switch” for hypoxia. HIF-1 is composed of two polypeptides: HIF-1 α and HIF-1 β [4]. HIF-1 β is expressed constitutively but HIF activity is regulated at the post-transcriptional level by oxygen-dependent hydroxylation of specific proline residues on the α subunit by the prolyl hydroxylase domain (PHD) proteins. When hydroxylated, HIF-1 α is targeted for proteasome degradation by complexing with the von Hippel–Lindau (VHL) tumor suppressor protein, a component of an E3 ubiquitin ligase complex [5]. In rapidly growing tumors or following a series of

X.-Y. Hong
Department of Vascular Surgery, China–Japan Union Hospital
Affiliated to Jilin University, Changchun 130031, China

J. Wang
School of Life Sciences, Jilin Agricultural University,
Changchun 130118, China

Z. Li (✉)
Department of Cardiovascular Surgery, China–Japan Union
Hospital Affiliated to Jilin University, Changchun 130031, China
e-mail: zhelicc@gmail.com

genetic mutations, VHL-mediated degradation of HIF-1 α can be lost. HIF-1 will then bind to hypoxia response elements (HRE) in the promoter and activate expression of a broad range of genes that mediate the adaptive responses to decreased oxygen concentration, such as enhanced glucose uptake and the formation of new blood vessels via proliferation and migration of endothelial cells toward the developing tumor [6].

Glioblastoma Multiforme (GBM) tumors are the most common type of brain tumors and despite recent advances in standard treatment, the prognosis still remains dismal. These malignant tumors are characterized by extensive angiogenesis that is mostly orchestrated by tumor hypoxia [7]. GBM has been a target for anti-angiogenic therapy as the tumor shows a high degree of endothelial cell proliferation and VEGF expression [8]. Blockade of VEGF-related pathways is a widely used strategy for GBM therapy, whereas resistance to anti-VEGF therapies eventually occurs. Therefore, the demand for new targets for anti-angiogenic therapy remains imperative.

Anterior gradient protein 2 (AGR2), also known as hAG-2 [9] or Gob-4 [10], is the human ortholog of the *Xenopus laevis* AGR protein, XAG-2. XAG-2 has a putative role in ectodermal patterning of the frog embryo and is regulated by a number of fundamental embryonic molecules like noggin and chordin [11]. In humans, AGR2 was first identified in breast cancers and forced expression of AGR2 cDNA conferred a metastatic phenotype on benign non-metastatic rat breast carcinoma cells [9]. AGR2 has been found highly expressed in malignant tumors [12–19]. Overexpression of AGR2 was found to attenuate p53 activation in UV-damaged cancer cells, leading to the suggestion that AGR2 functions as a survival factor through inhibition of p53 [20]. Recently, AGR2 was shown to act as an oncogene when overexpressed in NIH3T3 cells [16]. Tumor progression and metastasis depend on the ability of cancer cells to initiate angiogenesis. Whether AGR2 is a hypoxia-responsive factor and affects tumor progression via angiogenesis remains unknown. Our study was conducted to investigate the regulation of AGR2 expression and its role in growth and vascularity of GBM to potentially provide a novel target for anti-angiogenic cancer treatment.

Materials and Methods

Cell Culture

U87, LN18, and SW1783 GBM cell lines were purchased from American Type Culture Collection (ATCC). Normal human astrocytes (NHAs) were purchased from Lonza, Inc. Cells were growing in DMEM (Gibco), Ham's F12 (MediaTech) or Leibovitz's L-15 Medium (ATCC)

supplemented with 10 % FBS (Lonza) and antibiotics. HUVECs (Clonetics) were cultured in endothelial basal medium supplemented with 2 % FBS, 1 μ g/ml hydrocortisone, 10 ng/ml human epidermal growth factor (hEGF) (Cell Signaling), 5 ng/ml basic fibroblast growth factor (Bio Tang) and 10 μ g/ml heparin (Humedia-EG2; Kurabo).

Luciferase Assay

The cells were transfected with control DNA or a luciferase reporter plasmid containing the wild-type or mutated VEGF promoter (Gifted from Dr. Min Zhang, the University of Hong Kong) using Lipofectamine 2,000 (Invitrogen, Carlsbad, CA) and subjected to a luciferase assay (Dual-Luciferase Reporter Assay System, Promega, Madison, WI) in normoxia and hypoxia. Hypoxic conditions were generated by either growing cells in a 37 °C incubator in a sealed chamber containing a mixture of gases with 1 % oxygen.

Real-Time RT-PCR

Total RNA from the cells was isolated using Trizol reagent (Gibco) according to manufacturer's instruction. RNA was then converted into cDNA using the AMV reverse-transcription system (Promega) in the presence of random hexamers (Invitrogen). The cDNA was used for RT-qPCR with the following gene-specific primers: human AGR2 5'-GGGATGGAGAAAATTCCAGTG-3' (forward) and 5'-GGGTACAATTCAGTCTTCAG-3' (reverse); human GAPDH 5'-TGATGACA TCAAGAAGGTGGTGAAG-3' (forward) and 5'-TCCTTGGAGGCCATGTGG GCCAT-3' (reverse). A MYIQ RT-PCR detection system and SYBR green PCR mix (Bio-Rad, Richmond, CA, USA) were used to carry out the PCR. The relative quantization of targeted genes was determined by the comparative $\Delta\Delta C_t$ (threshold) method using GAPDH as an internal control.

Immunoblot Analysis

Cells were lysed, and 75 μ g of protein subjected to SDS-polyacrylamide gel electrophoresis. Following gel electrophoresis, a transfer was done onto a polyvinylidene difluoride membrane (Immobilon P, Millipore, Bedford, MA), which was incubated with the appropriate antibodies: anti-AGR2 (2 μ g/ml, Abcam), anti-HIF-1 α (1:2,000 dilution, Pierce), anti-HIF-1 β (1:2,000 dilution, Abcam), and anti-GAPDH (1:5,000 dilution, Abcam).

Migration Assay

A total of 5×10^5 HUVECs in the serum-free medium were added to the top chambers of Transwell systems (BD Biosciences; 8 μ m pore sizes). Conditioned medium from U87,

LN18 and SW1783 cells or the serum-free medium with 1 $\mu\text{g/ml}$ recombinant AGR2 (Novus Biologicals) was added to the bottom wells, with or without 100 $\mu\text{g/ml}$ AGR2 blocking antibody (Abcam). The migration was quantified after 18 h by staining the Transwell filters with crystal violet.

Tube Formation Assay

Growth factor reduced (GFR) Matrigel 10 $\mu\text{l/well}$ (in 15-well μ -angiogenesis slides, ibidi, Germany), was incubated for 15 min at 37 °C. HUVECs (10,000 cells/well) were resuspended in 0.5 % FBS MDCB131 medium containing 1 $\mu\text{g/ml}$ recombinant AGR2 or in conditioned medium from the indicated cell types, with or without 100 $\mu\text{g/ml}$ anti-AGR2 neutralizing antibodies. The cells were plated on the matrigel and incubated for 24 h at 37 °C to allow tube formation. The cells were then imaged for tube formation using an Olympus IX70 microscope equipped with a Hamamatsu camera using MetaView™ software. Quantification of the tubes was performed by taking six 4x images of each well. The tube formation was assessed by area of each image and was analyzed by Metamorph and averaged together.

Transient Transfection of Small Interfering RNA

GBM cells were plated on 100-mm dishes and transiently transfected with siControl, siAGR2, siHIF-1 α or siHIF-1 β (Dharmacon, Inc) at a final concentration of 10 nmol/L with HiPerfect transfection reagent (Qiagen, Inc), and lysates were prepared for immuno blot after 72 h.

Generation of Lentivirus-Expressing Short Hairpin RNA

For lentiviral-mediated gene silencing of AGR2, the following synthetic oligonucleotides were used: 5'-TGCTGTTGACAGTGAGCGAGGAATTGACC TTGTAATCGA TTAGTGAAGCCACAGATGTAATCGATTACAAGGTC AATTC CCTGCCTACTGCCTCGGA-3'. The oligonucleotides were ligated into the pLB lentiviral gene transfer vector [21], using *HpaI* and *XhoI* restriction sites. This pLB vector contains a U6 promoter that drives expression of short hairpin RNAs (shRNA), and a CMV promoter driving green fluorescent protein expression. Packaged lentiviruses were generated by transfecting HEK 293-FT cells (ATCC) with pLB transfer vectors in combination with plasmids encoding gag/pol and VSV-G envelope proteins.

In Vivo Tumor Growth in the Xenograft Models

All animal procedures were conducted under Institutional Animal Care and Use Committee-approved protocols.

NCR-nu/nu male mice were purchased from Animal Core Facility, Guangzhou University of Chinese Medicine. Human xenograft tumors were generated by injecting subcutaneously 1×10^6 SW1783 cells co-infected with control lentiviruses or lentiviruses coding for AGR2 shRNA and HIF-1 α oxygen-dependent degradation domain mutant mixed with matrigel into nude mice. Animals were sacrificed, and tumors were removed at 35 days after injection.

Immunofluorescence

Immunofluorescence on frozen sections was carried out after fixation with 4 % paraformaldehyde, washing with PBS, blocking in serum-free Protein Block (DAKO), followed by incubation with goat anti-CD31(1:100 dilution, Santa Cruz Biotechnology) at 4 °C for 12 h and secondary donkey FITC-conjugated antibody(1:150 dilution, Jackson ImmunoResearch) at room temperature for 1 h in PBS containing 0.05 % Tween-20. The slides were then washed three times with PBS for 3 min each. Coverslips were placed on top of the slides using glycerol in PBS with fluorescent mounting medium (propyl gallate, Acros Organics, Morris Plains, NJ). Images were captured with an Olympus microscope (BX-51) with an attached DP71 digital camera and processed with DP Controller and DP Manager software (Olympus, Center Valley, PA).

Statistical Analyses

Experiments were performed a minimum of three replicates. Statistical analyses were performed as a student's *t* test using GraphPad Prism software. *p* values of *p* < 0.05 were considered statistically significant.

Results

HIF-1 α is Elevated in GBM Cell Lines and Strongly Induced in Hypoxia

To characterize the hypoxic responses in GBM cell lines, we transfected U87 and LN18 GBM cell lines along with NHA with control DNA, a luciferase reporter plasmid containing the wild-type VEGF promoter, which is known to be activated in hypoxia, or the plasmid containing a mutated VEGF promoter [22], and subjected the cells to a luciferase assay under normoxic and hypoxic conditions. GBM cell lines expressing the wild-type construct exhibited an enhanced luciferase response under normoxic conditions and an increase in fluorescence in hypoxia compared with NHA controls (Fig. 1a). The results of this luciferase assay correlated well with the levels of HIF1 α

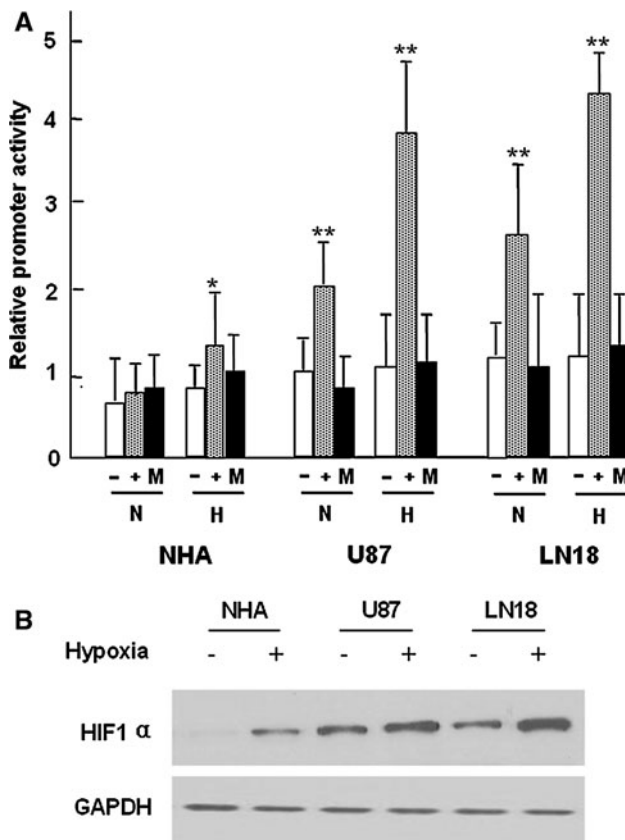


Fig. 1 GBM cells exhibited high levels of HIF-1 α protein and HIF-1 transcriptional activity (a), NHAs, U87 and LN18 cells were transfected with a luciferase reporter plasmid containing control DNA (-), wild-type (+) VEGF promoter, or a mutated (M) in normoxia (N) and hypoxia (H) for 24 h. Student's *t* tests were performed comparing U87 and LN18 cells expressing the wild-type luciferase reporter in normoxia and hypoxia with NHA under identical conditions (* $p \leq 0.05$; ** $p \leq 0.01$) (b), Immunoblot for HIF-1- α in NHA, U87 and LN18 cells in normoxia and hypoxia. GAPDH was used as the loading control

protein observed in an immunoblot of lysates of these cells, which showed constitutively high levels of HIF1 α even under normoxic conditions in GBM cell lines, but not in NHA, and a robust increase when the GBM cells were subjected to hypoxic conditions (Fig. 1b). Taken together, these results demonstrate that GBM cells have an enhanced hypoxic response compared with the NHA.

AGR2 is Highly Expressed in GBM Cell Lines and Strongly Induced in Hypoxia

We noted that AGR2 protein levels in GBM cells increased along with HIF1 α following hypoxia treatment (Figs. 1a, 2a). We then looked at AGR2 mRNA levels in GBM cells when compared with the response in NHA. Similar to immunoblot findings, the levels of AGR2 mRNA were significantly higher in U87 and LN18 cells in normoxia compared with NHAs (Fig. 2b). There was a greater than

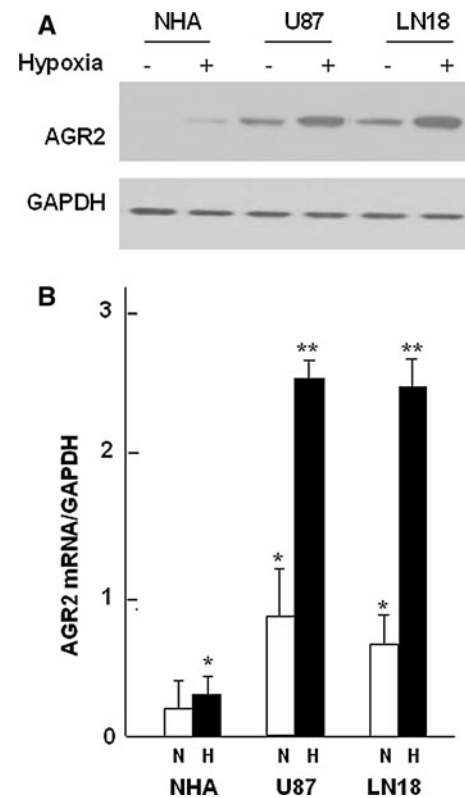


Fig. 2 Expression of AGR2 protein and mRNA were elevated in GBM cell lines and strongly induced in hypoxia (a), U87 and LN18 cells exhibited higher levels of AGR2 compared with AGR2 levels in NHA under normoxia or hypoxia for 24 h. GAPDH was used as loading control (b), NHA, U87 and LN18 cells were cultured in normoxia (N) or hypoxia (H) for 24 h, and total RNA was extracted to detect AGR2 transcripts in a real-time PCR analysis. GAPDH mRNA served as internal control. The bar graph represented the ratio of AGR2 mRNA to GAPDH mRNA. Student's *t* tests were performed for NHA, U87 and LN18 cells in hypoxia, compared with normoxia (* $p \leq 0.05$; ** $p \leq 0.01$)

two-fold increase in AGR2 mRNA levels in U87 and LN18 cells compared with only a slight elevation in NHA following exposure to hypoxic conditions for 48 h. These findings show that AGR2 expression is regulated by hypoxia in a response that is enhanced in U87 and LN18 cells.

AGR2 Expression is Regulated by HIF-1

To further determine if elevated levels of AGR2 in normoxia and its induction in hypoxia in GBM cells were mediated by HIF-1, we targeted HIF1 β , the binding partner for all forms of HIF [23] for knockdown. GBM cells transfected with HIF-1 β siRNA exhibited reduced levels of HIF-1 β protein in an immunoblot (Fig. 3a). When U87 and LN18 cells transfected with HIF-1 β siRNA or controls were exposed to hypoxic conditions, we observed increases in protein levels of AGR2 only in control transfected cells

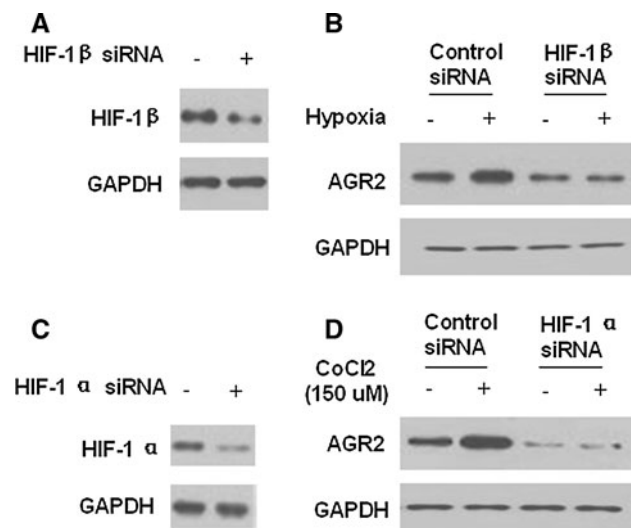


Fig. 3 AGR2 protein levels increased in hypoxia in a HIF-1-dependent manner (a), U87 cells exhibited reduced levels of HIF-1β protein when transfected with HIF-1-β siRNA oligos (b), HIF-1β siRNA transfected U87 cells and control cells were subjected to hypoxia for 24 h and immunoblotted for AGR2. AGR2 protein levels increased in control cells growing in hypoxia, but this response was lost in cells transfected with HIF-1-β siRNA oligos (c), transfection of HIF-1α siRNA oligos reduced levels of HIF-1α protein in U87 cells (d), increases in AGR2 observed in CoCl₂-treated U87 cells were lost in cells expressing HIF-1α siRNA oligos compared with controls. GAPDH was used as loading control in all immunoblots

and not in cells expressing HIF-1β siRNA (Fig. 3b). We then transfected U87 and LN18 cells with a control or HIF-1α siRNA oligos (Fig. 3c) and treated with CoCl₂, a hypoxia-mimetic agent. We observed an induction of HIF-1α and AGR2 protein levels in control cells growing in CoCl₂ but an attenuation of this response and even a reduction in AGR2 levels in cells transfected with HIF-1α siRNA oligos (Fig. 3d). These results show that AGR2 protein levels are under the control of the HIF-1 complex.

AGR2 Induce Migration and Tube Formation in Endothelial Cells

To examine whether AGR2 can contribute to angiogenesis, we did migration and tube formation assays in HUVECs. As shown in Fig. 4a, AGR2 was seen in medium conditioned by the GBM cell lines but not in medium conditioned by NHAs. Therefore, we asked whether medium from GBM cells can attract HUVEC in migration assays. The HUVECs were pretreated with IgG control antibodies or AGR2-blocking antibodies. Migration of HUVECs was seen toward positive control wells containing medium with 10 % FBS both with and without AGR2 blocking antibodies, but not toward serum-free medium containing 0.1 % BSA, which served as a negative control. We observed significant chemotaxis toward wells containing

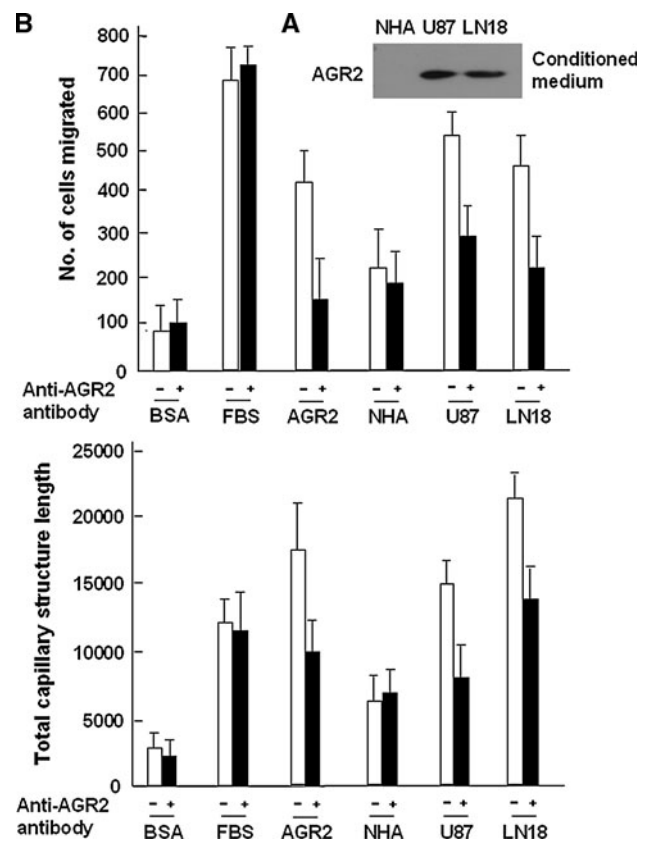


Fig. 4 AGR2 induced migration and tube formation of endothelial cells. AGR2 shed by GBM cells promoted chemotaxis and tube formation in HUVECs (a), AGR2 was seen in medium conditioned by U87 or LN18 cells but not in medium conditioned by NHAs (b), quantification of migration (upper panel) and tube formation (lower panel) in HUVECs. Conditioned medium from indicated cell types and serum-free medium containing 1 μg/ml AGR2, with or without 100 μg/ml AGR2-blocking antibodies, were used as the chemoattractants for HUVECs in migration and tube formation assays. 10 % FBS was used as positive control and 0.1 % BSA was the negative control

800 ng/ml recombinant AGR2 and toward the medium from U87 to LN18 cells containing AGR2, both of which were reduced upon incubation with the AGR2-blocking antibodies (Fig. 4b upper panel). These results suggest that AGR2 produced by GBM cells acts as a potent chemoattractant for endothelial cells. We then examined whether AGR2 could affect endothelial cell tube formation. Similar results were obtained by treatment of the HUVECs with conditioned medium from U87 or LN18, or recombinant AGR2, with or without 100 μg/ml AGR2-blocking antibodies (Fig. 4b lower panel). Taken together, these findings suggest that AGR2 may be a proangiogenic factor.

Stabilization of HIF-1α results in up-regulation of AGR2 and promotion of endothelial cell migration and tube formation in vitro.

To confirm that HIF-1α is responsible for AGR2 induction, we utilized lentiviruses coding for the HIF-1α

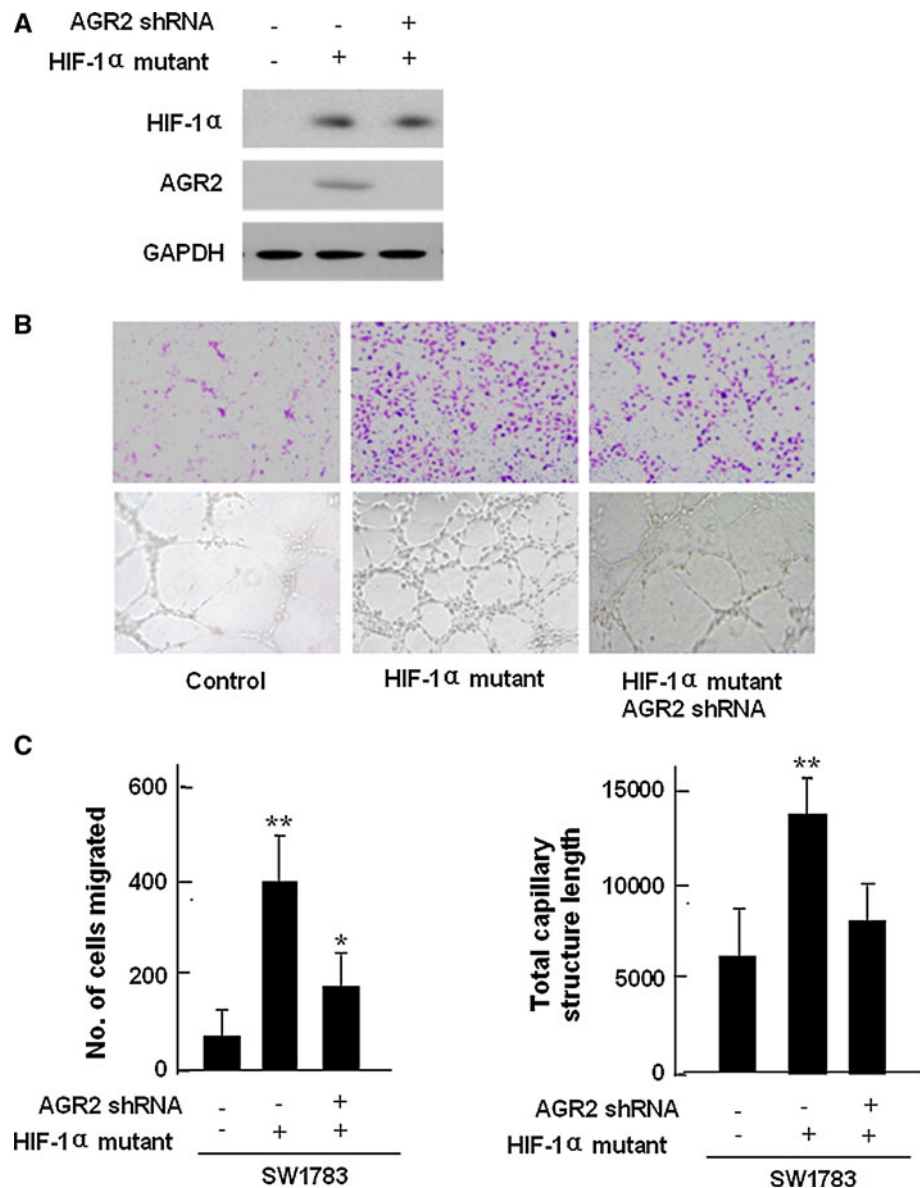
oxygen-dependent degradation domain mutant (Gifted from Dr. Min Zhang, University of Hong Kong) that is resistant to VHL-mediated ubiquitination and degradation because it lacks the prolyl residues that are targeted for hydroxylation by the PHD. When expressed in cells it results in a stable HIF-1 transcriptional complex even in normoxia. We expressed this HIF-1 α mutant in the SW1783 cells, which shows no detectable levels of AGR2 protein and does not exhibit a strong hypoxic response. As shown in Fig. 5a, when HIF-1 α levels increased due to the effect of the degradation-resistant mutant, these cells also began expressing high levels of AGR2 protein. To establish the biological significance of this effect, we looked for the ability of the SW1783 cells to serve as chemoattractants for HUVECs in a migration assay. We observed an increase in AGR2 protein levels in cell lysates as a result of HIF-1 α

mutant expression, but we blocked this increase by infection with lentiviruses expressing the AGR2 shRNA construct (Fig. 5a). When used as chemoattractants in migration and tube formation assays, we found that the conditioned medium from cells expressing HIF-1 α mutant could attract endothelial cells and enhance tube formation. However, this effect was partly blocked by infection with AGR2 shRNA-expressing lentivirus (Fig. 5b, c). These results establish that HIF-mediated increases in AGR2 are important for endothelial cell chemotaxis.

HIF-1-induced up-regulation of AGR2 results in promotion of endothelial cell migration in vitro, and is responsible for enhanced tumor angiogenesis and growth.

To determine whether HIF-1-induced up-regulation of AGR2 has biological significance on tumorigenicity in vivo, SW1783 cells infected with lentivirus were injected

Fig. 5 HIF-1 stabilization resulted in up-regulation of AGR2 and promotion of endothelial cell migration and tube formation in vitro (a), immunoblot analysis for AGR2 and HIF-1 α in SW1783 cells co-infected with control lentiviruses (–) or lentiviruses coding for AGR2 shRNA(+) and HIF-1 α oxygen-dependent degradation domain mutant (HIF-1 α mutant) (+). SW1783 cells overexpressing HIF-1 α , exhibited high levels of AGR2 protein in cell lysates. This response was abrogated in cells infected with AGR2 shRNA-expressing lentivirus. GAPDH was used as loading control (b), the conditioned medium from these cells was used as chemoattractants for endothelial cells in migration and tube formation assays. Enhanced endothelial cell migration and tube formation were induced by medium from HIF-1 α mutant - expressing SW1783 cells, an effect reduced in cells co-infected with AGR2 shRNA-expressing lentivirus. Quantification of the migration (left panel) and tube formation (right panel) assays was shown in (c). Student's *t* tests were performed (* $p \leq 0.05$; ** $p \leq 0.01$)



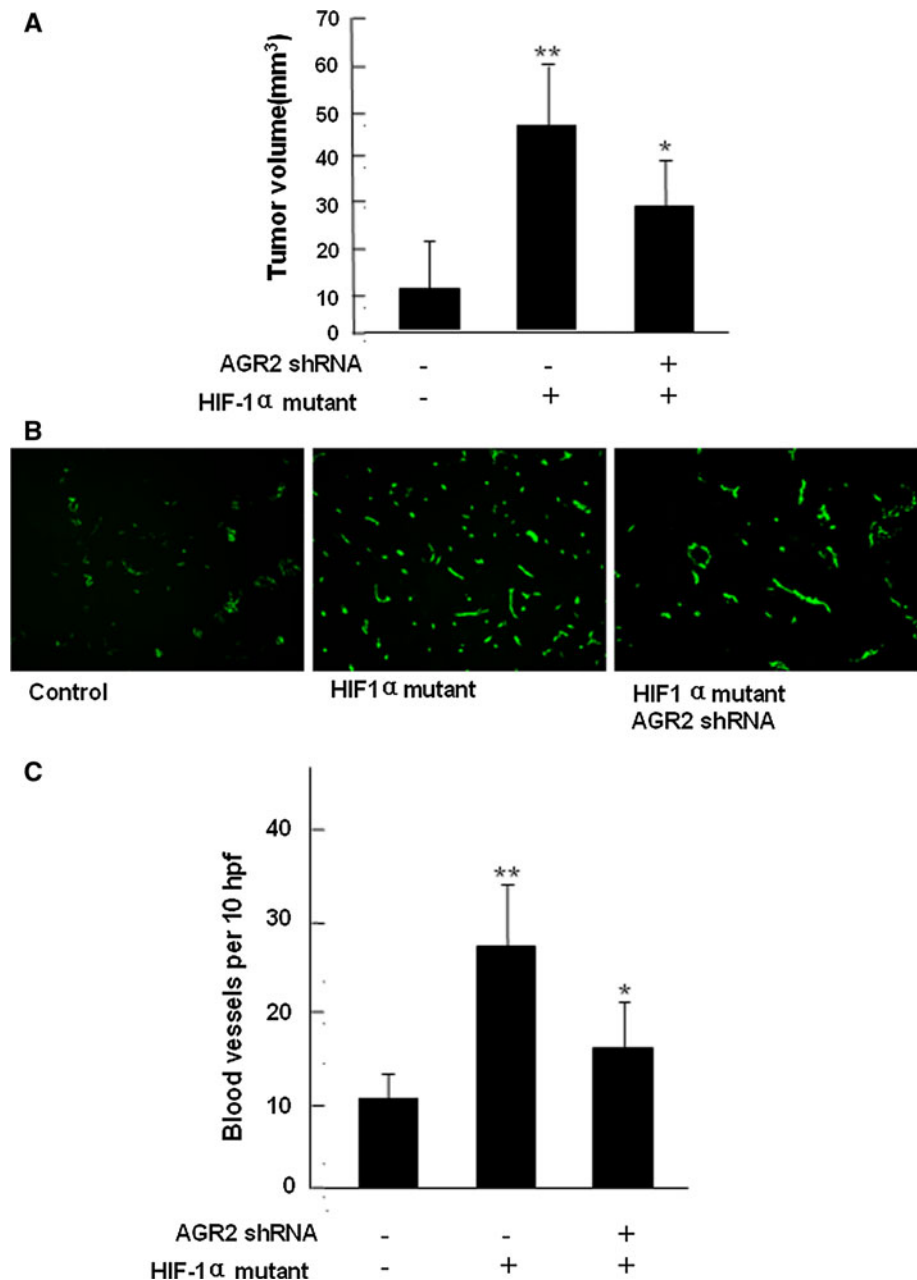
subcutaneously into nude mice and the tumors were harvested at day 35 postinjection. We found that when used as xenografts in nude mice, SW1783 infected with control lentivirus failed to grow in the mice. SW1783 cells were not tumorigenic [24], whereas tumors composed of cells infected with HIF-1 α oxygen-dependent degradation domain mutant lentivirus grew much larger and more rapidly than tumors from control infected cells, and growth was attenuated when the SW1783 cells were co-infected with the lentivirus coding for AGR2 shRNA. The results of tumor volume measurements were shown graphically in Fig. 6a. Immunostaining analysis revealed enhanced vascularity in tumors composed of cells infected with HIF-1 α mutant-

expressing lentivirus when compared with control tumors or tumors co-infected with HIF-1 α mutant and AGR2 shRNA, as demonstrated by anti-CD31 staining (Fig. 6b). These results, quantified in the bar graph (Fig. 6c), indicate that HIF-1-mediated up-regulation of AGR2 is important for enhanced tumor growth and vascularity.

Discussion

The role of the developmentally expressed AGR2 in tumor progression and metastasis is being increasingly studied in many cancer types. However, regulation of AGR2

Fig. 6 AGR2 knockdown reduced HIF-1 α -induced tumor growth and vascularity (a), SW1783 cells were co-infected with control lentiviruses(–) or lentiviruses coding for AGR2 shRNA(+) and HIF-1 α mutant(+). Quantification of tumor volume resulted from the three populations of cells at day 35 post-engraftment. Student's *t* tests were performed, comparing growth of control tumors with HIF-1 α mutant-expressing tumors and those co-expressing AGR2 shRNA ($*p \leq 0.05$; $**p \leq 0.01$) (b), CD31 stain of frozen sections of tumors derived from SW1783 cells infected with control lentiviruses, HIF-1 α mutant-expressing lentivirus, and the combination of HIF-1 α mutant and AGR2 shRNA lentiviruses (c), quantification of the number of vessels per 10 high-power fields in CD31-stained xenografts was shown in the bar graph. Student's *t* tests were performed, comparing the number of blood vessels from tumors expressing HIF-1 α mutant and co-expressing AGR2 shRNA to numbers seen in tumors derived from control cells ($*p \leq 0.05$; $**p \leq 0.01$)



expression and its action in tumor angiogenesis have not been analyzed extensively. This is the first report indicating that HIF-1 regulated expression of AGR2 is a strategy by which GBM tumors may promote angiogenesis.

GBM, the most common primary malignant brain tumor among adults, is a highly angiogenic and deadly tumor. The pathological characteristics of GBM tumors are exemplified by an active invasiveness, necrosis and a specialized form of angiogenesis, known as microvascular hyperplasia. These pathological features are thought to be due to tissue hypoxia. The mechanisms that regulate angiogenesis in hypoxia or hypoxic microenvironment are modulated by several pro- and antiangiogenic factors. Hypoxia-inducible factors, which are essential for the regulation of the expression of a large number of hypoxia-responsive genes, have been established as the basic and major inducers of angiogenesis [6, 8]. Angiogenesis in GBM, driven by hypoxia-dependent mechanism, is primarily mediated by VEGF, and generates blood vessels with distinctive features. The outcome for patients with recurrent glioblastoma is poor because of ineffective therapies. Although anti-VEGF strategies have been improving survival and quality of life, eventual tumor progression is the norm. Better understanding of resistance mechanisms to VEGF inhibitors and identification of effective therapy after VEGF inhibition progression are currently a critical need for patients with glioblastoma [25]. The GBM cell lines studied here exhibited high levels of HIF-1 α protein, even at baseline, and an enhanced activation of a hypoxia response in immunoblots and luciferase assays. AGR2 has been proven as an important tumor biomarker and negative prognostic factor potentially exploitable in clinical practice. AGR2 modulates the growth and metastasis of cancers [12–20]. Therefore, we attempted to look for a link between hypoxia and AGR2 expression in GBM cell lines.

AGR2 was markedly increased at both mRNA and protein levels in GBM cells compared with NHA in normoxia and hypoxia. We demonstrated that the observed increases in AGR2 levels were dependent upon HIF-1 and were biologically significant, as expression of the HIF-1 α degradation-resistant mutant lead to overexpression of AGR2 even in cells that normally express very little protein, and an enhanced ability of cells to act as chemoattractants for endothelial cells in a migration assay. When AGR2 levels were reduced with shRNA-expressing lentiviruses, this pro-angiogenic phenotype was greatly attenuated. Thus, HIF-1-regulated AGR2 may be a potential proangiogenic factor.

SW1783 cells do not express high levels of HIF-1 α and AGR2, induce only a slight pro-angiogenic response in HUVEC cells, and do not grow well as subcutaneous xenografts in nude mice in vivo. However, we could induce a more pro-angiogenic phenotype in SW1783 cells by

overexpressing the HIF-1 α degradation-resistant mutant. We show that hypoxia-driven expression of AGR2 is important in endothelial cell chemotaxis and tumor development, as this phenotype was attenuated by introduction of AGR2 shRNA. SW1783 cells expressing both HIF-1 α mutant and AGR2 shRNA exhibited an “intermediate” phenotype for induction of HUVEC migration and tube formation, probably due to the continued production of VEGF and other pro-angiogenic factors that are part of the HIF-1-mediated hypoxia response. AGR2 is a secretory molecule, and is present in the medium of cancer cells expressing high levels of AGR2. Secretion of AGR2 from the surface of GBM cells is likely not hindered by hypoxic growth conditions, since we observed HUVEC migration toward medium conditioned by SW1783 cells expressing HIF-1 α degradation-resistant mutant.

Here we show that AGR2 is elevated in hypoxia and influences tumor growth and angiogenesis. These results show that AGR2 may be part of the hypoxia adaptive response, which is dysregulated in tumor cells and thus could present a new target for anti-angiogenic therapy in the treatment of GBM tumors.

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Conflict of interest We have no conflict of interest to declare and informed consent was obtained.

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