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Hypoxia Responsive Vectors Targeting Astrocytes in Glioma

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1. Introduction

Gliomas are the most common brain tumor in the central nervous system (CNS). The majority of malignant gliomas arise from neoplastic transformation of resident astrocytes. Gliomas are very aggressive tumors since they are characterized by widespread invasion of brain tissue. The exact pathogenesis and underlying mechanisms for glioma cell infiltration are currently unclear. Cell-cell interaction and tissue microenvironment play an important role in tumor progression leading to modification and infiltration of surrounding tissue. The hypoxic microenvironment contributes to abnormal neovascularization of the glioma.

Uncontrolled cellular proliferation, abnormal angiogenesis and invasion of surrounding tissue make these tumors difficult to treat. Poor prognosis and ineffective treatments for glioma point to the necessity of developing new therapeutic strategies. The use of gene therapy to deliver a therapeutic gene may successfully overcome the failure of conventional therapies. Although non-cell specific and unregulated promoters have been used to target gliomas, it is possible that unregulated vectors elicit damage in cells that do not require the therapeutic protein. Thus it may be of value to design a regulated, tissue-specific vector to express a therapeutic protein in a specific cell type and to regulate it according to the local tissue environment.

The hypoxic microenvironment is known to play a major role in several conditions including cerebral ischemia, retinal angiogenesis, gliomas, and other cancers. Hypoxia inducible factor 1 alpha (HIF-1 α), the most common transcription factor induced during hypoxia, binds to a hypoxia regulated enhancer (HRE) region of oxygen sensitive genes leading to their transcription. By taking advantage of hypoxia for regulating foreign gene expression, a hypoxia regulated vector could be exploited to modify the cells that reside within the target tissue. Use of a cell-specific promoter would be an added advantage to restrict gene expression only in certain cells.

Our recently developed vector platform works in a manner such that in an hypoxic environment, HIF-1 α will bind to the HRE region and express the foreign protein in a cell-

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specific manner. These vectors were designed to prevent neovascularization in the eye. Using a hypoxia regulated, retinal pigment epithelial cell (RPE)-specific promoter, a vector was developed which leads to expression of a therapeutic gene only in RPE cells. By driving expression of the human endostatin gene, this vector prevented neovascularization in the laser induced mouse model of choroidal neovascularization. Another gene therapy vector was constructed using the human GFAP promoter along with several hypoxia regulated enhancer and silencer elements. Both of these vectors have been tested in different cell lines to show specificity and hypoxic-inducibility. The regulated promoter is active only during the conditions of hypoxia, and shows significantly enhanced induction of reporter genes, especially in primary rat astrocytes in culture.

Our hypoxia-regulated, astrocyte-specific vector has the capacity to restrict its expression to astrocytes and can be expressed only in the hypoxic microenvironment thus remaining inactive both under hypoxic and normoxic conditions in other cells. This vector has a unique potential for treating pathology in which astrocytes and an hypoxic environment play a major role in disease progression. Our goal is to use our regulated astrocyte-specific promoter in vectors designed to target gliomas in which both astrocytes and a hypoxic microenvironment are involved. Potential therapeutic proteins can be added to the downstream region of the promoter to prevent abnormal angiogenesis, induce apoptosis in tumor cells and/or prevent invasion and infiltration of the glioma. The following points will be discussed in section 2 through 6.

- a. Importance of gene therapy to target gliomas utilizing the hypoxic micro-environment.
- b. Design and testing of hypoxia-regulated astrocyte- specific promoter.
- c. Vector choice and route of delivery.
- d. Therapeutic window for treatment of gliomas.
- e. Future directions and limitations of proposed treatment.

2. Importance of gene therapy to target gliomas utilizing the hypoxic micro-environment

Gliomas are tumors of the CNS which arise from glial cells. They are divided into several types depending on clinical features and histopathology. According to the WHO classification, gliomas can be classified into four grades depending upon their malignancies: Grade 1 covers benign tumors (e.g. pilocytic astrocytoma), and group II to IV are malignant neoplasias which differ in aggressiveness. The most aggressive glioblastoma belongs to group IV. Histopathologically, gliomas are divided into astrocytomas, oligodendrocytomas and glioblastomas depending upon the type of macroglial cell. The most common form of gliomas in humans is the astrocytoma, and the most aggressive form of astrocytomas is glioblastoma multiforme (GBM.)

Gliomas are characterized by their active propagation through nervous tissue. There is a limitation of space for glioma growth in the CNS due to existence of firm boundaries (skull for the brain and vertebra for the spinal cord). In order to grow, gliomas must clear space by actively eliminating the surrounding healthy cells and actively propagate neoplastic cells in the brain parenchyma. Malignant gliomas produce space for expansion by secreting increased amounts of excitatory neurotransmitters which kill neighboring neurons. First, they express metalloproteinases which assist in breaking down the extracellular matrix and

producing migrating channels. Second, glioma cells are able to undergo substantial shrinkage, which helps them attain an elongated shape and thus penetrate into narrow interstitial compartments.

Poor prognosis, short survival time and poor response to chemotherapeutic drug intervention make glioma a devastating disease. The reason for the poor efficacy of these agents includes the selectivity of the blood brain barrier, the heterogeneity and low immunogenicity of gliomas and appropriate selection of chemotherapy-resistant clones. New therapeutic strategies are therefore needed to treat this devastating tumor. Since multiple genes determine the severity of glioma progression and invasion, theoretically the design of a therapeutic strategy could be targeted utilizing the tumor microenvironment. A major set of therapeutic targets could involve changes in the molecular pathways associated with the hypoxic environment.

2.1 Molecular basis of glioma

Molecular and genetic approaches have provided dramatic insights into glioma biology. As part of the intensive effort to determine the process of glioma formation, several molecular defects have been implicated in gliomas. These genes affect encoded proteins involved in several critical biologic processes, including signal transduction, cell growth, cell cycle control or proliferation, apoptosis and differentiation.

An hypoxic microenvironment is a frequent characteristic of GBM since these tumors exhibit abnormal neovascularization, irregular blood flow, and a high rate of oxygen consumption by the rapidly proliferating malignant cells. Regions of low oxygen concentration are a characteristic feature of growing tumors and are frequently found around necrotic areas. Hypoxic microenvironments are powerful stimuli for the expression of genes involved in tumor cell proliferation, angiogenesis and immuno-suppression. Tumor hypoxia activates a complex set of cellular responses which are differentially regulated by two members of hypoxia-inducible transcription factors family; hypoxia inducible factors 1 and 2 (HIF-1 and HIF-2). It has been shown that HIFs control tumor stem cell phenotype and the acquisition or maintenance of stem cell properties (Seidel et al., 2010).

2.2 Significance of hypoxia in glioma

Histological studies suggest that gliomas are abnormally vascularized due to highly proliferating tumor cells which exhibit slow or inefficient blood flow. Evans et al have shown that the physiological oxygen concentration varies between 2.5% to 12.5% in healthy brain tissue. The majority of GBMs examined showed mild to moderate/severe hypoxia with oxygen concentration ranging between 0.5% to 2.5% to (pO₂ = 20-40mm Hg) for mild hypoxia and 0.5%-0.1 % (pO₂ 0.75mm to 4 mm Hg) for moderate/severe hypoxia (Evans et al., 2004a, Evans et al., 2004b, Evans et al., 2008). Although some researchers do not include moderate hypoxia as major factor in the pathogenesis of glioma, an oxygen tension of 0.5% to 2.5% would likely be appropriate to model the hypoxic environment since HIF-1 elevation occurs in GBM patients (Kaynar et al., 2008).

There are several cellular responses to hypoxia and all are controlled by the expression of different transcription factors which are called hypoxia inducible factors (HIF-1-3 α). These

transcription factors produce dimerization with HIF-1 β which is expressed constitutively followed by translocation to the nucleus. Under normal conditions, HIF-1 α is expressed ubiquitously at low levels in all organs. The HIF-1 α subunit has an oxygen-dependent degradation domain which leads to rapid ubiquitination and degradation by the proteasome (**Figure 1**). During hypoxia, the hydroxylation of HIF-1 α is inhibited, leading to accumulation of HIF-1 α protein. The accumulated HIF-1 binds to hypoxia responsive regions (HREs) of several genes inducing their transcription. Many of these genes play a critical role in important aspects of cancer biology: angiogenesis, cell survival, chemotherapy, radiation-resistance, genomic instability, invasion and metastasis, and glucose metabolism (Bar et al., 2010).

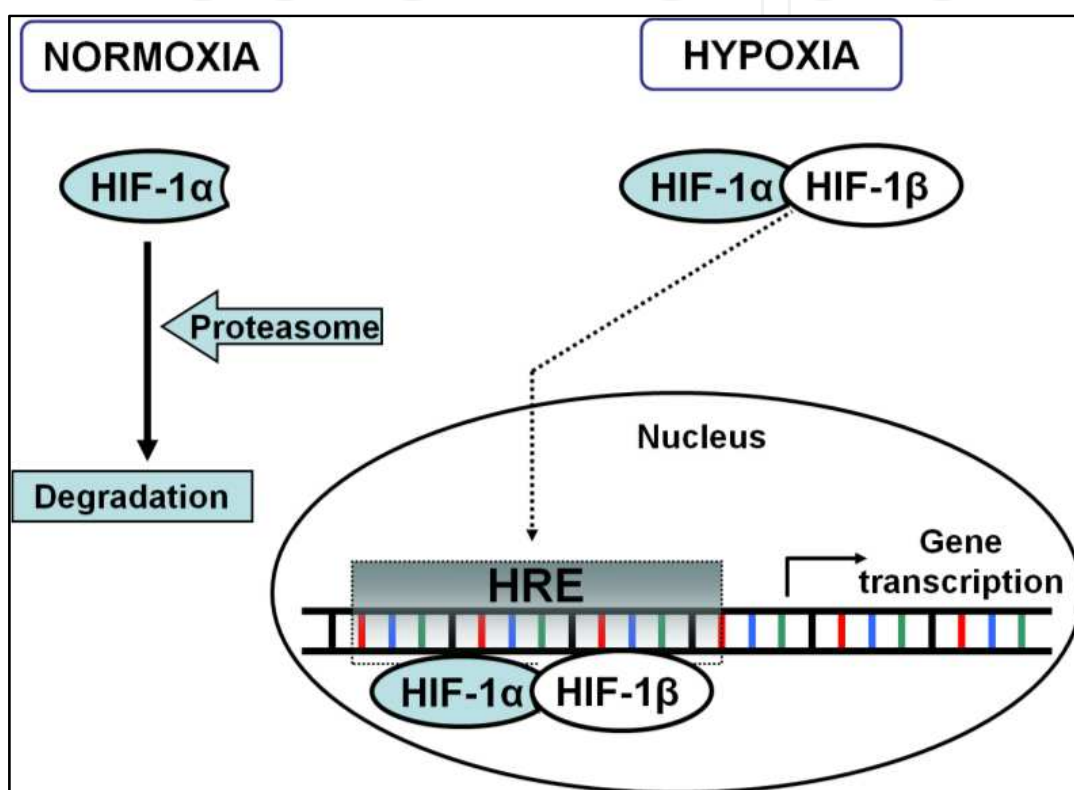


Fig. 1. Hypoxia induces the expression of hypoxia inducible factors (HIF-1) which are composed of two subunits HIF-1 α and HIF-1 β . During normoxia HIF-1 α is degraded by the proteasomal ubiquitination. In absence of oxygen HIF-1 α is stabilized by binding with HIF-1 β to form HIF-1. The HIF-1 translocates into the nucleus and binds to sequence of hypoxia responsive element (HRE) of several genes to induce the gene transcription.

2.3 Hypoxia promotes angiogenesis, glioma cell migration and immunosuppression

Hypoxia induced HIFs act as a proangiogenic switch to drive angiogenesis in gliomas. HIF is expressed inside tumor cells and astrocytes in response to changes in oxygen availability of the surrounding tissue. HIF-1 stimulates the expression of the potent angiogenic factor, vascular endothelial growth factor (VEGF). VEGF belongs to the family of growth factors which include VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and Placental growth factor. VEGF-A also exists with different splice variants (VEGF 121, 145, 165, 189 and 206). Different cell surface receptors for VEGF are expressed in endothelial cells. VEGF is

regarded to be the major factor for development of new vessels both in physiological and pathological angiogenesis. VEGFs not only stimulate the proliferation of endothelial cells, but they are also responsible for migration, vascular permeability and invasiveness during the angiogenic process. Production of high levels of VEGF is reported in cystic fluid and also around necrotic tissue in GBM patients (Brat and Van Meir, 2004).

Other than VEGF, HIF-1 also stimulates the expression of other angiogenic growth factors which include placental growth factor, basic fibroblast growth factor (bFGF), early growth factor, platelet growth factor Beta (PDGF- β), angiopoietin-1 (Ang1) and-2 (Ang-2). Along with the receptors for vascular proliferation, the endothelial cells express several receptor tyrosine kinases like Tie-1 and Tie-2 which regulate vessel remodeling, maturation and endothelial survival (Nawroth et al., 1993). Ang-1 facilitates the stabilization of new blood vessels and prevents leakiness, whereas in response to availability of VEGF, Ang-2 causes vascular remodeling and growth of new blood vessels. In the absence of Ang-2, regression of vessels occurs (Holash et al., 1999). The levels of angiopoietins and Tie-2 are greatly elevated in GBM samples indicating profuse tumor vascularity and invasiveness (Reiss et al., 2005).

Another potent growth factor, TGF- β and its receptor are highly expressed in gliomal tissues. These growth factors are activated by several proteases and integrins in the hypoxic microenvironment. TGF-beta regulates angiogenesis by activating several growth factors and their receptors including bFGF, PDGF, PDGFR, and EGFR. TGF-B affects angiogenesis by stimulating tumor cells to secrete VEGF and integrins which facilitates the motility of endothelial cells within the glioblastoma tumor.

Glioma cell migration and invasion depends on HIF-1 induced extracellular matrix proteases. Matrix metalloproteases (MMPs) play a major role in extracellular matrix (ECM) degradation and facilitate proliferation and migration of endothelial cells to produce new blood vessels. MMPs also modulate several growth factors and cytokines which affect the endothelial and tumor cell migration ultimately inducing angiogenesis. Lolmede et.al., 2003 reported that several MMPs, including MMP-1, MMP-1, MMP-7 and MMP-9, are activated by HIF-1 dependent pathways (Lolmede et al., 2003). HIF-1 activates TGF- β 2, which further regulates the activity of MMPs resulting in suppression of tissue inhibitor of metalloproteases (TIMPs) (Wick et al., 2001). The level of MMP-1 expression is upregulated in several cancers and activates the expression of MMP2 in gliomal cells. Tumor prognosis and vascularity are directly related to the expression of MMPs in gliomas.

Tumor hypoxia can activate the pSTAT3 pathway which triggers the downstream expression of HIF-1 α . In gliomas, several immunosuppressive cytokines, including TGF- β , soluble colony stimulating factor-1 (sCSF-1), chemokine ligand 2 (CCL2) and galectin expression, are induced by HIF during hypoxia. Macrophages (microglia) are the dominant infiltrating cells in gliomas. These macrophages become tumor-associated macrophages (TAM) when exposed to the hypoxic tumor microenvironment. The TAMs contribute towards tumor angiogenesis and invasion by activating the STAT3 pathway ultimately resulting in immuno-suppression and tumor supportive phenotypes. HIF -1 promotes expansion of CD133 positive glioma cancer stem cells (gCSCs) which play a role in immuno-suppression. Up-regulation of expression of macrophage migration inhibitory factor genes (MIFs) in glial cells is associated with glioma and promotes tumor angiogenesis and immunosuppression (Bacher et al., 2003).

2.4 Hypoxia as the therapeutic target

Since hypoxia modulates the glioma progression and metastasis, it can be targeted to minimize hypoxic effects on tumor cells. Several HIF-1 inhibiting agents have been used to inhibit the growth and spread of tumor cells (Jensen, 2009). Although activation of HIF-1 induces tumorigenesis, it is also necessary for normal cellular function including physiological angiogenesis, growth and survival. Therefore it is not wise to inhibit the activity of HIF-1 completely using anti-HIF agents. Several strategies have been developed to express therapeutic proteins under hypoxic conditions under the control of an HRE containing promoter (Ruan and Deen, 2001).

HIF-1 binds to the region of HRE elements and activates gene expression. An expression vector using hypoxia inducible cytosine deaminase has been used for cancer in a pro-drug activation strategy (Wang et al., 2005). During hypoxia, cytosine deaminase is expressed and converts a non-toxic 5-fluorocytosine to the highly toxic chemotherapeutic agent 5-fluoruracil. Several researchers are using this approach to activate enzymes required for apoptosis or tissue protection by the process of pro-drug activation (Binley et al., 1999, Ruan et al., 1999, Shibata et al., 2000, Cao et al., 2001). Along with hypoxia regulated expression, tissue-specific expression may increase the possibilities to turn on genes in a regulated and tissue-specific manner. McKie EA (1998) used a GFAP promoter to target astrocytes for expression of transgenes as a potential treatment strategy for glioblastoma (McKie et al., 1998). Hypoxia could be used to switch on a foreign gene in glioma cells or in astrocytes. We have designed an hypoxia-regulated, astrocyte-specific vector which will lead to gene expression only in hypoxic astrocytes while restricting expression in normoxia and other cell types.

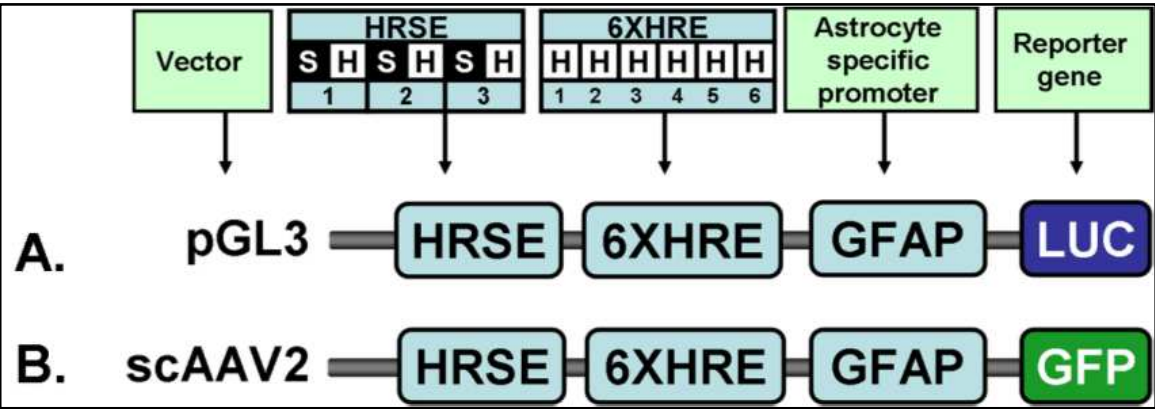


Fig. 2. Construction of hypoxia-regulated astrocyte –specific vector. Three copies of neuron restrictive silencer sequence (S) and three copies of hypoxia responsive element (HRE) sequence were alternated in a combination to make hypoxia responsive silencer elements (HRSE). This sequence provides conditional silencing in normoxia and induces gene expression in hypoxia. Similarly 6 copies of HRE sequence were combined together to make 6XHRE for enhanced hypoxic induction. A) Sequences of HRSE, 6XHRE and human glial fibrillary acidic protein (GFAP) promoter are incorporated into pGL3-basic vector to drive luciferase (LUC) gene expression. B) Sequences of HRSE, 6XHRE and human GFAP promoter are incorporated into self complementary Adeno Associated Virus plasmid vector (scAAV) to make scAAV2 virus which drives the green fluorescent protein (GFP) gene.

3. Design and testing of hypoxia-regulated, astrocyte- specific promoter

Astrocyte specific expression in transgenic mice was shown by Lee *et.al.* in 2008 (Lee et al., 2008). Different conserved domains of the GFAP promoter are used to demonstrate astrocytic specific reporter gene expression. A promoter domain designated Gfabcl1d (GFAP) consisting of selected subdomains of the GFAP promoter was shown to exhibit region and astrocyte specific transgene expression in the brain of a transgenic rat. Our lab developed an hypoxia regulated vector (Dougherty et al., 2008) in which a hypoxia responsive enhancer was used together with the retinal pigment epithelial cell (RPE65) (Dougherty et al.). This promoter cassette drives cell-specific and hypoxia responsive transgene expression in cell culture. It was used successfully to drive transgene expression of an anti-angiogenic agent to prevent the neovascularization in the mouse eye following laser treatment (personal communication). These positive results lead us to develop another cell-specific promoter to drive gene expression in astrocytes during hypoxia.

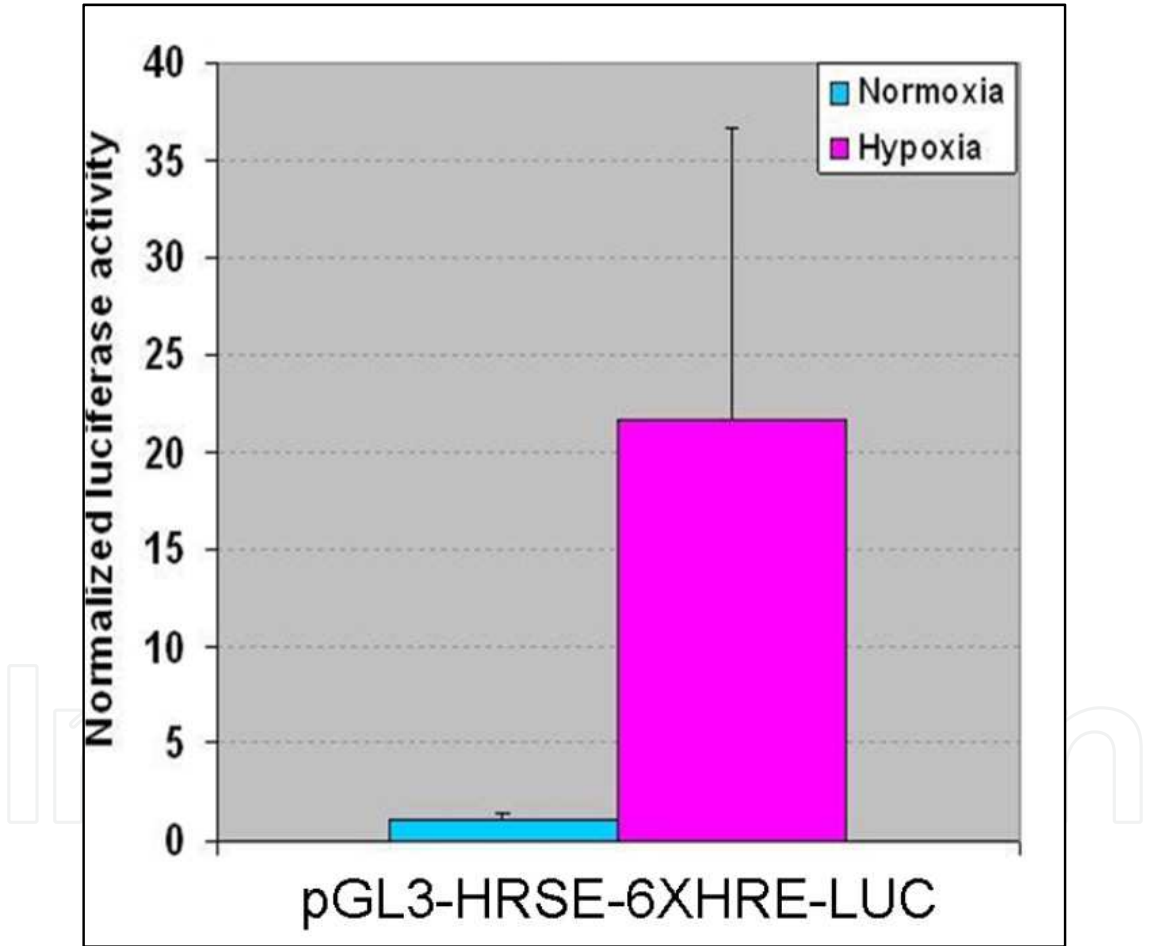


Fig. 3. Hypoxia responsive luciferase expression controlled by the regulated promoter in astrocytes. pGL3-HRSE-6XHRE-Luc plasmid was transfected to rat primary astrocytes. The transfected cells were exposed to either 0.5 % hypoxia or normoxia. After 40hrs of hypoxic or normoxic exposure, for testing the activity of the promoter, luciferase gene expression was measured using Promega’s Dual Glow luciferase assay kit (DLA). The activity of regulated promoter showed more than 15-fold induction in hypoxia compared to its normoxic counterpart.

We have designed astrocyte-specific promoters in a pGL3 based vector containing hypoxia responsive elements (HREs), silencer domains and the human GFAP promoter that provide both cell specificity and hypoxia inducible expression as well as normoxic silencing. The human GFAP promoter in a plasmid vector was a generous gift from Dr. Brenner (University of Alabama, Birmingham, AL). The promoter element was amplified and ligated to a pGL3 basic vector to drive the luciferase reporter gene. This construct was named pGL3-GFAP-Luc and is referred to as the unregulated plasmid vector. The addition of 6 copies of the HRE element (Dougherty et al., 2008) will be a target for HIF-1 to bind to and hence turn on gene expression during hypoxia. Incorporation of a silencer element (HRSE) (Dougherty et al., 2008) to the HRE will prevent the expression of the transgene in normoxia and maximize the expression of the transgene in hypoxia. The newly constructed plasmid pGL3-HRSE-6XHRE-GFAP-Luc is referred to as regulated plasmid vector (**Figure 2A**).

Rat primary astrocytes and other cell lines were transfected with the plasmids using lipofectamine. After 40 hours of hypoxic or normoxic exposure, the dual luciferase assay was performed to measure the activity of the promoters. Our preliminary results show that the GFAP promoter was modestly activated (< 3 fold), whereas the regulated GFAP promoter was induced by more than 15-fold in hypoxia and was tissue specific (**Figure 3**). Promoter constructs were incorporated into a self-complementary Adeno Associated Virus (scAAV) plasmid vector and produced in large quantities (**Figure 2B**). Vector specificity and hypoxia regulation were tested in cultures of primary rat astrocytes. Our results demonstrated that the regulated promoter construct was completely silenced in aerobic conditions in cultures of primary astrocyte. Hypoxic-exposure induced high levels of GFP expression in transduced astrocytes (**Figure 4**).

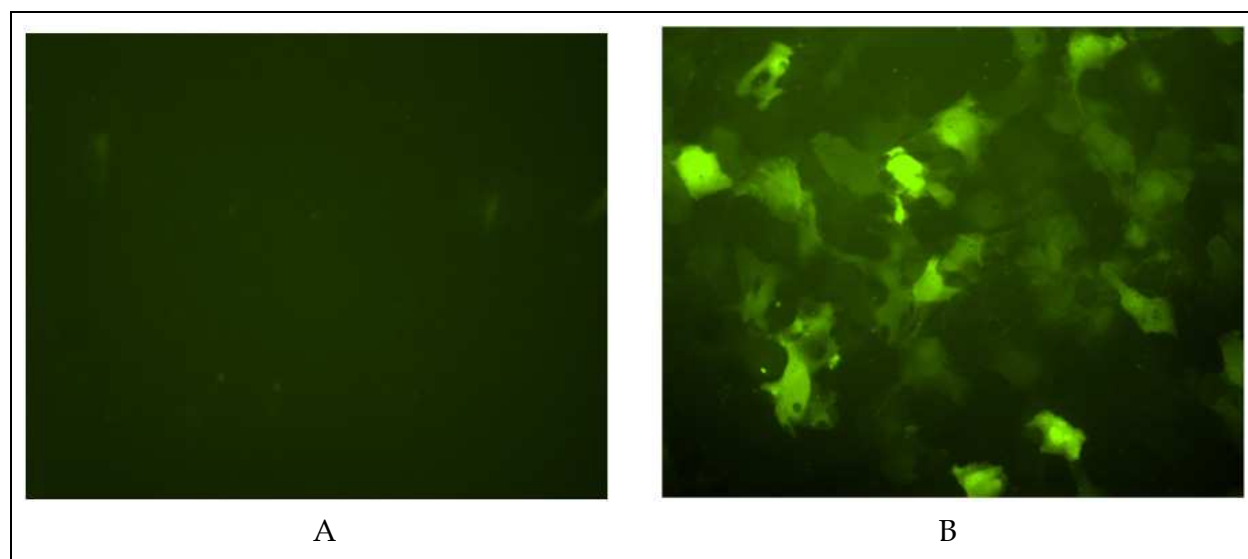


Fig. 4. Hypoxia responsive GFP expression from the regulated vector. scAAV2-HRSE-6XHRE-GFAP-GFP virus vector was transduced into primary rat astrocytes (1×10^3 viral particles per cell). After 3 days of transduction, the cells were exposed to either 0.5% hypoxia or normoxia for 6 days. In normoxia, the transduced astrocytes do not express GFP (A) whereas in hypoxia, the transduced astrocytes show high level induction of the promoter by expressing GFP (B).

4. Vector choice and route of delivery

Although current therapeutic interventions provide improvements in glioma therapy, these treatments do not result in long term cures. Development of gene therapy could be a potential strategy for long term treatment of GBM. Glioma is an ideal target for gene therapy because, unlike other solid tumors, it rarely metastasizes outside the CNS. Over 30 clinical trials using gene therapy vectors have been initiated with more than 400 glioma patients enrolled worldwide.

The major requirement of gene therapy is to develop vectors which are safe and efficient to deliver the therapeutic genes to a targeted cell type. Several viral and non-viral vectors are being used to transfer genes in both experimental models and clinical research applications. Although non-viral methods have advantages over the viral methods in terms of their large scale production and low host immunogenicity, low levels of transfection and expression of the gene limit their use. Mostly viral vectors have been used as vehicles to carry exogenous genes into human cells since they bind to the host cell and introduce genetic material into the cytoplasm or nucleus as a part of their replication cycle. Viruses used in glioma gene therapy generally fall into two categories: replication incompetent viruses (from which all or most of its genome has been removed) and replication competent viruses (where select viral genes are deleted or mutated so that viruses can replicate and lyse tumor cells selectively) to minimize toxicity and retain gene delivery efficiency (Iwami et al., 2010). Adenoviruses (AdV) or retrovirus (RVs) have both been used in clinical trials to treat gliomas.

Expression of a therapeutic protein depends on choice of vector and the route of delivery. There are several possible routes of delivery including systemic administration, stereotactic injection, intratumoral injection, intramuscular injection, and facial vein injection. Liposomal delivery of HSV-tk has been used to target gliomas using cationic liposomes carrying the human interferon beta gene (Yoshida et al., 2004) in a clinical trial. Adeno viral mediated HSV TK along with the pro-drug ganciclovir was used to treat human glioma (Immonen et al., 2004). Lenti-viral mediated transfer of GAS1 (Lopez-Ornelas et al., 2011) to human gliomas has been used to arrest cell proliferation, cause apoptosis and inhibit tumor growth. A combinational therapy using baculovirus-mediated expression of p53 along with sodium butyrate was also tested to treat human glioblastoma (Guo et al., 2011). AAV is one of the preferred vectors for gene therapy since it is less immunogenic and has an increased rate of transduction efficiency. Research using a variety of serotypes or mutations in the vector capsids has led to better vectors to target specific cell types for expression of the therapeutic protein. It may be preferable to produce a regulated or modest amount of transgene expression (McCarty et al., 2003) in a greater number of cells rather than overproduction in a small number of cells.

Despite its limited capacity in harboring larger size genes, development of the scAAV genome advances AAV gene therapy due to increased transduction efficiency. The scAAV genome is proven to be very suitable for both dividing and non-dividing cells in transgenic mice (Choi et al., 2006). The scAAV has also been used to target both neurons and glia using either intrathalamic or intraventricular injection (Chen et al., 1998, Chen et al., 1999). AAV mediated HSV-tk was targeted in mice bearing human glioma in conjunction with ganciclovir administration (Mizuno et al., 1998). Intramuscular injection of AAV has been used to deliver angiostatin to suppress glioma growth in nude mice (Ma et al., 2002).

Similarly AAV-8 was used to deliver soluble VEGF receptor to the CNS to trap the VEGF in orthotopic brain tumor models of GBM (Harding et al., 2006).

AAV mediated expression of the c-terminal fragment of the human telomerase reverse transcriptase gene (hTERTC27) has proven to be highly effective to reduce tumor growth in human glioblastoma xenografts mouse model (Ng et al., 2007). A chimeric AAV capsid was developed to enhance glioma cell transduction (Maguire et al., 2010). Stereotaxic injections of different AAV serotypes into the striatum of rat brain are reported to enhance glial delivery of the therapeutic protein. Intravenous or intracisternal injection of scAAV2 vectors have been used to study global distribution and dispersion of AAV in a mouse brain (Fu et al., 2003). Although several serotypes of AAV were used, it has been reported that AAV5 and AAV9 appears to best for enhanced glial transduction (Foust et al., 2009, Markakis et al., 2010).

5. Gene therapy strategies for glioma

An extensive range of genes and their signaling pathways contribute to glioma development and the level of severity. It may not be appropriate to consider only one gene or factor for prevention of glioma in a therapeutic strategy. Combinational therapy and pro-drug activation are now being used along with chemotherapy and radiation therapy. The most effective gene therapy strategy in use both in experimental and clinical gene therapy trials employs HSV-tk. While it may prevent the tumor growth initially, HSV-tk cannot reduce glioma growth at a later stage due to its dependence for cell killing on gap junctions between tumor cells. Ultimately a more effective strategy for cell killing may involve developing vectors which can target the local tumor environment.

5.1 Use of cytotoxic or suicidal approach

The suicidal gene therapy approach has been the most generally used in gene therapy clinical trials for glioma. The HSV-tk method which requires a pro-drug to elicit its effect is extensively employed for this purpose. HSV-tk enhances the sensitivity of transduced tumor cells towards the non toxic pro-drug ganciclovir, acyclovir or valaciclovir and then metabolizes the pro-drug into a cytotoxic agent which acts as a potent inhibitor of DNA replication (Tiberghien, 1994, Tiberghien et al., 1994). Several vectors have been used to drive expression of HSV-tk for killing of tumor cells. The effectiveness of suicidal gene therapy mediated by HRE cre/lox driving HSV-tk has been evaluated in a model of tumor xenografts implanted in nude mice (Greco et al., 2006). In a similar strategy a hypoxia regulated expression vector was used to drive the expression of cytosine deaminase gene which activates another pro-drug 5-fluorocytosine to form the highly toxic 5-fluoruracil (Chen et al., 2005, Wang et al., 2005). This cytotoxic compound was able to significantly kill glioblastoma cells in a hypoxia-dependent manner while exerting no toxicity on normal cells. Hypoxia regulated carboxyl esterase expression has been generated using an adeno viral based gene expression system, resulting in conversion of a pro-drug Irinotecan (CPT-11) to its active cytotoxic metabolite which is capable of killing glioma cells (Matzow et al., 2007). Based upon the above evidence of the feasibility of employing hypoxia regulated gene expression in a suicide gene therapy strategy, our vector may be highly applicable for delivering therapeutic genes in astrocytes for control over tumor growth.

5.2 Gene targets involving cell cycle arrest and apoptosis

Transfer of cell cycle genes could be a good strategy for blocking glioma growth (Fueyo et al., 2001). The E2F family of transcription factors contributes to the regulation of cell cycle and cell death genes in glioma. It has been shown that over expression of E2F-1 enhances the anti-glioma effect through increasing apoptosis in human glioma cells (Mitlianga et al., 2002). Another important strategy may be to induce cell cycle arrest and prevent the proliferation of the tumor cells. Soluble form of Gas1 protein is being used by lenti-viral gene transfer methods to arrest the cell cycle (Lopez-Ornelas et al., 2011), (Zamorano et al., 2003), (Zamorano et al., 2004) and preventing the proliferation of glioma cells in nude mice. Hypoxia regulated expression of these proteins has been proposed as a viable approach that can be applied to tumor killing.

The tumor cytokines TNF alpha and IFN beta are very good candidates for inducing apoptosis in hypoxic glioma cells when the respective genes are incorporated into our hypoxia regulated vector. The secretable form of trimeric Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL) has been employed for targeting gliomas and inducing apoptosis in glioblastoma (Jeong et al., 2009) and TRAIL therefore is also an excellent candidate for incorporation into a regulated gene therapy vector. Overexpression of bone morphogenetic protein (BMP4) was employed to induce apoptosis in glioma stem cells (Zhou et al., 2011) and therefore BMP4 represents a good candidate for enhancing tumor killing. Numerous studies have demonstrated detailed mechanisms by which BMP4 acts to induce BAX and inhibit BCL2 as well as Bcl-X family members. Glioma cells exhibit low endoplasmic reticulum (ER) stress. It is possible that over-expression of ER stress factors could be effective for inducing apoptosis in glioma. ER stress induces C/EBP homologous protein (CHOP) which is a key regulator for cellular apoptosis (Eom et al., 2010). Regulated expression of CHOP would also be valid for inducing apoptosis in tumor cells (Kim et al., 2011).

Gliomas can be targeted by overexpression of micro RNAs (miR). It has been demonstrated that miRs also play an important role in regulation of oncogenes and/or tumor suppressor genes at the post-transcriptional and/or translational level. Oligonucleotides mimicking miR-451 are used to inhibit cell growth, induce G0/G1 phase arrest and enhance apoptosis in different glioma cell lines. Targeting miR-451 also decreases the expression levels of Akt1, CyclinD1, MMP-2, MMP-9 and Bcl-2 genes while increasing expression of the tumor suppressor genes p27 in dose dependent manner (Nan et al., 2010). Adenoviral mediated co-expression of shRNAs for miR-221 and 221 have been used to induce apoptosis in tumor cells (Wang et al., 2011). Mir-34a was overexpressed in a p53 mutant glioma cell line with resulting induction of apoptosis.

Inhibition of MMPs may be a valuable strategy for suppressing glioma growth in regulated manner through induction of apoptosis. In a previous study siRNA mediated downregulation of urokinase-type plasminogen activator receptor (uPAR) and matrix metalloproteinase-9 (MMP-9) were employed to activate caspase-9 mediated apoptosis in glioma (Gondi et al., 2008). It is reported that miR-21 is involved in regulation of genes for glioma cell proliferation, migration and invasion through activation of several MMPs. Downregulation of miR-21 may be an effective strategy for blocking the expression of MMPs and for activating tissue inhibitor of metalloproteinases in glioma cells (Gabriely et al., 2008).

5.3 Anti angiogenic approach

Tumor hypoxia is implicated as a major stimulus for the growth of new blood vessels in tumor angiogenesis through inducing expression of most potent angiogenic factor VEGF. Tumor angiogenesis facilitates the invasion of tumor cells into normal tissue. Inhibiting pro-angiogenic factors such as VEGF, targeting VEGF receptors and over expressing endogenous anti angiogenic factors holds promise as a good strategy to prevent tumor angiogenesis. Since HIF-1 α mediates increased tumor aggressiveness in hypoxia, direct inhibition of HIF-1 α with siRNA may be effectively used to suppress glioma cell growth and reduce tumor invasiveness in a glioma model (Fujiwara et al., 2007). Targeting of VEGF signaling pathways and their receptors would provide an efficient treatment for glioma angiogenesis. Regulated expression of endogenous anti angiogenic factors (endostatin, angiostatin, pigment epithelial derived factor (PEDF), thrombospondin-1) could be beneficial in conjunction with other chemotherapeutic agents. Intra-arterial delivery of plasmids carrying the endostatin gene results in an 80% reduction in tumor volume, a 40% decrease in tumor angiogenesis and increases the survival time of up to 47% in a rat model of solitary intracerebral 9L tumors (Barnett et al., 2004). Co-delivery of a angiostatin-endostatin fusion gene and soluble VEGF receptor (sFLT-1) by a sleeping beauty transposon completely inhibited tumor growth and enhanced the survival of animals in an experimental GBM model (Ohlfest et al., 2005). AAV mediated delivery of angiostatin was used to treat the malignant brain tumor in a C6 glioma/Wistar rat model with a resulting inhibition of tumor growth and enhanced time of animal survival (Ma et al., 2002).

Antisense TGF-beta and soluble VEGF receptors have been exploited to reduce glioma growth (Harding et al., 2006). Overall it has been seen that glioma can evade anti angiogenic therapy by up-regulating alternative signaling pathways such as the ILK-1 pathway (Verpelli et al., 2010). Targeting both TGF-beta and VEGF pathways simultaneously shows promise for an improved treatment strategy. Over-expression of PEDF could also be exploited using our regulated vector and in this case the likely results would include activities that are neuroprotective and anti-angiogenic (Zhang et al., 2007). Over expression of PEDF has recently been shown to inhibit tumor malignancy in a rodent glioma model (Guan et al., 2004).

6. Future directions for development of hypoxia regulated anti glioma treatment

Gene therapy strategies have been broadly applied to treat all forms of glioma. Although expression of suicidal genes in tumor cells is effective to a degree in clinical trials, there is a risk that the suicidal genes can express in normal cells with detrimental effects. Use of cell type specific or cancer cell specific promoters may have advantages over universal promoters by restricting gene expression to the desired cells without damaging other cells collaterally. The use of vectors driving hypoxia-regulated astrocyte-specific expression of genes could be an effective strategy to target tumor cell apoptosis, cell cycle arrest and inhibition of key regulators for tumor angiogenesis.

One of the advantages of a hypoxia regulated promoter is it can restrict the expression of genes to hypoxic astrocytes within and in close proximity to the tumor core while avoiding production of the therapeutic protein in neighboring normal cells which may occur when

universal promoters are employed. Until now, surgical removal of tumor tissue in combination with radiotherapy has been a relatively effective strategy for treating glioma. However one of the major problems is the recurrence of the tumor which is associated with ROS induced HIF-1 expression. Upon recurrence of glioma the enhanced HIF-1 levels desensitize glioma cells to further treatment. The regulated vector could be employed to resensitize the tumor cells to be more responsive to chemotherapy. Some oncogenes induce HIF-1 in tumor cells in the absence of hypoxia (Lim et al., 2004). Our vector can be used to respond to HIF expression specifically in glial cells should HIF-1 be activated by any of the oncogenes.

Chapter summary

- The hypoxic microenvironment is known to play a major role in several diseases including age related macular degeneration, cardiac and cerebral ischemia, glioma, and other cancers.
- Many endogenous genes that are induced during hypoxia are activated by the binding of the transcription factor HIF-1 α to a hypoxia regulated enhancer (HRE) region in their promoter domain.
- For effective gene therapy, there is a great need for a vector that can be regulated according to the local tissue microenvironment.
- By taking advantage of hypoxia for regulating foreign gene expression, a hypoxia regulated vector including hypoxia regulated enhancer could be exploited to modify the cells that reside within the target tissue.
- Gliomas are the most common brain tumors in the central nervous system and the majority of malignant gliomas arise from neoplastic transformation of resident astrocytes.
- Our hypoxia-regulated, astrocyte-specific vector has the capacity to restrict its expression to astrocytes and can be expressed only in the hypoxic microenvironment thus remaining inactive both under hypoxic and normoxic conditions in other cells.
- A variety of therapeutic gene products could be delivered by our hypoxia-regulated promoter to prevent abnormal angiogenesis and induce apoptosis in tumor cells leading to prevention of invasion and infiltration of the glioma into surrounding tissue.
- One of the major problems in surgical removal of tumor tissue is the recurrence of glioma and the enhanced HIF-1 levels desensitize glioma cells to further treatment. The regulated vector could be employed to resensitize the tumor cells to be more responsive to chemotherapy.

As a combinational therapeutic strategy our hypoxia regulated promoter could be employed for expressing therapeutic genes encoding an anti-angiogenic factor in addition to a tumor suppressor gene. The scope and applications of our hypoxia -regulated gene therapy are shown in figure 5. Our strategy of hypoxia-regulated gene therapy may obviate the need for radiation therapy and chemotherapy and may be beneficial in terms of preventing tumor growth as well as eradication of residual cancer cells in instances where a glioma is removed surgically.

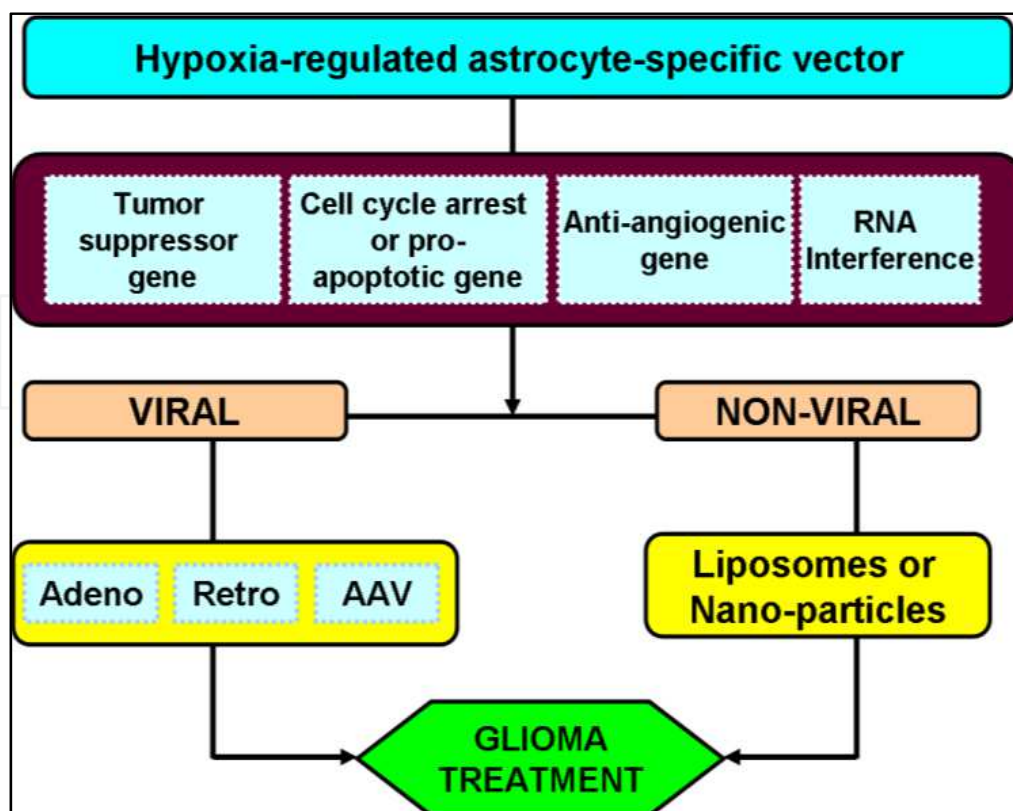


Fig. 5. Strategy for hypoxia-regulated astrocyte-specific gene therapy to treat glioma. The regulated-promoter can be applied to drive expression of genes responsible for tumor suppressor, cell cycle arrest, pro-apoptotic and inhibition of pro-angiogenic processes in astrocytes. The plasmid vectors can be packaged into either viral (Adeno, Retro and AAV) vectors or non-viral liposomes or nano-particles as a potential glioma treatment strategy.

7. References

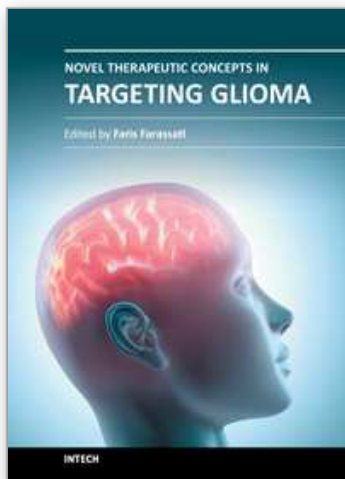
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