Activation of Vascular Endothelial Growth Factor A Transcription in Tumorigenic Glioblastoma Cell Lines by an Enhancer with Cell-Type Specific DNase I Accessibility

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Running Title: VEGF-A transcription in glioblastoma cells
Summary

Unregulated expression of vascular endothelial growth factor-A (VEGF-A) plays an important role in tumor growth. We have identified a cell-type specific enhancer, HS-1100, that contributes to VEGF-A transcriptional activation in tumorigenic glioblastoma cell lines. This enhancer exhibits increased accessibility to DNase I in glioblastoma cell lines that express high levels of VEGF-A but not in several other cell lines that express much lower levels of VEGF-A. HS-1100 contains a number of sequence elements that are highly conserved among human, mouse and rat, including the hypoxia response element (HRE). We show that the HRE contributes significantly to the cell-type specific enhancer activity of HS-1100 in U87MG glioblastoma cells. We use chromatin immunoprecipitation assays to show that endothelial PAS domain protein 1 (EPAS1) can efficiently bind to the endogenous HRE in U87MG cells but not in HEK293 cells in which the chromosomal HS-1100 is not accessible to DNase I. A dominant negative EPAS1 significantly reduces HS-1100 enhancer activity and VEGF-A levels in U87MG cells. Our results provide insight into the molecular mechanisms of VEGF-A up-regulation during cancer development.
Introduction

Numerous studies have shown that a tumor establishes its own vasculature in order to reach macroscopic size (1). Consequently, the up-regulation of angiogenic factors is often an important step in the development of aggressive metaplasia. Vascular endothelial growth factor-A (VEGF-A) is a potent angiogenic agent that is often up-regulated during cancer development (2). VEGF-A deregulation appears to play a particularly important role in the development of glioblastoma, a fast growing and highly vascularized brain cancer with poor prognosis. Up-regulation of VEGF-A is a common event in glioblastomas (3), and VEGF-A expression is correlated with tumor grade and vascularity in gliomas (4). Numerous studies in animal models have shown that the inhibition of VEGF-A using neutralizing antibodies (5), dominant negative VEGF receptor mutants (6,7) and antisense constructs (8,9) causes regression of blood vessels, inhibits growth of glioma cells in vivo and suppresses tumorigenicity of glioblastoma cells in immunodeficient mice (8-11). Taken together, these findings indicate that strategies to down-regulate VEGF-A can potentially be applied to treat highly vascularized glioblastomas.

VEGF-A expression is regulated by a wide variety of reagents, signaling pathways and external stimuli, including hypoxia, hormones, cytokines and oncogenes. The VEGF-A promoter contains a hypoxia response element (HRE) consisting of a hypoxia inducible factor-1 binding site and a hypoxia inducible factor-1 ancillary sequence (HAS) that mediates VEGF-A activation by hypoxia and nitric oxide (12-15). Two hypoxia inducible factors (Hifs), Hif-1α and endothelial PAS domain protein 1 (EPAS1, also called Hif-2α), have been shown to mediate
VEGF-A activation through binding to the HRE upon dimerization with the aryl hydrocarbon receptor nuclear translocator gene product (ARNT, also called Hif-1β). The transforming growth factor-β pathway and AP-1 protein have been shown to synergize or potentiate hypoxia induced VEGF-A transcriptional activation (16,17). AP-1 has also been implicated in the induction of VEGF-A by lead in human astrocytes and ionizing radiation in human glioblastoma cells independent of the hypoxia pathway (18,19). 17β-estradiol induces VEGF-A transcription through a variant estrogen response element on the VEGF-A promoter (20). Interleukin-1, epidermal growth factor, protein kinase C, Ras, mitogen-activated protein kinase and phosphatidylinositol 3-kinase pathways have all been implicated in stimulating VEGF-A expression in various cell systems (18,19,21-23). In addition to Hifs and AP-1, SP1 and AP-2 have also been shown to regulate VEGF-A transcription under various conditions (24).

Many glioblastoma cells constitutively express high levels of VEGF-A without any external stimuli, suggesting that they express intrinsic factors that activate VEGF-A expression to high levels independent of environmental signals. For example, U87MG human glioblastoma cells have been found to express much higher levels of VEGF-A than NIH3T3 fibroblasts, human fetal astrocytes and A172 glioma cells under tissue culture conditions (25,26). Given the prominent role of VEGF-A in tumor angiogenesis, it is critical to understand the molecular basis of high level VEGF-A expression in tumor cells. In this study, we investigated the cis-acting elements and transcription factors involved in constitutive VEGF-A up-regulation in U87MG cells. We identified a new regulatory region of VEGF-A that is selectively accessible to DNase I digestion in tumorigenic glioblastoma cell lines and functions as a potent enhancer in U87MG-based reporter assays. This new regulatory region, which we will refer to as ‘HS-1100’, spans
approximately four-hundred base pairs (bps) of the VEGF-A gene centered on bp -1100 relative to the transcription initiation site, and is highly conserved among human, mouse and rat.

We demonstrate that the HRE, which is located within HS-1100, plays an important role in up-regulating VEGF-A expression in U87MG glioblastoma cells, and that this is correlated with an enhanced binding of EPAS1 to the HRE. We further show that this enhanced binding appears to be due not to enhanced expression of EPAS1, but rather to the presence of a chromatin architecture that is more permissive for EPAS1 binding in U87MG cells than in other cell types.

These results suggest that constitutive chromatin accessibility may facilitate the binding of EPAS1 to the HRE within HS-1100, which acts to up-regulate VEGF-A expression in U87MG cells.
Experimental Procedures

Cell Culture- The human cervical carcinoma HeLa, the human embryonic kidney HEK293, the human glioblastoma U87MG, U118MG and A172 cell lines were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. The human erythroleukemia HEL and chronic myelogenous leukemia K562 cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. The human glioblastoma T98G cell line was cultured in Eagle’s minimum essential medium with Earl’s BSS supplemented with 10% fetal bovine serum. All cell lines were maintained in a 5% CO₂ atmosphere at 37°C.

Plasmids- The VEGF-A luciferase reporter plasmids pGL3P.-1100, pGL2P.-550, pGL3P.+500, pGL3P.-1083/-913, pGL3P.-1027/-913 and pGL3P. -1083/-959 were created by cloning the human VEGF-A genomic regions from bp -1302 to -913, -622 to -463, +400/+631, -1083 to -913, -1027 to -913 and -1083 to -959 into the pGL3-Promoter vector (Promega). PGL3P.-1083/-913ΔHRE was created by mutating the consensus Hif-1 binding site (TACGTGGG to TAAAAAGGG) in pGL3P.-1083/-913 using QuikChange™ Site-Directed Mutagenesis Kit (Stratagene). EPAS1(1-481) was created by cloning a fragment encompassing amino acids 1 to 481 from human EPAS1 plus a Flag tag sequence at 3’-end into pcDNA3.1 (Invitrogen). All cloned sequences were verified by DNA sequencing.

Quantitative RT-PCR Analysis of VEGF-A mRNA Level- Total RNA was prepared using the High Pure RNA Isolation Kit (Roche) and subjected to a real-time quantitative RT-PCR analysis using the Taqman assay as described previously (27). Each sample was analyzed in triplicates. The VEGF-A mRNA levels were normalized by the 18S ribosomal RNA levels.
**ELISA Measurement of Secreted VEGF-A**- Cells were replenished with fresh culture media 24 h before supernatants were collected. VEGF-A concentrations in cell supernatants were determined using a commercial ELISA kit (R & D Systems), following the manufacturer’s instructions. Each sample was analyzed in triplicates.

**DNase I Hypersensitive Site Mapping of VEGF-A**- Cells were grown to subconfluency and scraped off culture flasks following a brief wash with 1x PBS. Cell pellets were resuspended in DNase I digestion buffer containing 0.5% IGEPAL at 1-2x10^7 cells/ml buffer. 100 µl aliquots of the resuspended cells were mixed with equal volumes of DNase I buffer containing varying concentrations of DNase I. The DNase I digestion reaction was incubated at 23°C for 5 min before being stopped with the addition of 8 µl of 0.5 M EDTA and 2 µl of 100 mg/ml RNase A. Following 5 min of RNase A treatment, genomic DNA was isolated using the DNeasy Tissue Kit (Qiagen). 10 µg of each of the DNA samples was digested with EcoRI, and resolved in a 1.2% agarose gel by electrophoresis. The DNA from the gel was then transferred onto a nylon membrane. Southern blot was carried out with a radiolabeled DNA probe that corresponds to a region from bp -2274 to -1889 of the human VEGF-A gene, relative to the transcription start site. Following hybridization and washing, the blot was exposed to a phosphorimager screen.

**Transient Transfection and Luciferase Assays**- U87MG and HEK293 cells were transfected with the LipofectAMINE Plus Reagent (Life Technologies) in OPTI-MEM I reduced serum medium according to the manufacturer’s protocol. 24 h after transfection, cells were collected and analyzed for luciferase activities using the Dual-luciferase Reporter Assay System (Promega). The luciferase activities were normalized for transfection efficiencies measured by Renilla luciferase activities.
**Western Blotting of Nuclear Extracts**- 1x10^7 U87MG or HEK293 cells were pelleted, resuspended in 400 µl of buffer A (10mM Tris pH8.0, 1.5mM MgCl₂, 10mM KCl, 1mM dithiothreitol and 1mM phenylmethylsulfonyl fluoride) and incubated on ice for 10 min. Cells were lysed by adding 25 µl of 10% IGEPAL and vortexing vigorously for 10 seconds. After centrifugation at 13,000g for 30 seconds at 4°C, the pellets were resuspended in 100 µl of ice-cold buffer C (20mM Tris pH8.0, 420mM NaCl, 1.5mM MgCl₂, 0.2mM EDTA, 25% glycerol, 1mM dithiothreitol and 1mM phenylmethylsulfonyl fluoride) and gently shaken at 4°C for 30 min. After centrifugation at 4°C for 15 min, the supernatants were saved and protein concentrations were determined using the Bradford assay (Bio-Rad). 50µg protein from each nuclear extract was analyzed via electrophoresis on a 4-20% Tris-HCl gel (Bio-Rad) followed by western blotting using anti-EPAS1 and anti-ARNT antibodies (Novus Biologicals). The Western blots were visualized by SuperSignal West Dura Extended Duration Substrate (Pierce).

**Chromatin Immunoprecipitation Assay**- Chromatin immunoprecipitation (ChIP) assays were carried out using the Acetyl-Histone H3 Immunoprecipitation (ChIP) Assay Kit (Upstate Biotechnology) according to the manufacturer’s instructions. The antisera used are as follows: 1.5-2.0 µg/ml rabbit control serum (Santa Cruz), 1.5 µg/ml anti-EPAS1 antiserum (Novus Biologicals), and 2.0 µg/ml anti-E2F1 antiserum (Santa Cruz). After ChIP, the precipitated genomic DNA was analyzed by Taqman using the following primers and probes. For amplifying a DNA region containing the HRE on the human VEGF-A promoter, sequences of the forward and reverse primers are 5’-CAGGAACAAGGGCTCTGTCT and 5’-GCACTGTGGAGTCTGGCAAA, respectively; the sequence of the probe is 5’-
CCCAGCTGCCTCCCCCTTTGG. For amplifying a site centered on bp 1050 relative to the human VEGF-A transcription start site, sequences of the forward and reverse primers are 5’-GCCTCCGAAACCATGAACCTTT and 5’-TGGTGGAGGTAGACAGCAGCAA, respectively; the sequence of the probe is 5’-TGCTGTCTTGGGTGCATTGGAGCC. For amplifying the E2F1 binding site on p14ARF promoter, sequences of the forward and reverse primers are 5’-CATGTGGCCCCCAGCACA and 5’-CTCCCCGGTAGGTAGATTTCACT, respectively; the sequence of the probe is 5’-CACGGTGTTGGGTAAA. The genomic DNA measurements were normalized by 18S genomic levels.

Construction of Recombinant Adenoviruses- Recombinant adenoviruses were constructed using the Adeno-X Expression System (Clontech). The β-galactosidase cDNA fragment and the EPAS (1-481) cDNA fragment which contains a Flag tag sequence were cloned into pShuttle vector. The mammalian expression cassettes in pShuttle were then transferred to Adeno-X viral DNA by in vitro ligation. Recombinant adenoviral plasmids were packaged by transfecting HEK293 cells, and adenoviruses were harvested from transfected HEK293 cells lysed with three consecutive freeze-thaw cycles. Recombinant adenoviruses were amplified in HEK293 cells and titered by the TCID50 assay.

Sequence Alignment- Sequence alignments were performed using the Align X module of the Vector NTI Suite 6 software package with default settings. The sequences used are from GenBank # AF095785 (human VEGF-A), # U41383 (mouse VEGF-A), # U22373 (rat VEGF-A) and unpublished data (B. Chen and E.J. Rebar).
Results

Identification of a Cell-Type Specific DNase I Accessible Region in the VEGF-A

Promoter- VEGF-A expression levels in various human cell lines were measured by quantitative RT-PCR (Taqman) and ELISA assays (Table I). A human glioblastoma cell line, U87MG, exhibited 9 to 50 fold higher VEGF-A mRNA levels than several other cell lines, including HeLa, HEK 293, HEL and K562. Correspondingly, U87MG cells also secreted much higher amounts of VEGF-A protein into culture supernatants. The high endogenous VEGF-A levels in U87MG cells are consistent with the fact that these cells are derived from a highly vascularized tumor type.

To delineate the mechanisms of VEGF-A up-regulation, we set out to identify regulatory elements and transcription factors that activate VEGF-A transcription in U87MG cells. Since DNase I accessible regions often harbor cell-type specific enhancer elements, DNase I hypersensitive site mapping was performed in the above mentioned cell lines (Figure 1). Two hypersensitive sites centered at bp -550 and +1 relative to the transcription start site of the VEGF-A promoter were clearly observed in all cell lines. A third and weaker hypersensitive site centered at bp +500 could also been seen in all cell lines, which was more apparent under longer exposure (data not shown). These three hypersensitive sites have previously been described in a variety of cell types (27). A fourth and very intense hypersensitive site centered at bp -1100 of the VEGF-A promoter, which we will refer to as ‘HS-1100’, was only observed in U87MG cells. These data suggest that the -550, +1 and +500 DNase I hypersensitive regions may contain elements that are important for the normal regulation of VEGF-A in various cell types, while HS-1100 may contain cell-type specific regulatory elements that contribute to the extremely high level of VEGF-A expression in U87MG cells. An alignment of promoter regions of VEGF-A (Figure 2A) shows that there is a broad region of enhanced sequence conservation in the VEGF-A locus in the same location as HS-1100 (gray profile), which becomes more prominent when considered in terms of regulatory element-sized sequence blocks (black profile). Although this region encompasses the HRE (-975 to -957 relative to the human VEGF-A transcription
initiation site), it clearly extends further upstream and downstream of this element and includes many additional blocks of conserved sequence (Figure 2B). These results support the notion that regulators of VEGF-A transcription may operate via binding to cis-regulatory elements within HS-1100.

**Characterization of the Cell-Type Specific Enhancer Activity and EPAS1 Binding of HS-1100** - To further characterize HS-1100, a 400bp DNA fragment corresponding to bp -1302 to -913 was cloned upstream of the SV40 promoter in the luciferase expression vector pGL3P. The resulting plasmid, pGL3P.-1100 was tested for enhancer activity by transient transfection in U87MG and HEK293 cells. Figure 3A shows that pGL3P.-1100 exhibited over 10-fold activation of the SV40 promoter in U87MG cells but only 2-fold in HEK293 cells. For comparison, pGL3P.-550 and pGL3P.+500, which contain DNA fragments from the -550 and +500 DNase I accessible regions, respectively, exhibited no more than 3-fold activation of the SV40 promoter in both cell lines. Therefore, HS-1100 is a U87MG cell-specific enhancer for the VEGF-A gene.

Nested deletion analysis was performed to define the boundaries of HS-1100 (Figure 3B). A 5’deletion up to bp -1083 (pGL3P.-1083/-913) had no effect on the enhancer activity in U87MG cells, whereas it almost completely abolished the enhancer activity in HEK293 cells. A further 5’ deletion to bp -1027 (pGL3P.-1027/-913) reduced the enhancer activity in U87MG cells by 37%. A further 3’ deletion to bp -959 (pGL3P.-1083/-959), which abolished the HAS, reduced the enhancer activity in U87MG cells by 80%. Both pGL3P.-1027/-913 and pGL3P.-1083/-959 gave similar reductions of HS-1100 enhancer activity as pGL3P.-1083/-913 in HEK293 cells. Therefore, the minimal-sized fragment required for full function of HS-1100 as determined by reporter assays runs from bp -1083 to -913 in U87MG cells, and from bp -1302 to -913 in HEK293 cells. These results are consistent with the notion that HS-1100 functions in
a cell-type specific manner. In addition, these results suggest that multiple elements are required for full activity of HS-1100.

Since HS-1100 harbors the HRE, we tested whether the HRE contributes to the function of HS-1100. A 3-bp substitution in the Hif-1 binding site that has previously been shown to abolish the hypoxia response (14) caused ~ 70% reduction of HS-1100 enhancer activity in U87MG cells (Figure 3B), suggesting that the HRE accounts for a significant part of the enhancer activity of HS-1100 in these cells. Immunoblotting with nuclear extracts showed that EPAS1 and ARNT were expressed at comparable levels in U87MG and HEK293 cells (Figure 4), whereas Hif-1α was hardly detectable in both cell lines (data not shown). This result indicates that the different VEGF-A levels observed in U87MG and HEK293 cells cannot be explained by differential expression levels of Hifs. Next, we tested whether there is a difference in the binding of EPAS1 to the HRE in these two cell lines. Although EPAS1 can bind to the HRE in \textit{in vitro} gel mobility shift assays (28), the binding of EPAS1 to the endogenous chromosomal locus \textit{in vivo} has not to our knowledge been demonstrated. To determine this, chromatin immunoprecipitation (ChIP) assays were performed in U87MG and HEK293 cells. In U87MG cells (Figure 5A), immunoprecipitation with an anti-EPAS1 antiserum enriched a DNA region containing the HRE by 5.7-fold compared with a preimmune serum. As an internal control for specificity, a site centered at 1050-bp downstream of the VEGF-A transcription start site was not significantly enriched. In contrast, immunoprecipitation with the anti-EPAS1 antiserum did not significantly enrich the HRE site in HEK293 cells (Figure 5B). As a positive control in HEK293 cells, an anti-E2F1 antiserum could enrich an E2F1 binding site within a DNase I accessible region on p14 ARF promoter by 3.7-fold. These results show that EPAS1
can bind efficiently to the HRE in U87MG cells, whereas it cannot do so in HEK293 cells, most likely due to the less accessible nature of the HRE in this cell type.

**Inhibition of Endogenous VEGF-A Levels by a Dominant-Negative Mutant of EPAS1 in U87MG Cells** - To confirm that the HRE contributes to the high endogenous levels of VEGF-A in U87MG cells, a dominant-negative mutant of EPAS1 lacking the C-terminal activation domain, EPAS1(1-481), was constructed. This mutant EPAS1 retains the ability to heterodimerize with ARNT and to bind to the HRE, but it cannot transactivate the VEGF-A promoter (29). Previous studies have shown this mutant EPAS1 inhibits desferrioxamine induction of endogenous VEGF-A mRNA levels in human umbilical vein endothelial cells (29). When EPAS1(1-481) was co-transfected into U87MG cells, it was able to repress the enhancer activity of HS-1100 in a dose-dependent and HRE-dependent manner (Figure 6). EPAS1(1-481) was subsequently cloned into an adenoviral vector that was then used to infect U87MG cells. The EPAS1(1-481) adenovirus infected U87MG cells, which expressed a high level of the 53kD truncated EPAS1 protein (Figure 7A), showed ~ 50% reduction of the endogenous VEGF-A mRNA and protein levels compared with U87MG cells infected with a β-galactosidase control virus (Figure 7B). These data suggest that binding of the HRE by EPAS1 (or other Hifs) contributes to constitutive activation of VEGF-A in U87MG cells.

**Correlation of Tumorigenicity, VEGF-A Expression and the Accessibility of HS-1100 in Glioblastoma Cell Lines** - To determine whether the chromatin accessibility of HS-1100 is a general feature of glioblastomas, several additional glioblastoma cell lines with various degrees of tumorigenicity were tested for both their endogenous VEGF-A levels (Table II) and DNase I accessibility of the VEGF-A promoter (Figure 8). Similar to U87MG (8), U118MG, which is
tumorigenic in nude mice (30), expressed high levels of VEGF-A and exhibited a DNase I hypersensitive site over HS-1100. In contrast, A172 and T98G, which are not tumorigenic in nude mice (31,32), expressed much lower levels of VEGF-A and lacked a DNase I hypersensitive site over HS-1100. Therefore, the accessibility of HS-1100 correlates with tumorigenicity and VEGF-A expression levels of glioblastoma cell lines. These results suggest that the accessibility of HS-1100 could be one of the mechanisms for constitutive VEGF-A up-regulation in glioblastomas, which subsequently leads to malignant progression of these tumors.
Discussion

In this study we have identified and characterized a new enhancer region, HS-1100, that appears critical for the up-regulation of VEGF-A transcription in tumorigenic glioblastoma cell lines. HS-1100 is found in the tumorigenic U87MG and U118MG glioblastoma cell lines but not in cell lines expressing lower levels of VEGF-A. Correspondingly, a DNA fragment from HS-1100 exhibits a much higher level of enhancer activity in U87MG cells than in HEK293 cells, suggesting that HS-1100 contains cell-type specific cis-acting regulatory elements for VEGF-A transcription. Two mechanisms could contribute to the cell-type specific VEGF-A transcription in U87MG cells versus HEK293 cells. The first is that U87MG cells may possess unique transcription factors that bind to DNA elements within HS-1100. These factors may be absent or present at a lower level in HEK293 cells, which could explain the low enhancer activity of HS-1100 in HEK293 detected by reporter assays. Preliminary in vitro footprinting and gel mobility shift analyses indeed suggest that there is a differential binding of transcription factors to HS-1100 in nuclear extracts prepared from these two cell lines (data not shown). Secondly, for factors that are present at equal levels in both cell types, such as EPAS1, accessibility of HS-1100 could influence the efficiency of interactions of these factors with their cognate DNA binding sites in vivo. The combination of constitutive and cell-type specific transcriptional regulation of VEGF-A from HS-1100 locus could be responsible for the coordinated temporal and spatial regulation of VEGF-A transcription under both physiological and pathological conditions.

The minimally functional enhancer unit of HS-1100 defined by reporter assays spans a
region from bp -1083 to -913, relative to the transcription start site. Nested deletion analysis of this region suggests that multiple elements are required for the full enhancer activity. This is consistent with the observation that several conserved sequence blocks in this region contain consensus binding sites for known transcription factors. For instance, in addition to the HRE, there is an AP1 site at bp -937 to -931 (TGACTAA) that may also be involved in regulating HS-1100 enhancer activity. Transcription factors binding to these sites may activate transcription directly or may act primarily to remodel the chromatin surrounding HS-1100.

However, since transiently transfected templates do not acquire the repressed chromatin structure of endogenous genes (33), the deletion analysis by reporter assays may not identify those elements and associated trans-acting factors that are necessary to generate the DNase I hypersensitive site surrounding HS-1100. In this respect, sequences upstream of bp -1083 that also contain multiple conserved sequence blocks, although dispensable for enhancer activity in reporter assays, may be required for DNase I hypersensitive site formation surrounding HS-1100. Supporting this notion, preliminary DNase I footprinting and chromatin immunoprecipitation analyses show differential occupancy of transcription factor binding sites immediately upstream of bp -1083 in U87MG versus HEK293 cells (data not shown). Identification and characterization of these factors could shed light on how accessibility to chromosomal HS-1100 is regulated in U87MG cells, which would provide insight into the molecular mechanisms of constitutive VEGF-A up-regulation that leads to malignant progression of glioblastomas.

Many studies have demonstrated the pivotal role the HRE plays in VEGF-A activation by hypoxia. Our studies show that the HRE also plays an important role in constitutively up-
regulating VEGF-A transcription in cancer cell lines under normoxic conditions. Hypoxia increases EPAS1 and Hif1α protein levels (34) by preventing their degradation through an ubiquitination pathway. However, hypoxia does not induce the formation of a DNase I hypersensitive site over HS-1100 in HEK293 cells and only increases the VEGF-A protein level in HEK293 cells by 6-fold (data not shown). Therefore, the mechanism of VEGF-A up-regulation in U87MG cells appears to differ from the HRE-mediated activation of VEGF-A transcription by hypoxia. Our studies did not address the in vivo binding of Hif1α to the HRE, but it is conceivable that Hif1α would act similarly as EPAS1. The relative levels of endogenous EPAS1 and Hif1α in U87MG cells are unknown. We have observed that overexpression of EPAS1 is more potent than overexpression of Hif1α in activating reporter genes that contain the HRE in U87MG cells (data not shown), which is consistent with results from other studies (34,35). In addition, EPAS1 but not Hif1α is enriched in highly vascularized tissues, and significant up-regulation of VEGF-A is highly correlated with elevated expression of EPAS1 but not Hif1α in renal cell carcinomas and hemangioblastomas (35,36). These results suggest that EPAS1, rather than Hif1α, is the major player in activating VEGF-A transcription through the HRE in U87MG cells.

The identification of a cell-type specific enhancer whose chromosomal accessibility is correlated with high levels of VEGF-A expression in tumorigenic glioblastoma cell lines could have implications for the development of anti-angiogenic cancer therapies. Sequences within the accessible HS-1100 could serve as targets for transcription factor based therapeutics, such as engineered zinc finger DNA binding proteins (27). Activation of the HRE-mediated pathway has been demonstrated in many cancers and has been targeted for the development of cancer
therapeutics (37). Our present study demonstrates that the HRE is also required for the high level of VEGF-A expression in tumorigenic glioblastoma cell lines. Blockade of the HRE-mediated pathway by a polypeptide that disrupts the interaction between Hif1α and p300/CBP has been suggested as a new approach to cancer therapy (38). The potential drawback of this approach is that it is likely to interfere with other transcriptional systems requiring p300/CBP as well as the hypoxia pathway in normal tissues. In contrast, inhibition of the HRE-mediated pathway by DNA binding proteins that competitively bind to the HRE but lack any activation function would be selectively effective in tumor cells that have an accessible chromosomal HS-1100. Molecules that regulate the remodeling of chromatin structures at HS-1100 could also be interesting therapeutic agents with a selectivity for highly malignant glioblastomas. In summary, our studies on understanding how multiple elements and factors coordinate to regulate VEGF-A transcription could provide useful therapeutic strategies for treating cancer.

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Footnotes:

1. The abbreviations used are: VEGF, vascular endothelial growth factor; HRE, hypoxia response element; HAS, hypoxia inducible factor-1 ancillary sequence; Hifs, hypoxia inducible factor; EPAS1, endothelial PAS domain protein 1; ARNT, aryl hydrocarbon receptor nuclear translocator; RT-PCR, reverse transcription-polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; bps, base pairs; ZFP, zinc finger protein.

Figure Legends

Figure 1. Chromatin accessibility of VEGF-A promoters in various human cell lines. DNase I hypersensitive site mapping was performed as described in “Experimental Procedures”. DNase I concentrations used were as follows: 0, 3.75, 7.5, 15 and 30 units/ml. Arrows indicate the observed hypersensitive site bands on the gels, and their relative positions on the VEGF-A promoter are marked with vertical bars. Bent arrows denote the transcription start site, and shaded rectangles mark positions of the probe on the VEGF-A promoter. The cell lines tested were as follows: U87MG (human glioblastoma), HeLa (human cervical carcinoma), HEK293 (human embryonic kidney), HEL (human erythroleukemia), K562 (human chronic myelogenous leukemia).

Figure 2. Sequence alignment of VEGF-A promoters from human, mouse and rat. A. Sequence conservation of the VEGF-A promoter among human, mouse and rat. Each point in the gray profile indicates the fraction of human bases conserved in mouse and rat for a 50 bp sequence window centered on that point. The black profile is identical except that it indicates the fractional conservation of 5-bp sequence blocks. B. Alignment of the human VEGF-A HS-1100 sequence with corresponding regions of the rat and mouse VEGF-A promoters. Conserved bases are highlighted in gray. The Hif-1 binding site and HAS are boxed. Nucleotides -1083 and -913 are marked. Numbers at left indicate the location of the leftmost base of each line relative to the reported transcription start site of VEGF-A in each species.
Figure 3. **Enhancer activity of human VEGF-A HS-1100.** U87MG cells (gray bars) and HEK293 cells (black bars) were co-transfected with 500 ng of the indicated luciferase reporter constructs along with 10 ng of Renilla luciferase control vector (for normalization of transfection efficiency) as described in “Experimental Procedures”. The mean luciferase activity for the pGL3P vector was arbitrarily defined as 1.0. Each error bar represents the standard deviation of three or more independent transfections. The schematic illustration of the luciferase reporter constructs used in B is shown with the luciferase cDNA (luc), SV40 promoter (SV40P), the Hif-1 binding site and HAS marked. X indicates a 3-bp substitution within the Hif-1 binding site.

Figure 4. **EPAS1 and ARNT protein expression in U87MG and HEK293 cells.** 50 µg protein from nuclear extracts of U87MG and HEK293 cells were separated on a 4-20% Tris-HCl gel and immunoblotted with the anti-EPAS1 antiserum (upper panel) or the anti-ARNT monoclonal antibody (lower panel) as described in Experimental Procedures.

Figure 5. **Chromatin immunoprecipitation of the human VEGF-A promoter.** A. Chromatin was immunoprecipitated from U87MG cells with the indicated antisera. The enriched genomic DNA was measured by Taqman analysis amplifying a site either containing the HRE (gray bars) or centered at bp +1050 (black bars) on the human VEGF-A promoter as described under “Experimental Procedures”. B. Chromatin was immunoprecipitated from HEK293 cells with the indicated antisera, and the enriched genomic DNA was measured by Taqman analysis amplifying a site either containing the HRE on the human VEGF-A promoter (the middle bar) or containing an E2F1 binding site on p14ARF promoter (the right bar) as described under “Experimental
Procedures”. For both A and B, the mean values from preimmune serum immunoprecipitated samples were arbitrarily defined as 1.0. Each error bar represents the standard deviation of two independent Taqman measurements. The experiment was repeated three times and representative results are shown.

Figure 6. Inhibition of HS-1100 enhancer activity by a dominant negative EPAS1. U87MG cells were co-transfected with 500 ng of the indicated luciferase reporter constructs, 10 ng of Renilla luciferase control vector (for normalization of transfection efficiency) and the indicated amounts of pcDNA3 or EPAS1(1-481) expression plasmid as described in “Experimental Procedures”. The mean luciferase activity for pGL3P.-1083/-913 in the absence of EPAS1(1-481) expression plasmid was arbitrarily defined as 1.0. Each error bar represents the standard deviation of three independent transfections.

Figure 7. Inhibition of endogenous VEGF-A levels by a dominant negative EPAS1 in U87MG cells. U87MG cells were infected by AdEPAS1(1-481) or, as a control, Adβgal at a multiplicity of infection of 50 for 2 h at 37°C. At 48 h post-infection, a portion of infected cells was lysed directly in Laemmli sample buffer (Bio-Rad) and subjected to Western blotting with an anti-Flag monoclonal antibody (A). The rest of the infected cells were replenished with fresh culture media and incubated for another 24 h before being collected for analyses with Taqman (gray bars) and ELISA (black bars) assays (B). The mean VEGF-A mRNA and protein levels in Adβgal infected cells were arbitrarily defined as 1.0. Each error bar represents the standard deviation of three independent infections.
Figure 8. **Chromatin accessibility of VEGF-A promoters in various human glioblastoma cell lines.** DNase I hypersensitive site mapping was performed as described in “Experimental Procedures”. DNase I concentrations used were as follows: 0, 3.75, 7.5, 15 and 30 units/ml. Arrows indicate the observed hypersensitive site bands on the gels, and their relative positions on the VEGF-A promoter are marked with vertical bars. Bent arrows denote the transcription start site, and shaded rectangles mark positions of the probe on the VEGF-A promoter. The glioblastoma cell lines tested are indicated under each panel.
Table I. VEGF-A expression levels in various human cell lines

<table>
<thead>
<tr>
<th>cell line</th>
<th>VEGF-A mRNA[^a]</th>
<th>VEGF-A Protein (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>U87MG</td>
<td>0.713 ± 0.027</td>
<td>1166 ± 67.30</td>
</tr>
<tr>
<td>HeLa</td>
<td>0.059 ± 0.010</td>
<td>41.19 ± 2.84</td>
</tr>
<tr>
<td>HEK293</td>
<td>0.081 ± 0.010</td>
<td>5.20 ± 0.81</td>
</tr>
<tr>
<td>HEL</td>
<td>0.014 ± 0.002</td>
<td>2.23 ± 0.15</td>
</tr>
<tr>
<td>K562</td>
<td>0.062 ± 0.002</td>
<td>28.91 ± 3.48</td>
</tr>
</tbody>
</table>

[^a]: VEGF-A mRNA levels were measured by Taqman assays and normalized by 18S ribosomal RNA levels.
[^b]: Concentrations of VEGF-A in cell supernatants after 24 h incubation were measured by ELISA assays. The values were normalized by cell numbers counted with a hemacytometer.
Table II. VEGF-A expression levels in various human glioblastoma cell lines

<table>
<thead>
<tr>
<th>cells</th>
<th>tumorigenicity</th>
<th>VEGF-A mRNA *</th>
<th>VEGF-A Protein (pg/ml) *</th>
</tr>
</thead>
<tbody>
<tr>
<td>U87MG</td>
<td>+ a</td>
<td>0.733 ± 0.038</td>
<td>1681 ± 309.0</td>
</tr>
<tr>
<td>U118MG</td>
<td>+ b</td>
<td>0.341 ± 0.060</td>
<td>1185 ± 147.4</td>
</tr>
<tr>
<td>A172</td>
<td>- c</td>
<td>0.044 ± 0.009</td>
<td>&lt;3.40</td>
</tr>
<tr>
<td>T98G</td>
<td>- d</td>
<td>0.046 ± 0.003</td>
<td>23.38 ± 11.23</td>
</tr>
</tbody>
</table>

e. VEGF-A mRNA levels were measured by Taqman assays and normalized by 18S ribosomal RNA levels.
f. Concentrations of VEGF-A in cell supernatants after 24 h incubation were measured by ELISA assays.

The values were normalized by cell numbers counted with a hemacytometer.
Figure 2

A

B

<table>
<thead>
<tr>
<th>Species</th>
<th>Region 1</th>
<th>Region 2</th>
<th>Region 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>CAGG</td>
<td>TACG</td>
<td>TGGG</td>
</tr>
<tr>
<td>Mouse</td>
<td>CAGG</td>
<td>TACG</td>
<td>TGGG</td>
</tr>
<tr>
<td>Rat</td>
<td>CAGG</td>
<td>TACG</td>
<td>TGGG</td>
</tr>
</tbody>
</table>

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Figure 6

The figure shows a bar graph depicting the relative luciferase activity. The x-axis represents different concentrations of pcDNA3 and EPAS1(1-481), and the y-axis represents the relative luciferase activity. The graph includes two conditions: pGL3P.-1083/-913 and pGL3P.-1083/-913._ΔHRE. The bars indicate the activity at various concentrations: 500ng, 400ng, 250ng, and 100ng for EPAS1(1-481).
Activation of vascular endothelial growth factor A transcription in tumorigenic glioblastoma cell lines by an enhancer with cell-type specific DNAse I accessibility

Yuxin Liang, Xiaoyong Li, Edward J. Rebar, Peixiang Li, Yuanyue Zhou, Bingliang Chen, Alan P. Wolfe and Casey C. Case

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