Oncogenesis Recapitulates Embryogenesis via the Hypoxia Pathway: Morphoproteomics and Biomedical Analytics Provide Proof of Concept and Therapeutic Options

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Abstract. Background: Hypoxia (3 to 5% oxygen) is essential in maintaining the plasticity of embryonic stem cells and permitting their transformation via epithelial-mesenchymal transition (EMT) and mesenchymal-epithelial transition (MET) into tissues and organs of the developing fetus. Similarly, a relatively hypoxic microenvironment supports the development of tumor cells with stemness and epithelial-mesenchymal properties and capabilities. At the same time, such adaptation results in the tumor cells becoming relatively resistant to chemotherapy and radiation therapy and promotes invasation into blood vessels with metastasis. In this context, current therapeutic strategies designed to target tumoral angiogenesis could promote stemness and EMT by rendering tumor cells more hypoxic, leading to chemoradioresistance and metastatic and recurrent disease. Objective: The purpose of this report is to present a conceptual model that illustrates the impact of an hypoxic microenvironment on the signal transduction pathways involved in the hypoxia pathway. We will show the molecular connectivity and correlative association of these pathways with protein analytes in both embryogenesis and oncogenesis in order to strengthen our hypothesis that oncogenesis recapitulates embryogenesis. Finally, we propose to use the model as a basis for the construction of combinatorial, therapeutic options from existing pharmaceutical and nutraceutical agents that may obviate tumoral adaptation to hypoxia. Methods: Morphoproteomics and biomedical analytics. Application and Results: Archival data retrieved from morphoproteomic analysis of glioblastoma multiforme (GBM) cases revealed proteomic correlates of tumoral necrosis and associated hypoxia pathway signaling. Biomedical analytics using Ingenuity Pathway Analysis (IPA) showed comparative validation of the hypoxia pathway, as demonstrated by morphoproteomics in GBM, both with the hypoxia-induced genes in neuroblastoma and with the networks associated with embryogenesis. Additionally, therapeutic agents known to have activity against various components of the hypoxia pathway (identified by morphoproteomic analysis in GBM) were validated using UNIPROT identifiers entered into IPA and Path Designer. These therapies also connected with the hypoxia signature in neuroblastoma and embryogenesis. Conclusion: The application of morphoproteomics to define the presence of an adaptive hypoxia pathway in GBM accords with biomedical analytics in the demonstration of concordant interaction with the hypoxia signature in neuroblastoma and embryogenesis, providing proof of concept that oncogenesis recapitulates embryogenesis. This approach also validates a new combinatorial therapeutic strategy targeting the hypoxia pathway and designed to prevent tumoral adaptation, chemoradioresistance and recurrent disease.

Key words: oncogenesis, embryogenesis, hypoxia, morphoproteomics, biomedical analytics

Introduction

The parallels between the developmental processes of embryos and tumors were first postulated by Johannes Müller in his "law of correspondence" between embryogenesis and pathogenesis [1]. Virchow proposed that cancer cells arise from cells with embryonic characteristics such as plasticity [2]. Moreover, the importance of the adaptive tissue environment in the growth of both embryos and tumors has been known for more than a century [3], as well as the "intimate" morphological relations between embryos and tumors that can lead to mutual restructuring, as proposed by Rous.
in 1911 [4]. Additionally, the tumoral microenvironment has been implicated in metastasis to specific organs, starting with Paget’s “seed and soil” hypothesis that a malignant cell (“the seed”) proliferates in the favorable “soil” of a specific organ microenvironment [5]. Most recently, the National Cancer Institute (NCI) has suggested that signaling pathways associated with embryogenesis be investigated as therapeutic targets in cancer [6].

We propose that parallels in the microenvironment and, specifically relative hypoxia, can explain the stemness and transformation capabilities (plasticity) of both embryos and tumors, and that tumors and embryos share hypoxia-associated commonalities and parallels at the molecular pathway and genomic level indicative of the phenomenon of oncogenesis recapitulating embryogenesis (Figure 1). Importantly, such parallels, when identified, may be modulated by combinatorial targeted therapies designed to obviate adaptation in the tumor cells that could lead to chemoradioresistance and metastasis with recurrent disease. The purpose of this report is threefold: 1. to illustrate and define the characteristics of the hypoxia pathway using morphoproteomics as applied to glioblastoma multiforme (GBM); 2. to use biomedical analytics in the validation of the hypoxia pathway, as determined by the morphoproteomic findings in GBM with the hypoxia-induced genes in neuroblastoma and with the networks associated with embryogenesis; and 3. to connect the hypoxia signature in GBM, neuroblastoma and embryogenesis with therapeutic agents designed to target the hypoxia pathway using biomedical analytics.

Methods and Patient Study Population

**Morphoproteomics.** Morphoproteomics combines morphology using bright-field microscopy and proteomics by immunohistochemistry to help define the biology of tumors [7, 8]. Specifically, it uses chromogenic signals to detect, quantify and localize the protein analytes in the tissue specimen’s lesional and companionate cells. Non-cell cycle related protein analytes are measured visually within each subcellular compartment (i.e., plasmalemmal, cytoplasmic and nuclear) on a 0 to 3+ scale based on the signal intensity with the 3, 3’-diaminobenzidine tetrahydrochloride (DAB) chromogenic (brown) signal. Cell cycle-related protein analytes are visually quantified and reported as estimated percentage of immunopositive tumoral nuclei. Protein analyte activation is assessed based on phosphorylation, compartmental translocation, and functional grouping.

**Study Population.** Archival material obtained from IRB approved studies of the application of morphoproteomics to patients with GBM were used to identify morphoproteomic correlates of tumoral necrosis and signaling by the hypoxia pathway.

**Biomedical Analytics.** Biomedical analytics is the science of developing and applying computable algorithms based on mathematics, operations research, statistics and computer science to biomedical data in order to increase the information gleaned from the original data sets. For purposes of this review and comparative validation of the hypoxia signaling pathway in GBM and those of the hypoxia gene signature generated by Fardin and co-workers [9], morphoproteomic findings were entered into the Ingenuity Pathway Analysis(IPA) and connected using Path Designer (http://www.
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A computer-assisted search of the National Library of Medicine's MEDLINE database assisted in the connection of the pathways and interactions. Therapeutic agents known to have activity against the hypoxia pathway were tested for validity against the UNIPROT identifiers entered into the IPA and connected with the identified therapeutic agents using Path Designer.

Results

Morphoproteomics. Histopathologic features, linked to and considered to be criteria for the diagnosis of GBM, include some degree of coagulative-type tumoral necrosis and/or angiogenesis [10]. These reflect the consequence of tumoral hypoxia and the adaptive response of the remaining viable tumor cells to such insult, respectively (Figure 2). Coincident with such morphologic findings are the morphoproteomic correlates of a hypoxia-induced response in GBM to include: 1. mammalian target of rapamycin complex2 (mTORC2) with predominance over mTORC1 [11], as evidenced by nuclear translocation of phosphorylated (p)-mTOR (Ser 2448) and its downstream effector, p-Akt (Ser 473) [12-14] and the correlative expression of protein kinase C (PKC)-alpha [15] with occasional plasmalemmal expression; and 2. variable hypoxia-inducible factor (HIF)-1 alpha expression with nuclear translocation consistent with what has been previously demonstrated in tumors of patients with GBM [16] and dependent in part on the mTORC2 pathway [17], the signal transducer and activator of transcription (STAT) pathway (the latter evidenced by constitutive activation of the STAT3 pathway in the form of p-STAT3 [Tyr 705] with nuclear expression) [18-20], and an activated nuclear factor (NF)-kappaB pathway in the form of expression with nuclear translocation of p-NF-kappaBp65 (Ser 536) [21-23]. These protein analytes are illustrated in Figure 3.

Additional morphoproteomic correlates of HIF-1 alpha signaling in GBM include: overexpression of cyclo-oxygenase (COX)-2 [24,25]; c-Met (mesenchymal-epithelial transition) with constitutive activation in the form of cytoplasmic expression of p-c-Met (Tyr 1234/1235) [26,27]; overexpression of fatty acid synthase (FASN) [28] whose gene is up-regulated in the context of Akt activation and subsequent HIF-1 alpha signaling [29], vascular endothelial growth factor (VEGF)-A expression [30,31]; secreted protein acidic and rich in cysteine (SPARC) [32]; CD133 and CD44 expressions, stemness markers induced by hypoxia and associated with HIF-1 alpha [33-35] and with the chemoresistance of such populations in GBM and recurrent disease [36-40]. These are illustrated in Figures 4 and 5.

Biomedical Analytics.

Hypoxia and GBM: The hypoxia gene signature generated by Fardin and co-workers [9] and the UNIPROT identifiers of the measured protein analytes related to the hypoxia pathway in the above-mentioned morphoproteomic analysis of GBM were entered into Ingenuity Pathway Analysis and...
Figure 3. Phosphorylated (p)-mammalian target of rapamycin with predominant nuclear expression (DAB brown chromogenic signal) of p-mTOR (Ser 2448; frame A) and p-Akt (Ser 473; frame B) and cytoplasmic/plasmalemmal (cell membrane) expression of PKC-alpha (frame C) support mTORC2 over mTORC1 consistent with response to hypoxia in GBM. Downstream of hypoxia-associated mTORC2 expression is hypoxia-inducible factor (HIF)-1 alpha with variable nuclear translocation (frame D) and which correlates with nuclear p-signal transducer and activator of transcription (STAT3) (Tyr 705; frame E) in viable tumor cells and with nuclear expression of p-nuclear factor (NF)-kappaBp65 (Ser536; frame F) in supporting activation of the hypoxia pathway in GBM. (Original magnifications, x400 in frames A-F).
Figure 4. Nuclear expression of HIF-1 alpha (frame A) in GBM (DAB brown chromogenic signal) and downstream correlates of hypoxia pathway signaling that can be variably expressed in GBM include: cytoplasmic expression of cyclo-oxygenase (COX)-2 (frame B); cytoplasmic, phosphorylated (p)-c-Met (Tyr 1234/1235) including perinecrotic but viable tumor cells (frame C); cytoplasmic expression fatty acid synthase (FASN; frame D); plasmalemmal and cytoplasmic expression of vascular endothelial growth factor (VEGF)-isoform A (frame E), and secreted protein acidic and rich in cysteine (SPARC; frame F). (Original magnification x400 in frames A-D and x200 in frames E and F).
connected using Path Designer (http://www.ingenuity.com). The hypoxia signature in this model consists of 32 hypoxia-induced genes found in neuroblastoma; it has been shown to be a novel independent predictor of outcome in neuroblastoma patients [9]. Using this approach, the hypoxia-associated protein analytes measured by morphoproteomics had more than 100 interactions with the genomic hypoxia network reinforcing the validity of applying these to the hypoxia pathway in general and to GBM, specifically.

The protein analytes measured in GBM were normalized on a scoring scale from 0 to 100; the GBM profile scores, along with their UNIPROT identifiers, were entered into Ingenuity Pathway Analysis to generate the most likely biological pathway networks. An unscored profile with the same analyte names was used as a control to minimize selection bias.

The results were that each profile evoked one unique subnetwork with a major hub around huntingtin (HTT) and a minor hub around Ck2; however, each profile had different molecular interactions involved. Huntingtin is associated with Huntington’s disease (HD) signaling. HD is a neurodegenerative genetic disorder caused by mutation in the Huntingtin gene [41]. Although HTT is not typically associated with GBM, its emergence suggests that there may be some similar functionalities. In addition, there is a hypoxia connection. Mutant huntingtin is known to induce HIF-1 alpha mRNA, and it has been shown that hypoxia inducible factor prolyl-4-hydroxylases (HIF PHDs) inhibitors may be of clinical utility in preventing cell death in conditions such as Huntington’s disease [42]. Ck2 (Casein kinase 2) has been described as a switch involved in cell growth, proliferation and apoptosis suppression. It is predominant in the nuclear compartment in cancer cells and has been considered a target for some time [43]. Ck2 levels are elevated in GBM, and Ck2 inhibitors DRB (5, 6-dichlorobenzimidazole 1-b-D-ribofuranoside) and Apigenin have recently been shown to sensitize glioma cells to TNFa-induced apoptosis [44].

To evaluate the hypoxia influence, each profile subnetwork was connected with the hypoxia signature. The control subnetwork had 11 likely molecular interactions with the hypoxia network, whereas the
GBM subnetwork had 17. This suggests the greater influence of hypoxia in GBM. These are illustrated in Figures 6 and 7.

**Embryogenesis and GBM:** IPA was interrogated for lists of molecules that increase or decrease embryogenesis; these were compared with the control subnetwork and the GBM subnetwork. The results were that 1,005 molecules increase the process of embryogenesis, and 526 molecules decrease it; some molecules do both and are classified as modulators of embryogenesis. Of the molecules common to both control and GBM subnetworks, HTT is a modulator of embryogenesis and EDNRB and MBTPS1 increase embryogenesis. Of the molecules only in GBM, CNR1, FKBP4, NRF1, and STIP1 are associated with increased embryogenesis; none are associated with decreased embryogenesis. Of the molecules in the control, but not in GBM, CNR2 is a modulator of embryogenesis that may play a key role in early neuronal development [45]. CNR2 (also known as CB2) is a cannabinoid receptor gene. Although their mechanisms are not fully understood, the two cannabinoid receptors CNR1 (CB1) /CNR2 (CB2) are considered neuroprotective [46,47]. CNR2 seems to protect the blood-brain barrier during neuroinflammation [48]. The lack of CNR2 in the GBM subnetwork compared to the control suggests that there may be a defect or lack of CNR2 that may be targeted by therapy to produce sufficient receptors for an immune response.

**Therapeutic Options Targeting the Hypoxia Pathway Based on Morphoproteomics, Computer-Assisted Data Mining of the National Library of Medicine's MEDLINE Data Base and Biomedical Analytics.** Therapeutic agents that are reported to have activity against various components of the hypoxia pathway, identifiable in GBM by morphoproteomic analysis, include metformin, valproic acid, doxorubicin, melatonin and celecoxib. The specifics of their actions against the hypoxia pathway in the context of GBM are as follows:

**Metformin:** Metformin inhibits both mTORC1 and components of the mTORC2 pathway including downregulation of p-Akt (Ser 473) induced by the insulin-like growth factor (IGF) pathway [49], which has been previously documented in glioblastoma and implicated in its biology and stemness [50–57]. (Both mTORC1 and mTORC2 signaling have been shown to upregulate HIF-1 alpha expression [17].) Additionally, hypoxia can induce the IGF-II gene [58], which has been shown to be enhanced in glioblastoma [50]. Moreover, the correlates of IGF pathway signaling identified by morphoproteomic analysis of GBM include a constitutively activated STAT3 pathway in the form of nuclear p-STAT3 (Tyr 705) and HIF-1 alpha expressions [59]. Notably, metformin in other preclinical studies has been shown to inhibit STAT3 activation onTyr705 [60], to reduce NF-kappaB activity [61], and to inhibit HIF-1 alpha accumulation and the expression of HIF-1-targeted genes [62]. Finally, metformin selectively targets cancer stem cells including CD44+ cells [63], inhibits epithelial-mesenchymal transition [64], and is being proposed as a combinatorial agent with temozolomide in the treatment of glioblastoma [65,66].

**Valproic acid:** Valproic acid as an histone deacetylase inhibitor has been reported to decrease HIF-1 alpha protein levels and transcriptional activity in human tumor cells lines [67]. In glioma cells, valproic acid has been shown to: 1. inhibit proliferation and downregulate CD44 expression [68]; and 2. to up-regulate both melatonin receptor subtypes MT1 and MT2 and neurotrophic factors in C6 glioma cells [69,70]. The latter has implications regarding its combinatorial use with melatonin (vide infra). Clinically, valproic acid has been studied in pediatric patients with refractory solid or CNS tumors in a phase 1 study and on this basis has been proposed for future trials in combination with chemotherapy and/or radiotherapy for CNS tumors [71]. In separate clinical studies, valproic acid use in children with high-grade gliomas plus radiochemotherapy showed an encouraging response rate [72] and in patients whose glioblastoma was treated with temozolomide and radiotherapy, prolonged survival [73].

**Doxorubicin:** Doxorubicin inhibits the hypoxia pathway by blocking the transcriptional activation of the hypoxia response element by HIF-1 alpha [74,75]. One of the targets of doxorubicin is topoisomerase II alpha which can be variably expressed in GBM [76]. However, the expression of topoisomerase II alpha in human glioblastoma cell lines can be enhanced by valproic acid, an histone
Figure 6. Control (left). Green = absent from GBM. Right: Hypoxia Gene Signature (blue). There are 11 likely molecular interactions between them (orange lines).
Figure 7. GBM (left). Orange = present only in GBM. Right: Hypoxia Gene Signature (blue). There are 17 likely molecular interactions between them (orange lines).

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deacetylase inhibitor [77] (this appears to coincide with the observation that doxorubicin-induced apoptosis can be enhanced by another histone deacetylase inhibitor in glioblastoma cells [78]). Other combinatorial agents that appear compatible in this context include metformin which works together with doxorubicin in an experimental model to target both cancer stem cells and more non-stem tumor cells to block tumor growth and prolong remission while reducing the dose of doxorubicin [63,79] and melatonin, which reduces the cardio-toxicity of doxorubicin in preclinical studies [80]. Importantly, doxorubicin has been used in clinical trials with malignant glioma and the liposomal form has been reported to result in long term stabilization of disease [81].

**Melatonin:** Melatonin acts to inhibit both the transcription of VEGF and HIF-1 alpha and also their translation [82,83]. Additionally, melatonin has been shown to inhibit the stabilization of HIF-1 alpha in tumors that are undergoing hypoxia [84]. Clinically, melatonin has been reported to increase survival time in patients with glioblastomas when used in combination with radiotherapy [85]. Melatonin reduces the systemic toxicities of chemotherapeutic agents on marrow elements, particularly in ameliorating thrombocytopenia [86].

**Celecoxib:** Celecoxib inhibits hypoxia-related HIF-1 alpha recruitment/stabilization [87]. In GBM cells, celecoxib inhibits the activation of the prosurvival NF-kappaB pathway [88], which is a correlate of HIF-1 alpha signaling (*vide supra*) [22, 23]. It also enhances the radiosensitivity in glioblastoma-associated CD133-positive cells [88–89], the latter of which could be consequent to hypoxia and associated with the chemoresistance of such populations in GBM [37,38]. Clinically, celecoxib in conjunction with low dose temozolomide has shown activity against recurrent glioblastoma [90].

**Biomedical Analytics.** Comparative validation with graphic depiction of the interaction of the aforementioned therapeutic agents (metformin, valproic acid, doxorubicin, melatonin and celecoxib) was accomplished using UNIPROT identifiers of the measured protein analytes in the morphoproteomic analysis of GBM, that were entered into the Ingenuity Pathway Analysis (IPA) and connected with the five (5) agents using Path Designer. This revealed interaction of these therapeutic agents with the majority of the protein analytes measured by morphoproteomic analysis in GBM (Figure 8). Additionally, the UNIPROT identifiers of the hypoxia signature by Fardin and colleagues [9] were also entered into IPA and connected with the same therapeutic agents using Path Designer. This confirmed an interaction of the five therapeutic agents of metformin, valproic acid, doxorubicin, melatonin and celecoxib with the hypoxia signature (Figure 9).

**Discussion**

Embryonic stem cells reside in a relatively hypoxic environment at 3-5% oxygen, which appears to be preferential in the maintenance of a highly proliferative pluripotent population of human embryonic stem cells [91]. Exposure of human embryonic stem cell-derived embryoid bodies to ambient oxygen at or below 5% has been shown to result in the stabilization of HIF-1 alpha and increased transcription of hypoxic response genes [92]. Hypoxia has also been implicated in the epithelial-mesenchymal transition essential for embryogenesis and as a factor contributing to the stemness, metastasis and chemoradioresistance of cancer [93, 94]. Using morphoproteomics, we have illustrated the presence of an adaptive hypoxia pathway in GBM leading to stemness characteristics. Moreover, by coupling the morphoproteomic findings with biomedical analytics, we confirm a concordant interaction of such a hypoxia signature in GBM with that seen in neuroblastoma at the genomic level and in embryogenesis. This supports the thesis that oncogenesis recapitulates embryogenesis via the hypoxia pathway.

The therapeutic implications of such an adaptive relationship to hypoxia in oncogenesis are profound. The use of antiangiogenic strategies to target the tumoral vasculature at the level of vascular endothelial growth factor (VEGF) or its receptors potentially could exacerbate the hypoxic state leading to more EMT/stemness, chemoradioresistance and metastatic potential. For example, attempts to target angiogenesis with agents such
Figure 8. The azure interaction lines demonstrate that the measured protein analytes are widely affected by the proposed drug therapy of metformin, valproic acid, doxorubicin, melatonin and celecoxib. (The gray lines and notations may be ignored). © 2000-2012 Ingenuity Systems, Inc. All rights reserved.
as bevacizumab, an anti-VEGF monoclonal antibody, in human GBM xenograft models collectively have demonstrated reduced blood supply leading to induction of HIF-1 alpha, hypoxia-induced autophagy, tumor cell survival and increased tumor cell invasion, and adaptation to anti-angiogenic treatment [95, 96]. This coincides with the clinical experience of a transient benefit and then resistance with the use of bevacizumab in patients with GBM [97]. Targeting the adaptive hypoxia pathway represents an alternative approach and combinatorial therapeutic agents in individual tumors could be identified using morphoproteomic analysis and biomedical analytics, as demonstrated in this study.

In summary, we report on the application of morphoproteomics and biomedical analytics in defining the adaptive hypoxia pathway in tumors using GBM as a model. Moreover, we validate the hypoxia signature illustrated by morphoproteomics by showing concordant interaction with the genomic hypoxia signature in neuroblastoma and hypoxia in embryogenesis, providing evidence that oncogenesis recapitulates embryogenesis via the hypoxia pathway. Finally, we illustrate the potential of using morphoproteomics, biomedical analytics and computer-assisted mining of the National Library of Medicine's MEDLINE data base in developing combinatorial therapeutic strategies designed to obviate tumoral adaptation.

**Figure 9.** The five drug therapy of metformin, valproic acid, doxorubicin, melatonin and celecoxib (across the top of the graphic) span and interact with Fardin’s hypoxia signature. The solid azure lines are the direct interactions among the molecules; the dashed azure lines are indirect interactions. The dark blue line represents an interaction between doxorubicin and celecoxib. © 2000-2012 Ingenuity Systems, Inc. All rights reserved.
to hypoxia, stemness/EMT, chemoradioresistance and metastatic and recurrent disease.

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