2

# Impact of Metabolic and Therapeutic Stresses on Glioma Progression and Therapy

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## 1. Introduction

Glioma cells, both within solid tumors and during invasion, exist in surroundings that are subject to a variety of stresses, including metabolic and environmental stresses. Nonetheless, glioma cells survive and can even thrive under hostile conditions, such as hypoxia, nutrient deprivation, and therapeutic regimens. In order to survive, these tumor cells have to find a way to adapt to such an environment by activating certain growth factor and survival pathways while down-regulating cell death mechanisms. In fact, gliomas adapt so well that they not only survive but proliferate by creating a more hospitable environment through new blood vessel formation and dissemination, even as they endure additional stresses along the way. This chapter reviews the basic stresses that glioma cells encounter during the progression of tumor formation and therapeutic interventions.

## 2. Types of stress on glioma and their clinical implications

Internal stresses such as hypoxia, acidity, oxidative stress, and nutrient deprivation already exist within the cellular environment of tumors while external stressors like radiation treatment and genotoxic chemotherapy only worsen the internal factors. Encountering these stresses affects the process of carcinogenesis. Gliomas, especially GBM, are highly transformed tumors that react to stresses differently than less transformed cancers. Common markers of stress will be discussed along with their roles in induction of energy conservation and cell survival in glioma. Redistribution of energy resources towards survival pathways and away from energy-consuming processes is common.

Cellular stress can cause damage and mutations to numerous proteins, nucleic acid strands, and other macromolecules. The body has an innate reaction called the cellular stress response (CSR) to such damage. In the case of glioma and other cancers, the tumor is able to highjack the body's own machinery in order to help the cancerous cells survive usually by taking advantage of intrinsic or stress-related mutations. Thus, at times, stress may only further the growth and survival of tumor cells.

While the type of stress may vary, a common feature of many stresses is something referred to as the oxidative burst characterized by generation of oxidative stress and redox potential

changes. Reactive oxygen species (ROSs) can be produced from activation of NADPH oxidase or other ceullar oxidases in the cell's various membranes. Stressor-specific responses, on the other hand, may be induced differentially depending on the type, severity, and duration of the stress. The following sections will cover these stresses and the resulting responses by normal cells and cancerous glioma cells.

### 2.1 Types of stress

Although gliomas can vary by type and stage, more advanced gliomas are characterized by high rates of mitosis, hypercellularity, evidence of angiogenesis, and areas of necrosis. Gliomas are known to have relatively high cellular heterogeneity, much of which may be caused by different areas of a tumor encountering different stresses and growing conditions. Thus, stress may be a driving factor in tumor heterogeneity. Although, medical professionals, along with patients, are able to control to a certain extent the extrinsic stresses put on patients' bodies and tumors such as treatments and environmental stressors (*e.g.* smoking), intrinsic stresses still naturally affect the tumor as it progresses.

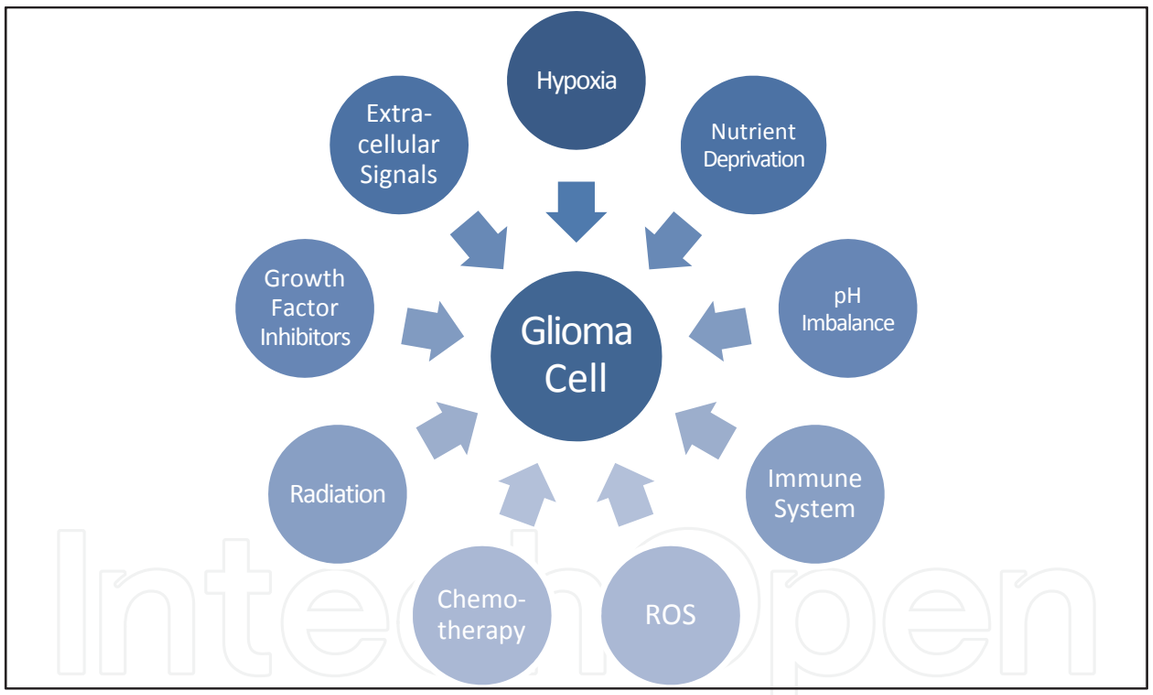


Fig. 1. Typical stresses encountered by glioma cells during tumor growth and progression.

#### 2.1.1 Stresses during tumor development and progression

While research on glioma cells in laboratories is conducted primarily under nutrient-rich conditions, the micro-environments for cancer cells are actually quite hazardous. Rarely do tumor cells find themselves in conditions of perfect nutrient balance with necessary blood flow and comfortable living spaces. More often, glioma cells are constantly inundated with a barrage of stresses (Fig. 1). The internal stresses faced by glioma cells are not unique to brain tumors, but are actually shared by the majority of solid tumors types.

Environmental and metabolic stress occurs during tumor growth and progression. As cancer cells divide, they take up more space. Normal cells would stop growing through contact inhibition, but malignant cells overcome the signals to inhibit growth and continue to divide. As the tumor expands, it continually outgrows its blood supply. Tumors larger than 1 mm in diameter can no longer subsist on passive diffusion of nutrients (Gimbrone, 1973). The glioma cells, thus, go through periods of severe nutrient deprivation and hypoxia until enough tumor cells are able to signal new blood vessel formation or neoangiogenesis. Even after angiogenesis, cells are still subject to stress as the new vessels are prone to collapse due to their abnormal state and harsh surrounding conditions (Vajkoczy & Menger, 2004). During this time, cancer cells must adapt to survive in conditions and intermittent periods of limited amino acids, salts, and oxygen. Some researches indicate that this is when cancer cells start to rely on glycolysis, which continues even after oxygen is available, a phenomenon known as the Warburg effect. This contributes to the high metabolic demand of proliferating tumor cells and is a relatively inefficient method of producing energy (Warburg, 1956). Therefore, the cells are put under enormous stress just to keep up with energy production needs and are subjected to further metabolic stress when nutrients become unavailable.

The metabolic demands of the glioma cells are partially responsible for the increased acidity or pH imbalance found in many tumors. Human brain tumors measured with electrodes had a mean pH of 6.8, with measurements as low as 5.9; the normal pH for the human brain is ~7.1 (Vaupel et al., 1989). Such pH imbalance is even found in well vascularized areas of gliomas, thus indicating that tumor cells reside within a highly acidic environment even when oxygen is present. It was originally hypothesized that that hypoxia caused the acid buildup, but these new findings mean that hypoxia and acidity are not always linked. The increased energy metabolism of the glioma cells produce hydrogen ions and metabolites like lactic acid and carbonic acid. All these products are actively pumped out of the cell through proton exchangers and other transporters (Chiche et al., 2010). In cases with decreased perfusion, poor circulation contributes to the buildup of an acidic extracellular environment.

As the tumor grows, the extracellular environment strives to slow down the progress of the cancer. Growth inhibition signals are sent that can either activate or deactivate cellular receptors depending on the need. Cancer cells survive by undergoing mutations in receptors like EGFR and PDGFR, changes that either stop the signaling cascades or rewire the signaling pathways to actually promote cancer cell growth. In this way, cellular proliferation is dissociated from nutrient availability by stress selection of surviving cells.

Hypoxia can play a major role in glioma development, with oxygen deprivation actually being necessary for tumor progression through alteration of gene expression, genomic instability, apoptotic dysregulation, and neoangiogenesis. In glioma, hypoxia is believed to be a key player due to the evidence of tumor necrosis in highly malignant forms like glioblastoma multiforme (GBM) (Brat & Meir, 2001). Brain tumors smaller than the previously stated 1 mm cutoff are found to be highly hypoxic and ill-perfused (Li et al. 2007). The oxygen deprivation is actually responsible for the growth of elaborate microvascular networks that indicate tumor progression in GBM. Even though larger GBM tumors are more vascularized, the blood vessels present are inefficient, and parts of the tumor environment remain hypoxic (Vajkoczy & Menger, 2004). Further transformation of the tumor cells occurs as reactive oxygen species (ROSs) increase during this time due to production by the mitochondria (Lui et al., 2008). Thus, hypoxia not only deprives cells of oxygen but leads to oxidative stress as well.

One of the hallmarks of a cancerous cell or tumor is its ability to invade through the basement membrane of one tissue into another type of tissue. The body has many stop guards in place to prevent this from happening, but somehow glioma cells overcome the challenge. Again, during this time, extracellular signals are sent to the cancer cells informing them to stop growing or to go through programmed cell death. Cell growth pathways are down-regulated by these signals causing severe stress to the cells. Without the normal nutrients or pathway activations, uncancerous cells would die, but glioma cells find a way to overcome the death signals. Cellular stress pathways that involve tumor suppressor p53 and metabolic stress pathways which activate the apoptotic protein Bim are often deregulated in human glioma (Tan et al.,2005). Therefore, the neoplastic cells are able to overcome invasion preventions.

The immune system is an added stress to cancer cells during all times of tumor progression, but is especially active during invasion and dissemination. While the immune system may ignore some cancer cells that stay in their own tissue, cells from different tissues are recognized by the markers or antigens they display. The innate immune system encounters the cancer cells first, with first-response cells like macrophages, granulocytes, and mast cells attacking foreign cells displaying unknown or altered markers. Even cancer cells that have managed to down-regulate these markers are subjected to hazardous surrounding environments due to the release of ROSs, metalloproteinases, chemokines, and cytokines created by the attack and death of neighboring cancer cells (Qian and Pollard, 2010). Dendritic cells transport the antigens from the neoplastic cells to the lymphoid organs in order to mount an adaptive response against the tumor. Yet somehow, in cases of cancer progression, tumor cells are able to survive these stresses and move to alternate locations. This is partially due to activated innate immune cells and paracrine signals from surrounding cells releasing soluble pro-survival molecules that initiated tumor cells can use to alter their levels of gene transcription, continuing the cell cycle and surviving (Egeblad et al., 2010). Even though the hazardous environment may kill some neoplastic cells, others may develop and thrive due to increased genomic instability from free radicals, creating additional, resistant cancer cells (Grivennikov et al., 2010). In fact, chronic inflammation has actually been linked to tumor development. Inflammatory cells can actually help in the angiogenesis and migration of glioma cells by promoting vasculature development and releases extracellular proteases that rebuild and mold the tumor environment (Colotta et al., 2009). The adaptive immune response eventually builds such that it can clear some of the neoplastic cells, but many of the cancer cells have further transformed so that they are not recognized by the cytotoxic T-cells. Even though the adaptive immune response may initially be helpful, as it continues it further promotes chronic inflammation and stress in the area, thereby contributing to cancer progression.

Those cells able to overcome the response of the immune system have a better chance of surviving invasion and migration into new tissues. Although very rare in glioma, occasionally cells do metastasize to other locations of the body, but more commonly disseminate to nearby areas of the brain. After breaking through the basement membrane, invasion may include entrance into nearby white matter tracks, and less frequently, blood vessels. By invading the white matter tracks, glioma cells are able to migrate along CNS developmental paths of the brain, taking up residence in new areas of ideal conditions, again through the process of invasion (Dai et al., 2001). In the uncommon case of hematogenous dissemination, the liver, lungs, pleura, lymph nodes and skeletal system are the most common sites of metastasis, although metastases outside of the brain are relatively

rare due to the specificity of programming of glial cells (Pasquier et al., 1980). The cells that do survive are again subjected to the stresses of the immune system, new ECM signals, and lack of designated tumor blood vessels.

With the above intrinsic stresses attacking gliomas throughout their progression, it is a testament to cancer cell adaptability that any cancerous cells survive. By adapting to these stresses, gliomas have selected for the most stress resistant cells, making cancer therapy a challenge. However, while many therapies do produce the same types of stresses already present in the body, the treatments cause more effective and sustained stress, especially when combined.

### 2.1.2 Therapeutic stresses

Tumor cells have already found ways to survive the numerous internal stresses covered in the previous section, so it is of no surprise that glioma cells are often able to find ways around common therapies due to the similarity of the mechanisms of action of the interventions with the mechanism of the body's natural defenses. Table 1 lists some of the common and experimental therapies used to treat glioma in addition to the type of stress they cause. While the mechanisms of action are diverse, the treatments cause the same stresses already encountered by the glioma cells during the body's intrinsic response to aberrant cell growth.

Surgery is almost always used on patients who are surgical candidates. Debulking of the tumor not only allows for better brain function but also allows chemotherapies to be more effective by working on a smaller population. While surgery should be undertaken in situations where critical structures will not be disrupted, the act is extremely stressful on the brain. Small areas will be cut off from the blood supply creating a hypoxic and nutrient deprived environment leading to metabolic stress for unremoved cancer cells. The death of neighboring cells along with the immune response will cause an increase in oxidative stress and ROSs production.

Radiation is another first-line therapy against gliomas. While there are many variations of radiotherapy, ionizing radation (IR) tends to work through two basic mechanisms that ultimately damage the DNA by either charged particles or photons. In the case of photon radiation, like in intensity modulated radiation therapy (IMRT), this technique causes indirect damage that occurs after water is ionized producing free radicals. Double-stranded DNA breaks (DSBs) are the most significant cause of cell death. Photon radiotherapy requires well-oxygenated tumors to create the damaging free radicals, which requires adequate blood supply to all areas of the tumor. Because many areas of gliomas are hypoxic, this technique is often relatively unsuccessful long-term in many brain tumors (Harrison et al., 2002). Particle therapy, on the other hand, works by directly damaging the DNA by charged particles. Direct damage can occur through transfer of energy from charged particles like proton, carbon or boron ions that do not require oxygen. These particles can cause DBSB themselves. In either case, there are free radicals and ROSs produced by radiation; the ROSs are necessary for the efficacy of the treatment, further injuring cells (Dal-Pizzol et al., 2003). However, these reactive molecules are released during cell death causing increased stress to surviving cells. The body mounts an immune response to repair and clear damaged cells. Although many patients with gliomas take steroids to reduce the swelling and inflammation produced by radiotherapy, remaining neoplastic cells are still subjected to large amounts of stress, killing many while further transforming others into radioresistant tumor cells.

| Therapy | Mechanism of Action | Form of Stress |
|---|---|---|
| **Surgery** | **Evacuation of tumor site** | **De-vascularization with subsequent hypoxia and nutrient deprivation, immune response** |
| Radiation<br>External beam radiation<br>Brachytherapy | Ionizing radiation | Oxidative stress, DNA damage |
| Genotoxic Chemotherapy<br>Temozolomide, Procarbazine<br>Carmustine (BCNU), Lomustine<br>*Cis*-platinum, Carboplatin<br>Vincristine<br>Etoposide, Irinotecan | <br>Nonclassical alkylating agents<br>Nitrosourea alkylating agents<br>Platinum DNA crosslinkers<br>Mitotic inhibitor<br>Inhibits topoisomerase I or II | DNA and organelle damage due to interruption of replication, induces metabolic stress and ROSs |
| Monoclonal Antibodies<br>Bevacizumab<br>EGFR – Cetuximab, Nimotuzumab | <br>Anti-angiogenic<br>Tyrosine kinase inhibitors | <br>Hypoxia<br>Growth factor signaling inhibition leading to oxidative stress |
| Immunotherapies/Vaccines* | Immune system response | Oxidative stress, DNA damage, metabolic stress |
| Small Molecule Targeted Therapy*<br>EGFR – Gefinitib, Erlotinib<br>PDGFR – Imatinib<br>mTOR - Everolimus | Tyrosine kinase inhibitors | Growth factor signaling inhibition leading to oxidative stress |
| | | |

Table 1. Common therapies and the stresses they cause. * Indicates experimental therapies

Chemotherapy is often used in conjunction with surgery and radiotherapy. Most of the common genotoxic chemotherapies for glioma produce their effects by disrupting the DNA strands. Alkylating agents like temozolomide (TMZ) and carmustine (BCNU) primarily work by alkylating the guanine base of DNA leading to cross-linking of the DNA strands which causes the strands to be unable to uncoil and separate. The platinum drugs, such as carboplatin and cis-platinum, work similarly by using the platinum ion to cross-link the guanine base pairs on the DNA strand. These therapies are more toxic to cells that replicate and proliferate faster, thus making cancer cells more sensitive than normal cells to genotoxic therapy. The stresses to the cell caused by chemotherapy are mostly due to interference of mitosis and induction of DNA repair mechanisms. When the cell is unable to unwind and repair its DNA, it causes apoptosis and metabolic stress. As apoptosis continues, ROSs are released into the ECM affecting nearby cells. Genotoxic stress through ROSs is dependent on

activation of SAPK/JNK pathway (Benhar et al., 2001). Thus, chemotherapy stresses glioma cells through different mechanisms.

A general chemotherapy-induced stress response is seen in many types of cancer cells. This is a response to anti-neoplastic agents that can destroy many cancer cells but induce survival and resistance mechanisms in others. In yeast, stress changed the cell cycle and lead to increases in *de novo* protein synthesis, proliferation, HSP90 expression, and proton pump levels. The first line of defense in severe shock is *de novo* synthesis of protective proteins (Miligkos et al., 2000). Increasing key membrane component proteins can up- or down-regulate their efficacy to restore ionic balance. Changes in the heat shock protein (HSP) population of the cell due to chemotherapeutic stress also increase HSP27 and HSP70 in resistant cells; these cells are translocated to the nucleus in response to stress, increasing protein synthesis necessary for resistance (Nadin et al., 2003). Whole body response is also important as hormones production levels can change during the stress response; such hormones can affect the cell cycle or gene transcription. On a smaller scale, cell-to-cell interactions occur between transformed and non-transformed cells involving the transfer of survival signals, thus indicating that the extracellular environment is important. The stress response to chemotherapy-induced hyperthermia can even lead to induction of drug resistance through a general increase in MDR P-glycoprotein production (Benhar et al., 2001).

Anti-angiogenic therapies are becoming more common in glioma and are used to combat the tumor vasculature. Most of the inhibitors, like bevacizumab, are monoclonal antibodies that work by antagonistically binding vascular endothelial growth factors (VEGFs), the factors responsible for signaling growth of blood vessels. Contrary to other cancers, it is thought that anti-angiogenic drugs in glioma could work by transiently normalizing the tumor vasculature (Nagy et al., 2010). As discussed previously, tumor blood vessels are abnormal and unstable due to the mixture of pro- and anti-angiogenic factors. An angiogenesis inhibitor would override many of these signals. Although, this might decrease blood vessel formation, it might also stabilize the existing vasculature. By normalizing the vasculature, there could be improved delivery of chemotherapeutic agents. Either way, the neoplastic cells would be subject to stress caused either by hypoxia and nutrient deprivation or increased concentrations of anticancer drugs.

Progress in targeted therapies for glioma has been made in recent years. Numerous small molecule inhibitors are being tested in clinical trials to antagonize the commonly mutated or over-expressed growth factor pathways. These inhibitors, like erlotinib which works on EGFR and imatinib for PGDFR, work by intracellularly binding the tyrosine kinase receptors, interrupting the downstream PI3K and MAPK signaling cascades. Monoclonal antibodies like cetuximab and nimotuzumab (EGFR inhibitors) work similarly, except they bind extracellulary to the growth factor receptors. Most of the stress caused by these antagonists is through decreased growth factor signaling and the resulting metabolic stress.

All these therapies cause stresses already encountered by tumor formation, but the duration and severity of the stresses during therapy is more extreme. Prolonged exposure to these stresses can induce cell death programming more effectively than short, intermittent periods. However, in most cases, some cancer cells do survive. They evade the immune system and death signals, selected for by their unique mutations leading to therapy resistance. It is therefore important to determine accurate markers to identify these cells and to classify the mechanisms through which they survive.

### 2.2 Markers of stress

While glioma cells may be adept at surviving cellular stress, they do show indicators of the stresses they endure. These indicators or markers may eventually be exploited to determine what stresses the cancer cells are under, and thus what types of stress they may be more susceptible to if subjected further. This section will cover the most common markers of general cellular stress and the specific stresses mentioned previously.

When cells encounter stress, certain elements of the stress response are universal. There is a highly conserved minimal stress proteome that is shared among species. In a paper by Dieter Kültz, a list of the 41 proteins needed for the minimal stress proteome was compiled (Table 2).

While this table is not exhaustive for all proteins involved in the stress response, nor does it list the most reliable markers, it does indicate that cells all have a fundamental basic response to stress. The response is referred to as the conserved stress response (CSR). Various stresses may induce different proteins and markers, but certain responses are unchanged between stresses, even amongst species.

The general response of cells to stress originally focused on three types of proteins: heat shock proteins (HSPs), glucose-regulated proteins (GRPs) and ubiquitin-associated proteins, all of which are inter-related (Feder & Hofmann 1999). Of these three types of proteins, HSPs have been studied the most thoroughly. HSPs are induced during stress as a protective mechanism. While HSPs ordinarily play a more mundane role in the cell, folding proteins into their appropriate tertiary structures and facilitating steroid hormone binding, the subjection of cells to stress activates heat shock transcription factors (HSFs), allowing the transcription of stress-related HSPs like HSP27, HSP70 and HSP90 (Calderwood et al, 2006). In many gliomas and other cancers, binding of HSP90 to p53 mutants in the cytoplasm can further the damage caused by stress (Goetz et al., 2003). This is because p53 functions in the nucleus, and it leads to enhanced HSP70 transcription which allows for cancer cell growth (Ciocca & Calderwood, 2005). These HSPs, along with others, have been linked to cancer therapy resistance.

The unfolded protein response (UPR) has gained increasing coverage as a fundamental stress reaction caused by changes in the celluar redox potential, energy status, or Ca2+ levels leading to unfolded or misfolded proteins within the lumen of the endoplasmic reticulum (ER). This is also known as ER stress (Herr & Debatin, 2001). ER stress is closely linked to hypoxia and glucose depletion. Misfolded proteins can be a problem due to their propensity to aggregate together and cause harmful accumulations. The role of UPR is to stop protein translation, arrest the cell cycle, and to signal pathways that increase activation of protein folding chaperones, some of which are HSPs. Ultimately, UPR leads to cell death through apoptosis if translation is halted for a prolonged period. GPRs are related to UPR and are actually just specialized HSPs that are found in the ER of the cell. In fact, Grp78 is the protein responsible for chaperoning the misfolded proteins and signaling downstream activators of the UPR. Another GRP, grp94 or HSP90B1, is actually essential for immune responses as it is a chaperone that regulates both innate and adaptive immunity through secretory pathways (Maki et al., 1990). Upregulation of these proteins is often seen during stress, and thus could represent markers for stress induction.

Many stresses signal through the stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK) pathway, which is activated by numerous extracellular signals and stresses.

| Minimal Stress Proteome | | |
|---|---|---|
| **Redox regulation** | **DNA damage sensing/repair** | **Fatty acid/lipid metabolism** |
| Aldehyde reductase | MutS/MSH | Long-chain fatty acid ABC transporter |
| Glutathione reductase | MutL/MLH | Multifunctional beta oxidation protein |
| Thioredoxin | Topoisomerase I/III | Long-chain fatty acid CoA ligase |
| Peroxiredoxin | RecA/Rad51 | |
| Superoxide dismutase | | |
| MsrA/PMSR | **Molecular chaperones** | **Energy metabolism** |
| SelB | Petidyl-prolyl isomerase | Citrate synthase (Krebs cycle) |
| Proline oxidase | DnaJ/HSP40 | $Ca^{2+}/Mg^{2+}$-transporting ATPase |
| Hydroxyacylglutathione hydrolase 6 | GrpE (HSP70 cofactor) | Ribosomal RNA methyltransferase |
| NADP-dependent oxidoreductase YMN1 | HSP60 chaperonin | Enolase (glycolysis) |
| Putative oxidoreductase YIM4 | DnaK/HSP70 | Phosphoglucomutase |
| Aldehyde dehydrogenase | | |
| Isocitrate dehydrogenase | **Protein degradation** | **Other functions** |
| Succinate semialdehyde dehydrogenase | FtsH/proteasome-regulatory subunit | Inositol monophosphatase |
| Quinone oxidoreductase | Lon protease/protease La | Nucleoside diphosphate kinase |
| Glycerol-3-phosphate dehydrogenase | Serine protease | Hypothetical protein YKP1 |
| 2-hydroxyacid dehydrogenase | Protease II/prolyl endopetidase | |
| phosphogluconate dehydrogenase | Aromatic amino acid aminotransferase | |
| | Aminobutyrate aminotransferase | |

Table 2. The minimal stress proteome as described by Kültz, 2005.

These kinases are part of the larger superfamily known as mitogen-activated protein kinases (MAPKs), which control many intracellular events. SAPK is activated by SEK1 or MKK4. The SAPK/JNK pathway is activated by stresses like hypoxia, radiation, drug therapy, ROSs, and inflammatory molecules (Benhar et al., 2001). They signal through a variety of receptors, including G-protein coupled receptors (GPCRs), cytokine receptors (TNFα), death

receptors (Fas), and antigen receptors. SAPK/JNK pathways can control proliferation, apoptosis, transformation and differentiation along with migration. In response to many types of stress such as radiation and hypoxia, this pathway signals for mitochondrial-dependent apoptosis (Sanchez-Prieto et al., 2000). Thus, the SAPK/JNK signaling cascade is a protective mechanism for cells. However, any mutations or aberrant signaling could also lead to further glioma progression. Up-regulation of proteins involved in these pathways is a good indicator of cellular stress.

Additionally, other markers of general stress have also been found. The MDR1 (multi-drug resistance 1) gene, which encodes the P-glycoprotein responsible for reducing drug accumulation in cancer cells, is actually induced by stresses like acidity, drug treatment, and radiation (Szabo et al., 2000). Thus, cancer cells under stress have created multiple mechanisms to evade cell death. The original intent for non-transformed, normal cells was for them to be able to pump out toxins encountered in their environment for survival purposes. Transformed cancer cells have adapted those responses to their own needs.

Some indicators of specific stresses have also been revealed. An example of a marker for specific stress can be found in hypoxia. The transcription factor HIF-1 (hypoxia-inducting factor-1) is a major regulator of the cellular hypoxia response, which binds to hypoxia-responsive elements (HREs) leading to the transcription of genes involved in cell survival, metabolism, angiogenesis and invasion. It can increase expression of glycolysis genes and VEGF protein (Jiang et al., 1996). The expression of HIF-1 is increased in glioma usually through induction by EGFR signaling the PI3 kinase pathway and loss of the tumor suppressors p53 and PTEN. HIF-1 expression, and thus hypoxic stress, in tumors can be determined by immunohistochemical staining. Another indicator of stress linked to HIF-1 is NF-κB induction. NF-κB activation leads to the rapid transcription of important genes involved with the stress response. Because it is a transcription factor, it is often thought of as a first line of defense, especially against activators of the immune system (Garg & Aggarwal, 2002). NF-κB is able to regulate many proteins involved in proliferation and survival, including HIF-1. Another isoform, HIF-2α, appears to be a specific marker for pH imbalance as it is increased with exposure to acidic stress (Hjelmeland et al., 2010).

Overall, there are numerous markers of stress in glioma cells. Many are the result of a general response to stress, but as research continues, better markers for specific stress, like HIF-1 in the case of hypoxia, will be developed as our understanding continues to grow. These markers may eventually help clinicians to positively identify the stresses the tumor is under, which will inevitably lead to more effective glioma treatment.

### 2.3 Mitigation of metabolic and therapeutic stress by autophagy

Glioma cells are able to employ several ways to overcome stress and the resulting energy depletion. Autophagy, the catabolic recycling of the cell's own components, takes advantage of this idea as a survival mechanism. The autophagic process allows the tumor cells to reallocate amino acids, fatty acids and other macromolecules for energy and go into a hibernation-like state until the surrounding environment is more favorable. Glioma therapies, like temozolimide and etoposide, have been shown to induce autophagy as have various metabolic stresses. While autophagy is also viewed as a cell death mechanism, new research indicates that autophagy can actually be responsible for glioma cell survival and resistance to therapies and stresses. Molecules like beclin-1 and elongation factor-2 kinase (EF-2K) have been shown to play a critical role in the autophagic response in glioma.

### 2.3.1 Role of autophagy in stress response

Autophagy was first discovered as a Type II programmed cell death (PCD) mechanism with distinct differences from apoptosis in yeast. Most initial research was done in yeast, and later, it was found that many of the proteins involved are conserved in higher eukaryotes with human homologues. During autophagy, double membrane autophagosomes or autophagic vacuoles engulf cytoplasm along with long-lived proteins and damaged organelles through a process of self-digesting. The enveloped contents of the autophagosome are degraded by fusing with the lysosome. Their constituents of fatty acids and amino acids are recycled into new molecules or shunted into the synthesis of ATP to meet energy needs. If left unchecked, autophagy does indeed lead to cellular destruction and canabolitic cell death. Because autophagy occurs in many cells immediately preceding cell death, it was initially seen as a cell death mechanism (Levine & Yuan, 2005). However, cells can also utilize autophagy as a means to go into a cellular "hibernation" state where they have decreased energy needs as a strategy of survival (Kuma et al., 2004). Thus, autophagy can also be seen as a temporary protective response of cells.

Autophagy is known to occur in the brain during neurodegenerative processes such as Alzheimer, Parkinson, and Huntington disease. However, its role as a protector or cause of disease is debated (Nixon, 2006). As for its role in glioma, cancer cells were shown to have decreased levels of autophagy compared to non-malignant cells, while nutrient deprivation upregulated its autophagic activity in cancer cells (Wu et al., 2006). This could be due to the early stages of tumorigenesis needing increased protein synthesis and proliferation, where autophagy would impede the growth of the cells. Also, since autophagy removes damaged organelles, it can decrease the mutation rate of the cancer cells, leaving them at a disadvantage during early stages of the disease. Later stages of glioma progression see an increase in autophagy to protect against the numerous cellular stresses present at this stage like nutrient deficiency.

An important mechanism of regulation of autophagy is through the PI3K-Akt-mTOR pathway, which is activated in many cancers. Suppression of autophagy occurs through class 1 PI3K, while class III PI3K promotes autophagosome development (Lum et al., 2005). This is because class III PI3K binds to a molecule known as beclin-1 (BECN1). Beclin-1, the homologue of yeast Atg6, is part of an early-autophagy complex that participates in the autophagosome formation (Liang et al., 1999). It is also known to interact with the anti-apoptotic protein Bcl-2, binding the molecule and preventing cell death (Erlich et al., 2007). Bcl-2 may regulate the balance between autophagy and apoptosis, since it plays a role in both. Down-regulation of Bcl-2 or up-regulation of its binding protein, BNIP3, induces autophagy. Beclin-1 expression has been shown to be aberrant in cancers, including glioma (Liang et al., 2006). When beclin-1 binds class III PI3 kinase, the complex can activate autophagy. This complex is located in the cell cytoplasm and trans-Golgi network where it can sort the necessary autophagosome components. It, in turn, can be regulated through miRNA miR-30a which is able to down-regulate beclin-1 and thus regulate autophagy. miR-30a expression in glioma was initially implicated in a miRNA screen for differential expression during conditions that induce autophagy and was found to be decreased during autophagy. Further studies with the miRNA showed that expression of miR-30a in glioma cells decreased beclin-1 expression and decreased autophagy (Zhu et al., 2009). This was the first report of miRNA regulating autophagy, so it is certainly possible that other miRNA's may play a role in this process.

The formation of the autophagosome is mediated by a series of autophagy specific genes (ATGs) originally identified in yeast like Beclin-1. Other important human counterparts of regulators of autophagy have been found, such as LC3, a mammalian homologue of yeast gene Atg8, which is one of the primary markers of autophagy. It is a ubiquitin-like protein which cooperates with Atg4 protease (Mizushima, 2004). Other autophagy proteins include another system of an Atg12-Atg5-Atg16 complex in addition to gene products like Atg5, Atg7, and Atg10, which also play roles in activating autophagy.

Another regulator of autophagy is the protein synthesis inhibitor, elongation factor-2 kinase (EF-2K). EF-2K, a $Ca^{2+}$/calmodulin kinase, halts translation by phosphorylating elongation factor-2 (EF-2), a protein responsible for moving the peptide strand along the ribosome during elongation. It does so through hydrolyzing GTP to GDP, which provides the energy for elongation. Phosphorylation of EF-2 negatively reduces its affinity for the ribosome (Ryazanov et al., 1991). Regulating this energy-consuming step in translation is important to cell survival during periods of stress and reduced nutrients, so it is unsurprising that EF-2K protein and activity levels are found to be increased in glioma and other cancers.

The first link of EF-2K to cellular stress was found in hibernating squirrels. During hibernation, decreased respiration and blood flow along with abstinence from food intake, greatly reduces the amount of oxygen and nutrients that are available to cells. In tissues with high metabolic rate, both p-EF-2 and EF-2K levels were found to be increased(Chen et al., 2001). Further studies in cells and mouse models showed increases in EF-2K during nutrient deprivation, hypoxia, radiation exposure, and drug treatment. As EF-2K is a calmodulin kinase known to be activated by calcium flux, ER stress was also found to be dependent on EF-2K status (Py et al., 2009). Unsurprisingly, it was later discovered that EF-2K regulates autophagy. Down-regulation of EF-2K reduces autophagy and increases cell death during stress in glioma (Wu et al., 2006).

EF-2K is regulated through the PI3K/mTOR/S6 kinase pathway by nutrient and growth factor availability, which links together cellular stress and the autophagic response, as they are regulated by the same pathway (Hait et al., 2006). In fact, disruption of the PI3K/mTOR/S6K pathway is known to induce autophagy, probably through EF-2K activation (Fig. 2). Nutrients and growth factors activate mTOR that in turn activates S6 kinase. Both mTOR and S6 kinase negatively regulate EF-2K by phosphorylating it on Ser 78 and Ser366, respectively (Browne & Proud, 2004). This inhibits EF-2K activity and its induction of autophagy, since nutrients and cellular building blocks are plentiful. Nutrient deprivation not only inhibits mTOR signaling and regulation of EF-2K, but it also increases AMP kinase activity due to the depletion of ATP. AMP kinase can positively regulate EF-2K activity by phosphorylating it on a different site, Ser 398, leading to its activation and induction of autophagy, as measured by autophagic markers, like LC3 and acidic vacuole organelle staining (Browne et al., 2004).

It is through the mTOR/S6 kinase pathway that cellular stress can cause the induction of autophagy. Some stresses, dependent on severity and duration, can instantaneously cause both apoptosis and autophagy, while the same stress under different conditions make cause one or another. While it is not yet clear why one pathway is chosen over another, new studies into the induction of autophagy have helped to determine the conditions under which it is stimulated. Disruption of the PI3K/Akt pathway has been associated with autophagy induction as well as stimulation of the AMP kinase pathway. mTOR inhibits autophagy through activating one of its downstream targets, S6 kinase (Abeliovich, 2003).

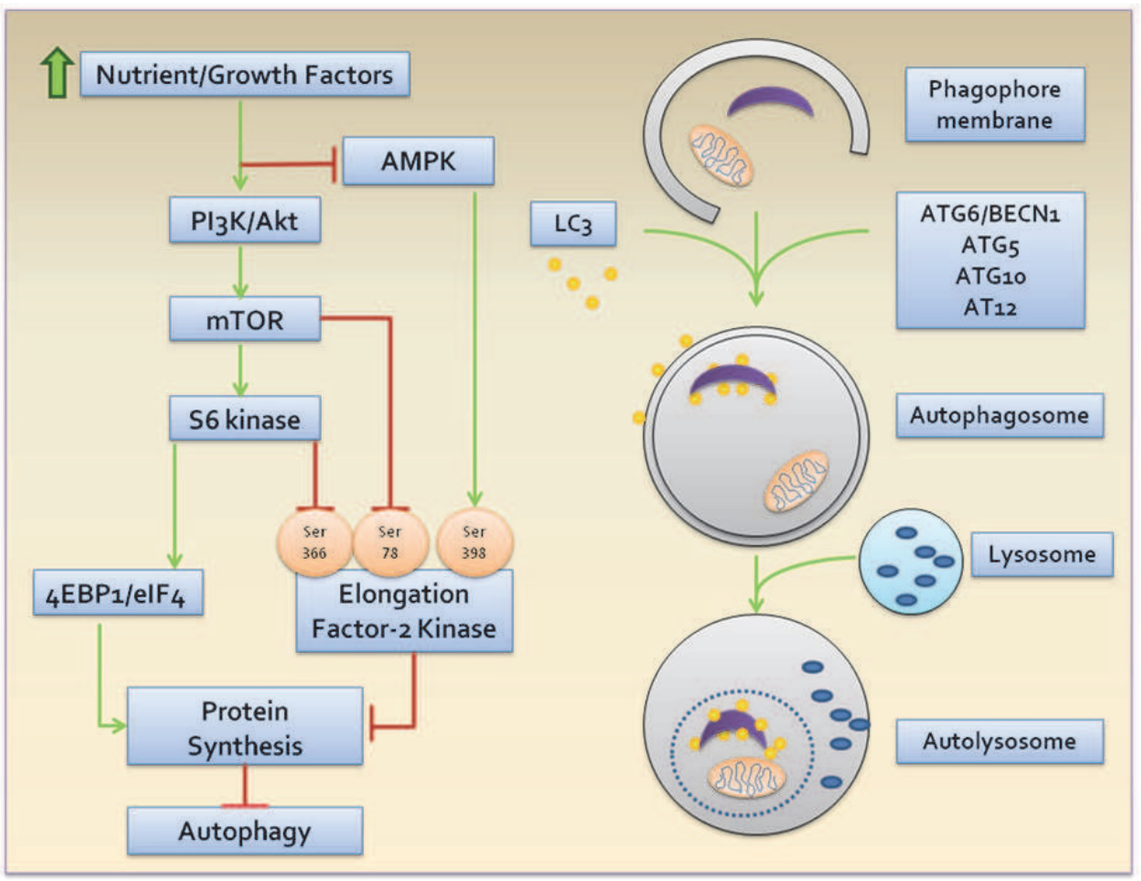


Fig. 2. Autophagy machinery and its regulation by EF-2K.

Many neoplastic cells, including glioma cells, survive metabolic stress through autophagy. Autophagy can originally act as a tumor suppressor due to the inactivation of apoptosis and subsequent immune reaction. In fact, autophagic markers localize i*n vivo* to areas of tumors that are undergoing metabolic stress (White, 2007). Autophagy supports metabolic functions during periods of starvation by cannibalizing and recycling need elements, in short, providing alternative energy sources. Initially, autophagy in non-malignant cells can help prevent tumorigenesis through the removal of damaged organelles and defective proteins, since accumulation of these particulars can lead to oxidative stress. In fact, research shows that it even protects the genomic stability (White, 2007). However, as genetic mutations from other sources accumulate, autophagy allows compromised cells to survive. Defective autophagy can lead to cell death through apoptosis and necrosis, which further stresses neighboring cells through the recruitment of inflammatory molecules. Damage can occur to cellular DNA, creating further genomic instability. Increased mutation rate can further tumor progression at the expense of some cells.

Stresses known to induce autophagy include starvation, ER stress, mitochondrial damage, protein aggregation, radiation, hypoxia, and pathogens stimulation. Failure of autophagy has been reported to be the mechanism behind cell damage accumulation and aging.

Starvation is readily linked to autophagy through activation the mTOR pathway. Other stresses regulate autophagy in different manners. During hypoxia, autophagic clearing of damaged mitochondria is advantageous to cells. The source of pro-apoptotic signals and ROSs is removed, and thus the cancer cell is able to survive even under oxygen deprived conditions. Bcl-2 family protein, BNIP3, is known to induce autophagy during hypoxia, while others believe it induces apoptosis especially after the cellular environment becomes too acidic from hypoxia (Azad et al., 2008). Metabolic stress activates the p53 pathway, which can normally induce apoptosis through proteins like Puma and Noxa, but metabolic stress increases Bim instead, which signals through Bax and Bak (Vousden & Lane, 2007). Therefore, autophagy can suppress apoptosis during both hypoxia and metabolic stress in glioma.

In short, autophagy plays a critical role in glioma cell survival during various stresses. While autophagy can lead to cell death if not properly regulated, neoplastic cells can also use it as a protective mechanism. The autophagic response is activated by the typical hazards encountered by glioma cells during tumorigenesis, helping the cells to survive periods of limited oxygen and nutrients. Thus, exploiting autophagy as a therapeutic intervention is a subject that has been actively explored.

### 2.3.2 Glioma treatments and autophagy

Since autophagy can serve as a cell survival mechanism, it is unsurprising that cancer cells would adapt to use it to their advantage. Not only can neoplastic cells survive intrinsic stresses through autophagy, but they can use it to evade therapeutic interventions. Autophagy-associated therapy resistance is gaining recognition as a key resistance mechanism. Glioma cells tend to undergo autophagy rather than apoptosis, perhaps due to their advanced nature created by genomic instability. Autophagy has been shown to be induced in a wide range of glioma therapies.

Radiation was the first therapy shown to cause glioma cells to undergo autophagy. As stated previously, the main mechanism of damage caused by radiation is through DNA double-strand breaks (DBSs), which can lead to translocation, misrepair, and even loss of chromosomes. Gamma radiation is known to induce autophagy in human glioma cells, but there is some controversy as to whether it causes cell death or if it protects cells (Paglin et al., 2001). This could be due to autophagy playing different roles at different times, acting as a cell death mechanism during early stages and acting as a protective mechanism later in tumor development after the accumulation of more advantageous mutations. Autophagy itself is also regulated on many levels and at different stages of induction, producing differing effects in glioma cells. Inhibiting autophagy is sometimes protective and other times destructive, indicating that autophagy is a sensitive modulator of cell survival. Even with glioma cells using autophagy as a way of survival, prolonged radiation may eventually switch the autophagic program from cell survival to cell death as too many damaged proteins accumulate. Glioma cells treated with autophagic inhibitors were radiosensitized, and radiation was able to create more DBSs (Ito et al., 2005).

Many chemotherapies cause autophagy in glioma. Mainstay treatment, temozolomide (TMZ), is used for high-grade gliomas (late stage). It is a small, lipophilic agent that easily passes through the BBB. Although referred to as an alkylating agent, TMZ does not actually cause cross-linking but instead adds a methyl group to a guanine which gets

mispaired with thymine instead of cytosine during the next cycle of DNA synthesis. Initial research into the mechanism of cell death by the drug indicated that TMZ causes cell death through autophagy not through apoptosis (Kanzawa et al., 2004). . However, not much cell death occurred, and it was eventually discovered that glioma cells were using autophagy initially as a protective mechanism as glioma cells were able to start proliferating again after a week of TMZ treatment. This could be due to the previously stated idea that inhibiting autophagy at different stages of induction leads to different outcomes. Before the recruitment of LC3, cells can be rescued from autophagy by treatment with 3-MA (inhibitor of PI3K) (Kanzawa et al., 2004). Bafilomycin A1, an inhibitor of lysosomal ATPase and atuophagy, in combination with TMZ induces apoptosis.

While TMZ induces autophagy and not apoptosis in glioma cell lines, another alkylating agent cisplatin induces both apoptosis and autophagy. Apoptosis is further activated if autophagic inhibitors were added to cisplatin-treated glioma cells due to the release of Bcl-2 from beclin-1. Thus, cisplatin also utlizes autophagy as a protective mechanism (Harhaji-Trajkovic, 2009). Additional studies were done on TMZ and etopside showing that autophagy clearly protects cells from the multimicronucleation and cell death normally associated with TMZ and etoposide treatment. This was discovered through the detection of a concomitant ATP-surge that occurred with treatment. The associated unsustained ATP surge was not through glycolysis but through a brief period of oxidative phosphorylation. This was due to an induction of autophagy that increased catabolic metabolism to increase ATP levels (Katayama et al., 2007). Many chemotherapeutic agents have also been shown to cause hyperthermia in areas of tumors due to increased inflammation and stress in glioma cells. Hyperthermia itself is also known to induce autophagy, adding another mechanism by which glioma chemotherapies are able to activate autophagic response (Sanchez-Prieto et al., 2000).

Growth factor inhibitors are in the experimental stage of glioma therapy development. Platelet-derived growth factor receptor (PDGFR) antagonists, such as imatinib, and epidermal growth factor receptor (EGFR) antagonists, like erlotinib, have been developed to inhibit the growth signals transmitted through these pathways. Autocrine signaling of growth factors can occur, with glioma cells over-expressing both the growth factor and its receptor together to signal through PI3K pathway. Inhibition of PDGF and EGF signaling induced autophagy but not apoptosis (Takeuchi et al., 2004). This result could be due to inhibitory effect of class I PI3K and/or stimulatory effect of class III PI3K. Downstream targets are also available for inhibition of growth factor pathways. Rapamycin, the inhibitor of mTOR, was able to induce autophagy along with suppressing proliferation. Since mTOR regulates both cell proliferation and autophagy, this could be a good target for future combined therapies. Combining rapamycin with an Akt or PI3K inhibitor increased glioma cell death, and future studies will look at the combination of rapamycin with growth factor inhibitors (Takeuchi et al., 2005).

Another category of experimental therapies, glycolytic inhibitors, work similarly to nutrient deprivation as they have preferential uptake by glioma cells that are normally dependent on high levels of glucose to satisfy their rapid glycolysis needs. 2-deoxy-D-glucose (2-DG) is a glycolytic inhibitor that blocks the effects of glucose on metabolic pathways. It had previously been shown to inhibit growth of cancer cells and enhance the efficacy of other glioma treatments. 2-DG causes oxidative stress in glioma cells which lead to the discovery

of its induction of autophagy. The glycolytic inhibitor activated EF-2K and thus autophagy in a PTEN-independent manner. Inhibition of EF-2K blocked autophagy induction by 2-DG, thereby sensitizing the cells to 2-DG cell death through caspase-3 apoptosis. Cells under additional stress like hypoxia were further sensitized by concurrent treatment of 2-DG and EF-2K inhibition (Wu et al, 2009).

Thus, it appears that glioma cells have used autophagy to resist a wide range of current glioma therapies. Although originally thought to be a mechanism of cell death, autophagy obviously plays a major role in protecting glioma cells from therapeutic intervention. Studies do indicate, however, that inhibiting autophagy may re-sensitize cancer cells to currently used treatments, providing a way around tumor resistance.

### 2.4 Stress and glioma cancer stem cells

Neural stem cells (NSCs) are specific to the central nervous system and are multipotent able to generate neurons, astrocytes, and oligodendrocytes. Like other stem cells, they are self-renewing, proliferative, and quiescent until needed. NSCs are common during human embryonic development but are reduced in number and sequestered to specific regions of adult brains. These tiny subpopulations of cells can be recognized by their CD133+ status. In recent years, there has been a new consensus that gliomas contain a glioma cancer stem cell (GCSC) population in addition to other precursor and differentiated cancer cells. Thus, gliomas can express both neuronal and glial markers. There is accumulating evidence that NSCs are key players in tumor initiation and progression along with angiogenesis and dissemination. Thus, their presence is starting to redefine how therapy outcomes are determined and understanding their role in tumor progression and therapy resistance may be pivotal in improving patient prognoses.

For years it was thought that humans were born with all the brain cells that they were ever going to have and that mitosis of neural and glial cells only occurred during early development. While most cells in the CNS do exit the cell cycle as terminally differentiated cells early in life, it has come to light that neurogenesis continues throughout life in small areas of the brain including the subventricular zone (Lois & Alvarez, 1993) and the dentate gyrus (Kuhn et al., 1996). These locations are home to NSCs that exhibit the normal stem cell markers and are capable of migration and multipotency. The existence of these NSCs that are normally present in the brain provides precedence for the idea of mutipotent cells in the CNS and gliomas. As gliomas are known to be highly heterogeneous tumors with cells from multiple neural lineages, cancerous neural stem cells could explain this finding. Poor prognosis has been linked to glioma tumor heterogeneity (Pallini et al., 2008), which could be the result of GCSCs.

#### 2.4.1 Stress-induced stem cell markers

Although, at present, there are no universally accepted markers of GCSCs, this section will cover known NSC markers along with frequently studied glioma stem cell markers (Fig. 3). Small side populations (SP) of glioma stem cells (0.01-5% of total cells) exist that rarely divide despite elevated proliferation potential (Hirschmann-Jax et al, 2004). These were first found through flow cytometry studies where a small SP of tumor cells could be sorted and differentiated from the rest of the population. These cells were shown to efflux the fluorescent nucleic acid-staining dye, Hoechst 33342 (Pattrawala et al, 2005). When isolated, this small percentage of cells was able to generate neurospheres and xenografts, which are

key stem cell properties. The SPs were able to efflux both the dye and chemotherapeutic agents through up-regulation of an ATP-binding cassette (ABC) member, BCRP, which is involved in multi-drug resistance (Eramo et al., 2006). These SPs were the first indicator that there was probably a cancer stem cell population in gliomas.

The two most common markers of neural stem cells are CD133 and nestin. CD133, also known as prominin-1, is a cell membrane glycoprotein which is present on different types of stem cells and cancer cells while being down-regulated on differentiated cells (Uchida et al., 2000). CD133+ cells can be isolated from human brain tumors and are able to demonstrate stem cell properties *in vivo* like accelerated tumor growth and invasion (Singh et al., 2004). They pass the gold standard for determining stem cell properties, which is that cells must be able to initiate formation of tumor similar to the patient's and is able to undergo serial transplantations. These cells have increased levels of stem cell genes such as nestin, Msi-1, MELK, and CXCR4 (Lui et al., 2006). Recent studies indicate that CD133 expression may be linked to periods of angiogenesis or times of stress. In fact, hypoxia can induce a CD133+ brain tumor stem cell population. CD133+ cells are resistant to drug treatment and apoptosis with increased expression of several ABC transporters and DNA repair machinery (Eramo et al, 2006). These cells also show an increase in chemokine receptor CXCR4 that directs NSC migration and thus GSCS movement (Lui et al., 2006). Even though it appears that CD133+ status is indicative of a NSC, CD133- cells can still exhibit stem cell properties (Wang et al., 2008). Therefore, CD133 status is not the final determinant of stem cellness for glioma cells.

The another common marker, nestin, is an intermediate filament protein produced by NSCs that controls cellular morphology, proliferation, and adhesion. Differentiated cells down-regulate nestin and increase other neurofilaments involved in neurons and glial cells that have exited the cell cycle (Zimmerman et al., 1994). Nestin expression increases during stress like ischemia, traumatic brain injury, inflammation, and tumor progression (Holmin et al., 1997). As a glioma marker, it indicates an increased malignant potential in invasion and motility abilities associated with poor prognosis (Stronjnik et al., 2007). It was one of the first discovered NSC markers but is not ideal due to its cytoplasmic location. Thus, sorting methods like flow cytometry cannot be used to separate stem cells from non-stem cells according to nestin status.

Other stem cell markers may be more useful due to their location on the cell surface. A2B5 is cell surface marker of neural progenitor cells. It is a ganglioside normally found in cells in the subventricular zones that host NSCs (Nunes et al., 2003). It has recently been reported that cells in GBM have been found that are A2B5+ and exhibit stem cell properties like tumor initiation. In fact, A2B5+/CD133- cells are also capable of initiating tumors and forming neurospheres (Tchoghandjian et al., 2009). Thus, A2B5 status is a potential useful marker for stem cells in gliomas and should be added to initial screenings. Stage-Specific Embryonic Antigen -1 (SSEA-1), which is also known as CD15 or Lewis-X Antigen, is another cell surface antigen. It is a carbohydrate moity that associates with glycoproteins and glycolipids. SSEA-1+ cells have increased stem cell gene expressions and properties. The majority of GBM tumors analyzed for the marker were SSEA-1+, and SSEA-1+ cells are highly tumorigenic while SSEA-1- cells are not (Son et al., 2009). Therefore, these two surface markers may play an important role in determining a GCSC population.

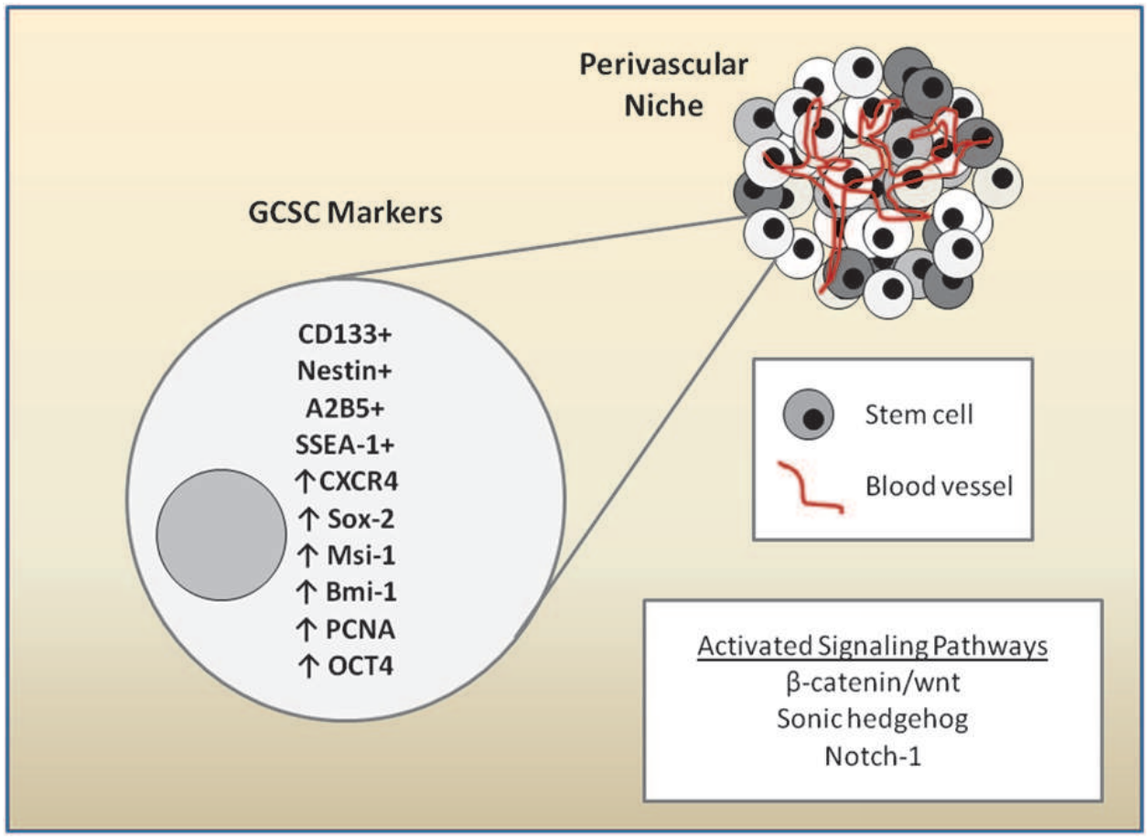


Fig. 3. Glioma cancer stem cell population in the perivascular niche. Common markers are listed.

Developmental pathways and other signaling pathways are also up-regulated in SPs, which later became indicators of stem cell activity (Hadnagy et al., 2006). The Wnt/β-catenin and the sonic hedgehog (SHH)/Gli1 pathways are both upregulated in GCSCs (Rich, 2007). Wnt signaling increases β-catenin activity. Over-expression of Gli1 and β-catenin have recently been shown to be correlated with poor prognosis (Pu et al., 2009), while EGFR and p53 were not predictive (Rossi et al., 2011). Some GCSCs have increased Notch-1 signaling, as do their healthy NSCs counterparts. Even PDGF status is linked to stem cells, as signaling of the growth factor is upregulated during oligodendrocyte proliferation and differentiation (Nait-Oumesmar et al., 1997).

Other stem cell pathways and molecules have been found in these populations. The Notch receptor pathway has also been implicated in GCSCs, as this signaling cascade mediates differentiation and proliferation (Koch and Radtke, 2007). Over-expression of Notch-1 leads to formation and proliferation of neurosphere-forming stem cells that are nestin-positive, while down-regulation leads to apoptosis (Hitoshi et al., 2002). Additional traditional stem cell markers such as Sox2, Bmi-1, PCNA, NANO, Msi-1, and OCT4 have all been found in these cells, indicating that gliomas do in fact have populations of cancerous cells that have stem-cell like properties and are probably GCSCs (Hemmati et al., 2003). This finding has a tremendous impact of glioma cell survival and important implications for new therapies regimens to treat patients with glioma.

### 2.4.2 Glioma cancer stem cells are obstacles to effective treatment

It remains a matter of debate whether GCSCs are derived from original NSCs or other progenitor cells. Some argue that cancer cells can actually transition from a differentiated cell back into an undifferentiated cell through epithelial-mesechymal transition (EMT) (Singh & Settleman, 2010). This would mean that signals that trigger EMT could also trigger cancer stem cell formation. Either way, it has been shown that stem cell pathways including Wnt/ β-catenin, SSH, and Bmi-1 can all be activated by common treatments used in glioma therapy, like ionizing radiation and TMZ, which may lead to resistance to radiation and chemotherapy (Bell & Miele, 2011).

Thus, regardless of the origins of the GCSCs, the presence of cancer stem cells would explain the seemingly inevitable recurrence of advanced gliomas. If cancer cells can either revert to a de-differentiated state or if original mutated stem cells from a tumor could survive a therapy, then the cancer could proliferate again. Stem cells are designed to survive assaults. They are a form of cellular dormancy that can wait until the cell encounters a more favorable environment, and then self-renew, proliferate, and create differentiated progeny that are suited to the present conditions (Hambardzumyan et al., 2008). GCSCs could shed light on why patients seem to have been cured of their cancer only to have it return months or even decades later after having undergone numerous surgeries, chemotherapies, and radiation treatments. Mounting evidence indicates that cancer stem cells are key to the survival or recurrence of glioma. In fact, current treatment regimens could worsen matters by putting the cancer cells under the exact stresses that actually select for GCSCs (Tetyana et al., 2010).

Stresses in different areas of a tumor could select for distinct subpopulations of normal tumor cells and CSCs. Since CSCs by definition are plastic, they are able to adapt to their current environment, at times lying dormant and at others, proliferating and differentiating cancer cells that have adapted through genomic instability that is inevitable with cancer progression. These cells are even able to create stromal support layers recruiting host cells to make some of the necessary growth factors and signals (Bao et al, 2006).

The GCSCs either migrate or produce their own local microenvironments or niches that created by cells and an ideal extracellular matrix (ECM) (Fig. 3). Niches shield the GCSCs from harsh environments present in the rest of the brain (Valshi et al., 2009). They support a specific mix of necessary growth factors, signaling molecules, and nutrients to sustain a stem cell population. Perivascular niches are also commonly home to healthy NSCs (Calabrese et al., 2007). In GBM, the niche is composed of vasculature that contacts the cells and allows secretion of factors that help to maintain stem cell quiescence. This increased density of microvessels is highly associated with GCSCs niches, which tightly regulates the availability of oxygen and nutrients while allowing the cells a means of migration to other areas if necessary. The stems cells continue to modulate this extracellular environment – they secrete VEGF and increase the number of endothelial cells, which in turn leads to increased GCSC and tumor growth (Gilbertson & Rich, 2007). Niches are abnormal in GCSCs because they cause the stem cells to renew and proliferate.

Protected by their ideal environment, GCSCs are easily able to resist common treatments. This is conceivable since even normal stem cells need to survive years of stress under normal circumstances. As mentioned previously, GCSCs express adenosine triphosphate

(ATP)-binding cassette transporters (ABC-transporters) such as MDR1 and breast cancer resistance protein (BCRP) that are able to efflux chemotherapeutic drugs. This allows stem cells to survive regardless of the type of chemotherapy delivered. Even when the cells cannot efflux all of the agent, GCSCs derived from patient tumors, when treated with common therapies, are able to show resistance within 48 hours with continued ability to proliferate (albeit at a lower rate) in the presence of the drugs (Eramo, 2006). Many of these cells also show increases in DNA-mismatch repair with over-expression of methyl guanine methyl transferase (MGMT) which is common in resistance to alkylating or alkylating-like agents (Jullierat-Jeanneret et al., 2008). Anti-apoptotic proteins like Bcl-2 and Bcl-XL were found to be over-expressed in the increased population of CD133+ cells GCSC treated with TMZ, carboplatin, or taxol, as were members of the inhibitor of apoptosis (IAP) family such as surviving (Lui et al., 2006). Numerous mechanisms and pathways have been associated with treatment resistance of GCSCs.

Not only are GCSCs able to survive therapeutic insults, but many interventions actually select for the cancer stem cells. Radiation has been shown to enrich for GCSC. These stem cells have increased survival advantages over their non-radiotreated counterparts. Radioresistant tumors showed increases in CD133+ status. Notch-1 signaling has also been shown to be activated upon radiation exposure (Scharpfenecker et al., 2009). All cells had the same amount of initial damage to DNA and organelles, but GCSCs were able to repair damage more quickly than matched non-stem cells (Bao et al., 2006). CD133+ cells are able to evade radiation damage by preferential activation of DNA damage checkpoints. Bao determined that the resistance to radiation can be partially circumvented by inhibiting cell cycle proteins, Chk1 and Chk2. Treatment with alkylating agents also increased GCSC populations, as determined by stem cell markers (Kang and Kang, 2007).

Due to the ability of gliomas to either induce stem cell dedifferentiation or to select for already present GCSC populations during treatment and stress, glioma cancer stem cells present a considerable problem for future therapeutic regimens. While common therapies can reduce the size of gliomas to microscopic levels, these small populations of stem cells remain within the tissues, resistant to therapies and selected due to their ability to survive. Recurring tumors will therefore be more malignant and progress more quickly than the original glioma. Therefore, focus on eliminating GCSCs should be pursued during brain cancer research treatment.

### 2.5 Targeting stress response as glioma therapy

The preceding sections have focused on how glioma cells manage to evade the deleterious effects of both intrinsic and extrinsic stressors. Future developments in glioma therapy should take into account these survival pathways. New treatment options should take advantage of the stress response and new survival mechanisms such as autophagy, while others could target GCSCs in order to completely, and hopefully, permanently eliminate glioma tumors. Novel treatments can be used to sensitize glioma cells to traditional therapies by exacerbating the stresses the cells are under (Table 3).

Most importantly, gliomas need to be characterized for the proteins that they express. This can then inform clinicians what therapies with which that particular tumor may be treated. For example, glioma cells or tumors that are shown to be deficient in autophagic proteins might be more susceptible to traditional apoptotic therapies or radiation. Tumors void of any stem cell markers might be less likely to recur, and those patients can be offered more simple treatment

plans. The key is to make the most of the information available to personalize the therapies given to each patient. Therefore, a compilation of an assortment of markers and screening panels needs to be created to fit into the era of personalized medicine.

| Category | Compound | Target |
|---|---|---|
| **Autophagic Inhibitors** | 3-MA | Pre-autophagosomal structure inhibition |
| | Bafilomycin A1 | Block autophagosome and lysosome fusion |
| | HCQ | Block autophagosome and lysosome fusion |
| | Monensin | Block autophagosome and lysosome fusion |
| | siRNA against BECN1,ATG5, ATG7, ATG10, etc. | Blocks translation of autophagic response |
| **Autophagic Enhancers** | NH125 | Inhibits EF-2K |
| | Rapamycin | Inhibit mTOR pathway to increase stress |
| **Agents Affecting Tumor Stem Cells** | Arsenic trioxide | Targets mitochondria for destruction to sensitize to autophagy inhibition |
| | Bevacizumab | Anti-angiogenic to sensitize stem cell population to therapy |
| | Sodium Bicarbonate | Increase cellular pH to sensitize stem cell population to therapy |
| | siRNA against β-catenin/wnt, SSH, and Notch-1 pathways | Reduce stem cell signaling |

Table 3. Proposed therapies for sensitization of gliomas to current treatments.

Since the majority of current glioma therapies induce autophagy in glioma cells, including radiation and chemotherapy, inhibition of autophagy would decrease the survival of these cells. Tumor cells may undergo apoptosis when autophagy is started and then disrupted. Adding the autophagy inhibitor bafilomycin A1 ($H^+$-APTase inhibitor) to TMZ lead to cell death via apoptosis through caspase-3 and mitochondrial permeabilization. Autophagy can be blocked at multiple levels, allowing for adjustment according to whether cells are using it as a protective or cell death mechanism. The P13K inhibitor, 3-methyladenine (3-MA), can be used to inhibit autophagy before formation of the autophagosome (Kanzawa et al., 2004). Other agents like bafilomycin A1 blocks the fusion of the autophagosome with the lysosome, as do several other drugs like hydroxychloroquine (HCQ) and the proton exchanger, monensin. The natural product arsenic trioxide targets mitochondria and induces autophagy in glioma cells, and when combined with bafilomycin A1, is able to

eliminate all remaining tumor cells (Kanzawa et al., 2005). Drugs like rapamycin could be used to further the stress caused by nutrient starvation, activating autophagy, thereby leading to cell self-digestion. Synergistic killing of glioma cells has been shown with treatment of rapamycin and either Akt or PI3K inhibitors (Takeuchi et al., 2005). These are just a few examples of how therapies can be combined to sensitize and eliminate glioma cells through autophagic inhibition or enhancement.

In the future, genetic modification of cells will be more feasible, so eventually the autophagic response could also be targeted with small interfering RNA (siRNA) against autophagy proteins such as BECN1, ATG5, and ATG10 along with many others. EF-2K presents a novel target for inhibiting autophagy and sensitizing cells to other therapies. NH125 is the preclinical inhibitor of EF-2K and could be developed into a bioavailable agent for humans or an siRNA could be used against the kinase as well.

Glioma cancer stem cells are another cell type that could be targeted as glioma therapy for complete abolishment of the malignancy. Bmi-1 is an E3-ubiquitin ligase that is up-regulated in GCSCs and other cancer stem cells. GSCSs have low proteosome activity, which can be used to track stem cells through Bmi-1 degradation (Vlashi et al., 2009). Eliminating GCSCs by targeting Bmi-1 expressing cells was sufficient for causing regression of the solid glioma indicating that ridding the tumor of cancer stem cells may actually be curative.

Several possibilities exist for targeting stem cells. As mentioned previously, anti-VEGF treatments like bevacizumab might help to normalize the vasculature and allow for efficient delivery of chemotherapies (von Baumgarten et al., 2011). In xenotranpslants, bevacizumab synergizes with radio- and chemo-therapy to effectively kill glioma cells (Vredenburgh et al., 2007). Anti-angiogenic therapy could help reduce the stem cell niches. Bevacizumab blocked the GSCSs ability to induce the migration of endothelial cells necessary for neoangiogenesis and cell migration (Ailles & Weissman, 2007). Thus, anti-VEGF treatment might work in multiple ways.

GSCSs could be targeted by additional approaches. Treatment of tumors with sodium biocarbonate increased tumor pH and reduced invasion, while reducing stem cell markers in breast cancer (Robey et al., 2009). While not yet used in glioma, this could restore the non-acidic pH environment, targeting the tumor microenvironment. Also, developing agents against the stem cell signaling pathways will be important in eliminating tumors. The β-catenin/wnt and SHH pathways along with Notch-1 signaling are all good candidates for targeting. Markers, CD133 and nestin, could be used to identify potential responders.

Overall, new therapies should be used to take advantage of the stress that glioma cells already encounter, in addition to targeting their means of survival. Those gliomas that use autophagy as a protective mechanism could be treated with autophagic inhibitors to enhance efficacy of therapy. These therapies could be used to sensitize tumors to treatment with standard radiation and chemotherapies. Also, any gliomas that have markers indicative of cancer stem cells might be considered to be treated with agents that target stem cells to circumvent that avenue of cancer cell survival. Autophagy and GCSCs represent attractive, novel targets for future glioma therapy that can be combined with conventional therapy to fully eradicate glioma cells.

## 3. Conclusions/perspectives

The preceding chapter sought to introduce the concept that glioma cells are constantly under stress, a factor which needs to be taken into consideration during the treatment of

brain cancers. Both intrinsic and extrinsic stresses impact the development and progression of glioma. Stresses like nutrient deficiency, hypoxia, acidity, and the immune response are present during normal tumor growth and throughout treatment. Tumor cells that survive these stresses are more adept at surviving hostile conditions and are more resistant to current therapies. Stress, therefore, shapes the tumor cell population. The autophagic response and glioma cancer stem cells are two of the prevalent survival and resistance mechanisms in glioma, and both can be induced by cellular stresses.

New treatment regimens should take advantage of various stresses and stress responses present in glioma. Stresses like hypoxia or acidity could be exacerbated and sustained with new agents, allowing traditional therapies, such as TMZ or radiation, to permanently eliminate the sensitized cells. Autophagy is a fragile state for cancer cells, and inhibition of the autophagic process at the level of EF2K, beclin-1, and other proteins may combat this glioma cell survival mechanism. Targeting the autophagy pathway has already been shown to render glioma cells and other types of cancers more susceptible to currently available treatments. Recognition of GCSC markers could lead to better diagnostic and prognostic tools in addition to targeted cancer stem cell therapy. Combining current therapy with inhibitors that interrupt the stress response of glioma cells is an attractive approach for future glioma treatment.

## 4. Acknowledgment

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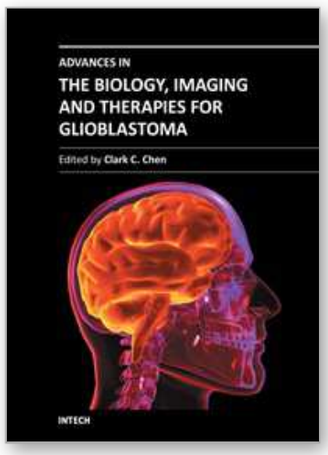


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This book is intended for physicians and scientists with interest in glioblastoma biology, imaging and therapy. Select topics in DNA repair are presented here to demonstrate novel paradigms as they relate to therapeutic strategies. The book should serve as a supplementary text in courses and seminars as well as a general reference.

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