ROS Signaling in Brain Tumor

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An Emerging Paradigm for Cancer Stem Cell Therapy

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Abstract

The heterogeneous cell population of the brain tumor and its microenvironment is a significant challenge to the current treatment modalities of maximal surgical resection followed by radiation and adjuvant chemotherapy in gliomas. A distinct subpopulation of quiescent brain tumor stem cells escape these therapeutic interventions that majorly target the rapidly dividing cells. It is now well established that these brain tumor stem cells are responsible for relapse. Thus, elucidation of resistance mechanisms mediated by the stem cell population in brain tumors is an area of active research to prevent recurrence. Both radio and chemotherapies induce cell death via the production of pleiotropic agents like Reactive Oxygen Species (ROS). Unfortunately, the tumor stem cells can modulate their redox balance to maintain their viability, quiescence, and self-renewal. This chapter will focus on ROS signaling in brain tumors, various mechanisms of low ROS level maintenance in brain tumor stem cells that help in their therapy

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escape, and how efficient approaches to increase oxidative stress in such cells might facilitate their selective elimination.

Keywords

Brain tumors · Glioblastoma stem cells · ROS · Mitochondrial function · Cancer stem cell signaling · Therapy resistance

Introduction

In a cell, reactive oxygen species (ROS) are formed as by-products of metabolic pathways involving molecular oxygen. Under, normal conditions the cellular redox balance is maintained by the equilibrium of ROS production and ROS scavenging via regulation of enzymes like superoxide dismutase (SOD), catalases, glutathione reductase, glutathione peroxidase, thioredoxins, peroxiredoxins, and glutaredoxins (Poli et al. 2004). However, elevated ROS levels lead to genomic instability by causing irreversible damage to essential biomolecules like proteins, lipids, and nucleic acids, thereby promoting oncogenesis through regulation of cytoskeletal remodeling, epithelial to mesenchymal transition (EMT), invasion, and metastasis (Liou and Storz 2010). Moreover, ROS also acts as second messenger in signaling cascades regulating transcription factors involved in maintaining self-renewal, proliferation, and differentiation of stem cells facilitating cancer promotion and progression.

Over 80% of diagnosed brain and central nervous system tumors are accounted for by the aggressive and heterogeneous gliomas arising from glial cells of the brain. Histopathologically, according to World Health Organization (WHO) guidelines, gliomas can be classified into ependymomas, astrocytomas, or oligodendrogliomas (Louis et al. 2016). The current treatment modalities for gliomas include surgical resection followed by radiotherapy and chemotherapy using Temozolomide (Davis 2016). As the presence of brain tumor stem cells plays a significant role in tumor recurrence due to their quiescent nature and resistance to the current therapeutic interventions, it is essential to understand how the balance between quiescence and stemness is maintained in these cells. Maintenance of low ROS levels is one of the crucial strategies followed by these brain tumor stem cells to survive therapy. Thus, therapeutic approaches to increase ROS levels can facilitate their elimination and improve the response rate and patient outcome. Here, we will discuss the involvement of ROS in glioma progression, its role in maintaining the brain tumor stem cell population, and therapeutic interventions to increase oxidative stress in brain tumor stem cells.

Reactive Oxygen Species and Stem Cells

Reactive oxygen species (ROS) generated from molecular oxygen by incomplete reduction contains unpaired electrons leading to their highly reactive nature. The first evidence of ROS production in a cell came from the neutrophils that used ROS to

protect themselves against bacteria (Babior 1978). Superoxide (O_2^-), hydroxyl (·OH) radicals, and non-radicals like hydrogen peroxide (H_2O_2) are predominant ROS that facilitates the integration of intracellular response to external cues by acting as second messengers. Mitochondrial respiration, involved in 0.1–0.2% of O_2 production, is the primary source of ROS. Electron transport chain in the inner mitochondrial membrane involved in oxidative phosphorylation for ATP production generates ROS through complexes I and III (Bell et al. 2007). In addition to mitochondrial source, NADPH oxidases (NOX) located in the plasma membrane, endoplasmic reticulum, and nucleus are also involved in ROS production by the rapid conversion of NADPH to superoxides which further get converted to hydrogen peroxide by the action of superoxide dismutases (Bedard and Krause 2007).

ROS plays a major role in determining stem cell fate by modulating transcription factors, epigenetic modifiers, kinases, and phosphatases involved in maintaining stemness (Bigarella et al. 2014). For instance, a polycomb repressor family member, BMI-1, determines stem cell fate by controlling ROS levels and mitochondrial function (Liu et al. 2009). Expression of NOX4 by hydrogen peroxide helps differentiate Embryonic Stem Cells (ESCs) to cardiac lineage and mesenchymal stem cells (MSCs) to adipocytes and neural lineage (Bigarella et al. 2014). ROS levels also maintain the levels of intermediates required for epigenetic modifications involved in determining stem cell fate. Levels of acetyl CoA required for acetylation of histones, NAD required for deacetylation by sirtuins, and S-adenosyl methionine (SAM) required for methylation are all produced in metabolic pathways modulated by ROS (Bigarella et al. 2014).

Embryonic stem cells (ESCs) maintain low ROS levels due to the presence of immature mitochondria, wherein uncoupling of electron transport chain (ETC) occurs. For efficient self-renewal and DNA replication, these cells rely on glycolysis and pentose phosphate pathway for ATP production and nucleotide synthesis, respectively. Zhou et al. have shown that in hypoxic conditions due to increased HIF-1α, the proliferative potential of ESCs increases via accelerated glycolysis. Accordingly, a decrease in oxidative phosphorylation leads to decreased proliferation (Zhou et al. 2012). Similarly, in adult hematopoietic stem cells (HSCs), upon genetic knockdown of MEIS1 homeobox protein, which regulates both HIF1α and HIF2α, ROS production is increased due to higher oxidative phosphorylation leading to loss of quiescence and self-renewal (Unnisa et al. 2012). Loss of the M2 isoform of pyruvate kinase M and lactate dehydrogenase caused glycolytic metabolic defects in HSCs by increasing ROS levels (Kocabas et al. 2012). Additionally, increased glycolysis upon lipid phosphatase, PTPMT1 knockdown was associated with an increase in self-renewal and decrease in the differentiation of HSCs. Similarly, a correlation between ROS levels and maintenance of self-renewal properties of neural stem cells and spermatogonial stem cells is also observed (Bigarella et al. 2014).

In most solid cancers, there exists a discrete population of therapy-resistant cancer stem cells (CSCs) first discovered in leukemia in 1994 (Lapidot et al. 1994). These cancer stem cells can arise from normal stem cells, progenitor cells, mature cells, or fusion of stem and mutant cells (Morrison and Kimble 2006). These cells show properties of indefinite self-renewal and differentiation with immunosuppressive and

migratory activities. These cells possess the capacity to repopulate the entire tumor, which primarily results in resistance to chemo and radiotherapies, ultimately leading to recurrence. Like normal stem cells, the low reactive oxygen species level is a crucial survival strategy of these cancer stem cells as well (Zhou et al. 2014).

Brain Tumors

Brain tumors are one of the most frequently occurring malignancies of the central nervous system, affecting children and adults. According to the World Health Organization, brain tumors are classified into astrocytic, oligodendroglial, mixed, ependymal, neuronal and mixed, neuronal/glial, embryonal, and primitive neuroectodermal types based on the cell origin. Among all the central nervous system tumor types, gliomas have an incidence of approximately 45% and are further graded into type I–IV based on the degree of malignancy (Behin et al. 2003). Glioblastoma multiforme, a grade IV astrocytoma, is the most frequent, heterogeneous, aggressive, and invasive form of all gliomas (Louis et al. 2016). Despite aggressive radio and chemotherapy of gliomas, the highly heterogeneous nature owing to the plethora of cells with distinct molecular and genetic profiles constituting the brain tumor microenvironment makes the therapeutic interventions challenging.

Various reports suggest the involvement of ROS in cancer initiation and progression by regulating signaling molecules like mitogen-activated protein kinase (MAPK), transforming growth factor-beta (TGF-β) and SMAD7 to constitutively activate downstream kinases like extracellular-signal-regulated kinase (ERK) and Jun N-terminal kinase (JNK) (Son et al. 2011; Krstić et al. 2015; Zhang et al. 2016; Su et al. 2018). The constitutive signaling promotes epithelial to mesenchymal transition by modulating E-cadherin, Snail, and various matrix metalloproteinases like MMP-3 and MMP-9. Hypoxic condition is predominant in brain tumors due to its aberrant metabolism, high proliferative rate, and increased angiogenesis, leading to increased ROS production. Thus, ROS-mediated downstream signaling leading to lipid peroxidation and protein oxidation triggers glioma progression (Rinaldi et al. 2016). For instance, ROS production by serine protease, tissue-type plasminogen activator (TPA) administration leads to increased migration and invasion of U87 cells by activated ERK-mediated cyclooxygenase-2/prostaglandin E2 and metalloproteinase 9 activity (Chiu et al. 2010). ROS plays a vital role in all aspects of brain tumors, which are discussed in detail subsequently.

Brain Tumor Stem Cells

Brain tumor stem cells, first reported by Singh et al. based on CD133+ marker sorting, are a leading cause of resistance to the current treatment modalities (Singh et al. 2004). Since the radio and chemotherapeutic agents target the rapidly dividing

cells, these quiescent stem cells with efficient DNA damage repair, hypoxia modulating capacities in the G0 phase escape their toxicity (Wang 2015; Kaur et al. 2020). These brain tumor stem cells mostly reside in the proliferative germinal layer and not in the non-proliferative brain parenchyma (Singh et al. 2004). In pediatric tumors like medulloblastoma, the cerebellar granular layer originating from the undifferentiated germinal region of the developing and early pre-natal central nervous system is the origin of tumor stem cell (Singh et al. 2004; Vescovi et al. 2006) whereas glioblastoma stem cells (GSCs) with self-renewal, differentiating, and proliferative capacities are seen in specific niches like the subventricular zone (SVZ). The neural progenitor cells and neural stem cells residing in SVZ, dentate gyrus of hippocampus, and subcortical white matter on acquiring mutations post-irradiation give rise to glioma stem-like tumor-initiating cells which evolve to glioblastoma stem cells (GSCs). Since these GSCs are formed due to the dedifferentiation of differentiated GBM and progenitor cells with distinct transcriptomic profiles, they make the GBM tumor heterogeneous. The GSCs have genetic, molecular, and metabolic profiles different from neural stem cells. CD49f+, CD90+, CD44+, CD36+, EGFR+, A2B5+, LICAM+, and CD133+ are distinct molecular markers that define GSCs while glycerol-3-phosphate dehydrogenase (GPD1) is a prognostic GSC marker which is expressed in GSCs post-chemotherapy (Dirkse et al. 2019). Of all the markers of GSC, a surface glycoprotein with five transmembrane domains, and two glycosylated extracellular loops, CD133 is most abundant. Therefore, in almost all studies, CD133 has been used to isolate GSCs. CD133+ cells have been shown to initiate tumors in NOD/SCID mice (Brescia et al. 2013). CD133+ GSCs secrete more VEGF as compared to CD133- cells, thereby forming angiogenic tumors. Thus, an antibody against VEGF, bevacizumab, prevents angiogenesis and decreases migration in GBM (Liebelt et al. 2016; Iranmanesh et al. 2021). In addition, CD133+ cancer-initiating precursors are also identified from medulloblastomas, a frequently occurring cerebellar tumor in children (Bahmad and Poppiti 2020). Furthermore, cells from human ependymoma, when cultured as neurospheres, show characteristics of multipotent radial glial cells, which give rise to ependymal and adult neural stem cells (Vescovi et al. 2006). Together, these reports demonstrate that the brain tumor stem cell population is a leading cause of its progression.

Regulation of Stemness by ROS As discussed above, mitochondria play a significant role in maintaining the stem cell properties by modulating the reactive oxygen species (ROS) levels in the cell. Mitochondria of the brain tumor stem cells are few in number, fragmented, less mature, having tubular shape, inactive, and thus help maintain quiescence and low self-renewal by keeping low ROS levels. Mitochondrial fusion helps in the maintenance of self-renewal property, whereas fission favors differentiation (Khacho et al. 2016; Iranmanesh et al. 2021). Depending on the nutrient availability, hypoxic conditions, and tumor microenvironment, stem cells switch between glycolysis and oxidative phosphorylation to maintain their stemness. Oxidative phosphorylation is predominant in the quiescent cells, whereas the proliferative cells perform glycolysis. GSCs prevent ROS increase by activating

Unfolded Protein Response (UPR) and Heat Shock Response to relieve proteotoxic stress (Hetz 2012). Ca^{2+} homeostasis by mitochondrial store-operated Ca^{2+} entry channels also helps in maintaining the quiescence of GSCs (Iranmanesh et al. 2021), whereas serum addition induces differentiation of GSCs by upregulating ROS levels, leading to altered metabolism as seen by the decrease in Sox2, Olig2, and Not1 and increase in superoxide dismutase (Iranmanesh et al. 2021).

The presence of hypoxic conditions is a significant cause of dedifferentiation of GBM cells to stem-like cells. Hypoxia leads to activation of HIF1α, HIF2α, and histone methyltransferase and mixed lineage leukemia (MLL1) activation, which in turn increases expression of Notch, involved in maintaining survival and self-renewal of cancer stem cells (Heddleston et al. 2009). Further, nutritional stress leads to stemness induction by upregulation of CD133, nuclear translocation of Sox2, Nanog, and Oct4 and upregulation of Wnt and Hedgehog signaling pathways. For instance, the Bone Morphogenetic Factor signaling pathway induces differentiation, whereas the JNK pathway prevents it (Caja et al. 2018). Thus, GSCs secrete BMP antagonist gremlin to inhibit the signaling cascade, and inhibition of JNK induces differentiation and reduces tumor initiation (Yan et al. 2014). Tight regulation of the cell cycle also plays a pivotal role in controlling the switch between quiescence and self-renewal of GSCs. BMP pathway maintains quiescence by regulating p21. Cyclin B1, D1, 4, and 6 are shown to be downregulated in GSCs, maintaining quiescence and protecting these cells against chemoradiotherapy. For the transition of GSCs from quiescence to self-renewal, GINS helicase complex comprising of Sld5, Psf1, Psf2, and Psf3 subunits associates with Cd45 and Mcm 2–7, which are involved in DNA replication (Iranmanesh et al. 2021).

ROS-Mediated Signaling Pathways in Brain Tumor Stem Cell Self-Renewal Wnt/β-catenin, along with its role in normal brain development and astroglial lineage differentiation, is also involved in the proliferation and differentiation of stem cells. ROS plays a significant role in inhibiting Wnt/β-catenin pathway by abrogating the association of dishevelled and nucleoredoxin, as shown in murine-derived embryonic fibroblasts (Funato et al. 2006). Aberrant Wnt signaling helps in the nuclear translocation of β-catenin where interaction with FoxM1 induces gliomagenesis by modulating transcription of c-Myc and other targets (Zhang et al. 2011). Wnt/β-catenin, also by inducing expression of PLAGL2, inhibits differentiation and promotes self-renewal of GSCs. Additionally, EGFR plays a significant role in modulating proliferation, differentiation, migration, and survival of GSCs via β-catenin translocation (Pei et al. 2012; Liebelt et al. 2016). Wnt/β-catenin, basic helix-loop-helix, and HIF1-α interaction activates bone morphogenetic protein (BMP) which then induces differentiation of astroglial cells. BMP2 overexpression prevents GSC proliferation and sensitizes GSCs to TMZ by destabilizing HIF1-α. BMP4 delivery in vivo has also been shown to decrease brain tumor growth. In addition, the use of BMP antagonist, Gremlin1, inhibits differentiation and helps in the maintenance of the self-renewal property of GSCs.

Notch signaling involved in proliferation, apoptosis, differentiation, and cell lineage decision-making is also upregulated in GSCs. Notch signaling aids in the survival of GSCs by increasing antioxidants and decreasing oxidative stress. Conversely, ROS like nitric oxide helps in the activation of Notch in glioma cells (Charles et al. 2010). Notch and its ligands Jagged one and Delta-like 1 knockdown decrease the oncogenic potential of GSCs implying the role of Notch in brain tumor proliferation and survival via maintaining stemness and preventing differentiation. Additionally, activation of the Notch pathway in vascular endothelial cells helps in maintaining the stemness of GSCs which in return maintain cell growth and angiogenesis in endothelial cells (Zhang et al. 2013).

Sonic Hedgehog (Shh) is another important pathway that plays a pivotal role in the ventral patterning of neural stem cells. Shh increase is associated with increased ROS levels and HIF1- α stabilization independent of hypoxia in cerebellar granule neuron precursor. Shh is upregulated in SVZ, which is the prime niche of GSCs. Shh knockdown led to a decrease in self-renewal and in vivo tumorigenic potential of GSCs (Liebelt et al. 2016).

Transforming growth factor-β is also an important factor in ROS production (Wu 2006). Transcriptomic analysis of GBM cells cultured from two patients in stem cell media with or without TGF-β identifies upregulation of LIF, ID1, and NOX4. Increased NOX4 has been associated with poor prognosis, and a positive correlation exists between TGF-β and NOX4. TGF-β mediated increase in NOX4 further increases ROS levels. Deletion of NOX4 further leads to decreased self-renewal, stem cell markers like CD133/PROM1, OLIG2, Nestin, and increased differentiation marker GFAP. TGF-β knockdown leads to decrease of only Nestin but not Sox2, whereas knockdown of NOX4 leads to the decline of both, implying NOX4 to be a better modulator of GSC maintenance. In addition, activation of NOX4 via TGF-β activates nuclear erythroid 2-related factor 2 (Nrf2) to ultimately control the transcriptional activity of Heme oxygenase 1 (HO-1) and positively regulate pentose phosphate pathway, fatty acid oxidation. Further, Nrf2 controls the expression of glutathione transferase, glutathione peroxidase, catalase, and NADPH: quinone oxidoreductase by binding to the antioxidant response element (ARE) of the target gene promoter. MicroRNAs like miR-153 also regulate Nrf2 by binding to 3′ UTR. GSCs contain low levels of miR-153. On overexpressing miR-153, a decrease in Nrf2, and other redox enzymes like GPX, has been observed, and there has been an increase in ROS levels which was shown to increase radiosensitivity by inducing apoptosis. Also decrease in neurosphere formation accompanied with decrease in expression of stemness markers like CD133 and Nestin and increase in differentiation markers like GFAP and Tuj-1 by induction of p38 MAPK signaling have been observed. Likewise, in vivo studies have also shown decreased tumorigenicity upon miR-153 overexpression. Additionally, Nrf2 knockdown leads to ATP depletion, which in turn activates 5′ AMP-activated protein kinase, thereby inhibiting mTOR signaling and decreases glioma cell proliferation by inducing differentiation of GSCs (Yang et al. 2015). Furthermore, NOX4 regulates the expression of Glut1, a glucose transporter that helps in the switch from oxidative phosphorylation to glycolysis. Taken together, TGF-β increases ROS levels via

NOX4 and thus is involved in maintaining cell proliferation, self-renewal, and glucose metabolism in GSCs. These results indicate NOX4 to be a potential target in GSCs (García-Gómez et al. 2019).

ROS-Mediated Epithelial to Mesenchymal Transition Neural stem cells and oligodendrocyte precursor cells mainly contribute to gliomas (Lindberg et al. 2009). However, the mesenchymal phenotype in the neuronal development process is different from the one exhibited by ectodermal cells. Hence the classical EMT is not observed in GBM cells; it is termed "EMT-like" or "glial-mesenchymal" transition. E-cadherin absent in neural tissues is only present in GSCs. Upon growth factor stimulation, ROS activates MAPK, PI3K, and Wnt signaling pathways in cancer stem cell. Activation of Wnt/β catenin, TGF β, tyrosine kinase receptor, and SDF/CXCR4 promotes EMT and increases migration and invasion of cells. Wnt/β catenin in invasive front promotes migration by *Zeb1*, *Twist1*, and *Slug* expression. Transcription factors like *Slug*, *Snail*, *Zeb1*, *Twist1*, and *Twist 2* are expressed upon metabolic stress induction of ROS. The first marker, Snail, causes repression of tumor suppressor gene and induces stemness leading to metabolic aberrations. Transcription factors E12/E47 also repress E-cadherin transcription by binding to E-box. These factors are involved in cell-cell interaction and epithelial organization, wherein they cause cytoskeletal rearrangement leading to ECM degradation (Mladinich et al. 2016).

Additionally, TGF-β also promotes transcription and nuclear translocation of Zeb1. Knockdown of Zeb1 causes increased sensitivity to therapies as Zeb 1/2 expression is correlated with invasive potential and survival of GBM cells (Joseph et al. 2014). Further, Twist 1/2 controls the stemness of GSCs by modulating the expression of MMP2, Slug, and Hepatocyte Growth Factor (HGF). HGF, in turn, binds to c-Met, which is highly expressed in GSCs and causes transcription of EMT factors to increase invasion and migration (Cruickshanks et al. 2017).

Additionally, elevated ROS levels induce fatty acid oxidation, resulting in increased fatty acid, which further promotes metastasis by activating MAPK signaling after epithelial to mesenchymal transition (Wang et al. 2019). MAPK, the critical signaling pathway to a wide range of external cues, plays a significant role in promoting differentiation of glioma tumor-initiating stem cells via ROS induction and causes loss of self-renewal. On TGF-β stimulation, p38 MAPK gets phosphorylated in the Thr-Gly-Tyr motif resulting in p38 translocation to the nucleus, where it regulates transcription factors that repress stem cell maintenance. miR-141 and miR 200a help in maintaining GSC phenotype by inhibiting MAPK signal (Dolado et al. 2007).

Together, as summarized in Fig. 1, various stimuli induce ROS production which controls various signalling pathways involved in the maintenance of brain tumor stem cells, thereby favoring brain tumorigenesis. Therefore, it is crucial to understand how the current brain tumor therapies can target these quiescent cells based on the knowledge of these signaling molecules to devise new therapeutic interventions to overcome the current challenges.

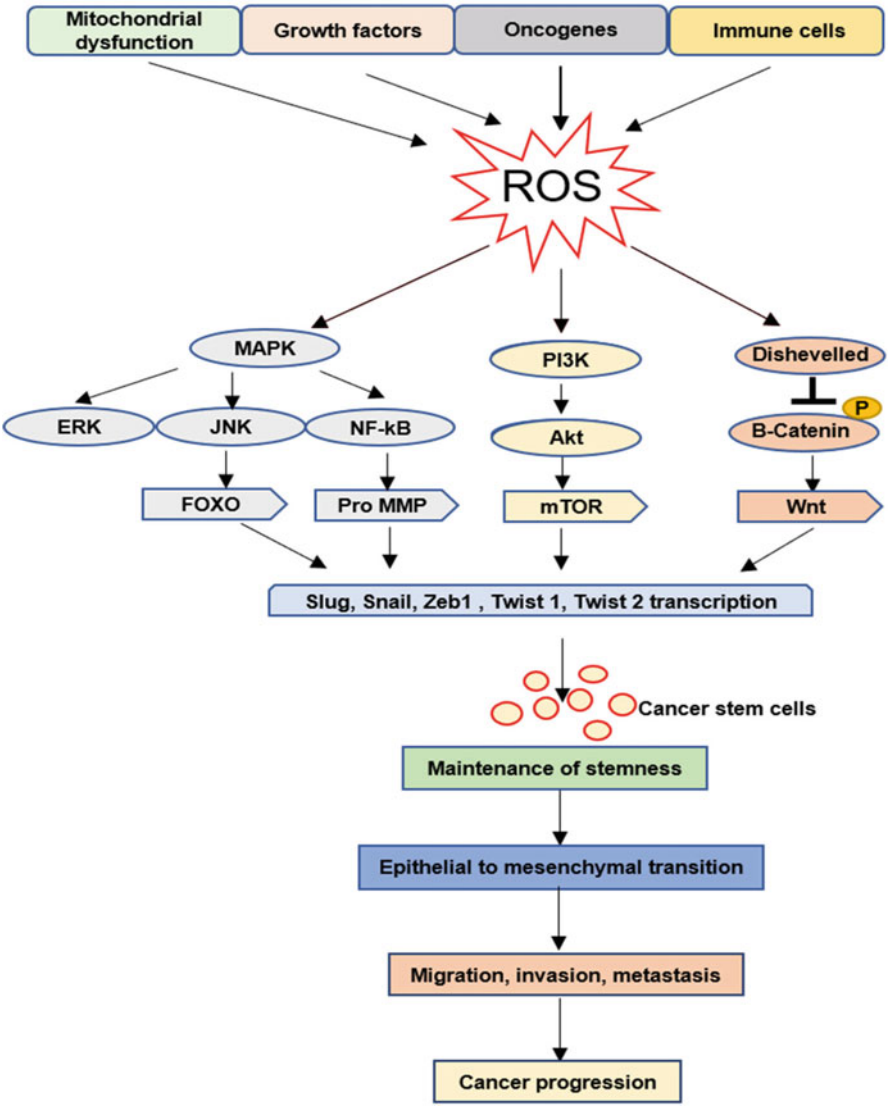

Fig. 1 ROS-mediated stem cell signaling pathways involved in cancer progression

Current Brain Tumor Treatment Modalities Involving ROS

Radiation and Temozolomide Radiotherapy is one of the most widely used treatment modalities in brain tumors, which induce DNA damage primarily via the production of Reactive Oxygen Species (ROS). Upon irradiation, ROS arising from extracellular hydrolysis of water has a short life of 10^{-9} s, and that generated

from metabolic changes, or mitotic damage has a life span of 24 h. IR mainly induces partial deactivation of complex I and complex III of mitochondrial electron transport chain leading to ROS generation (Tulard et al. 2003). ROS-induced EMT upon irradiation is associated chiefly with the regulation of transcription factors and signaling molecules like Snail, Zeb1, Wnt/β-catenin, Notch, HIF1, and TGFβ (Lee et al. 2017). Pharmacological inhibition of ROS scavengers leads to a decrease in clonogenic capacity and increased radiosensitivity in GBM cells.

Administration of temozolomide (TMZ) is another treatment modality in gliomas. Alkylating agent TMZ undergoes pH-dependent hydrolysis to 5-3-(methyl)-1-(triazen-1-yl) imidazole-4-carboxamide, which is highly reactive and methylates O6 and N7 positions of guanine. These adducts are repaired by DNA repair protein, Methyl Guanine Methyl Transferase (MGMT). Methylation of *MGMT* promoter leads to an increase in progression-free and overall survival post alkylating agent treatment in GBM. TMZ has majorly been shown to mediate apoptotic cell death by ROS production via the AMPK-mTOR axis (Zhang et al. 2010).

Radiation Resistance The brain tumor stem cells are intrinsically radioresistant as they depend on oxidative phosphorylation for energy production and escape most therapies that target glycolysis. As shown in Fig. 2, brain tumor stem cells mediate resistance to IR mediated radiotherapy due to a heightened DNA damage repair response, modulation of tumor microenvironment like hypoxia, metabolic

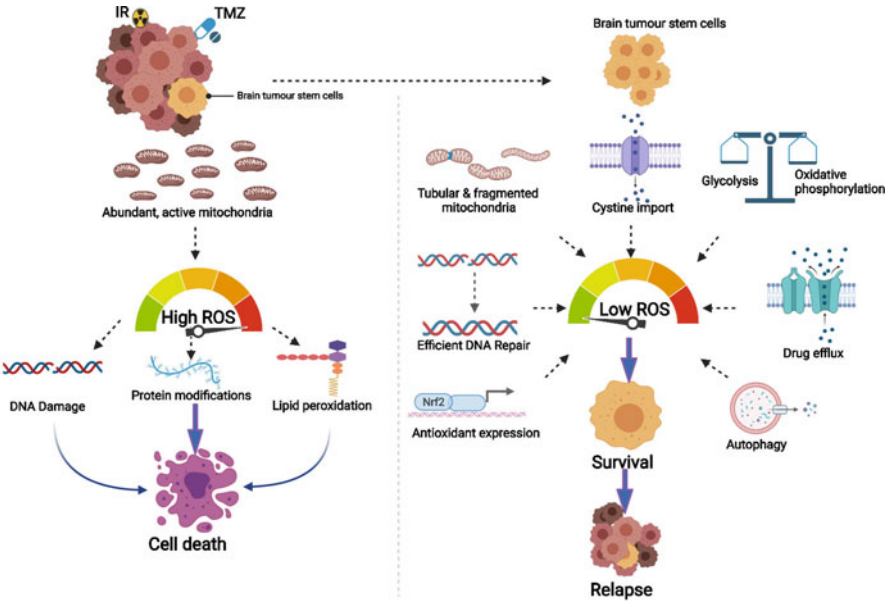

Fig. 2 Mechanism of low ROS level maintenance in brain tumor stem cells leading to therapeutic resistance. (Created with BioRender.com)

alterations, and increased autophagy (Tang et al. 2018; Mudassar et al. 2020). Both in vitro studies in cell culture and in vivo studies in the brain of immunocompromised mice show upregulation of Prominin (CD133) expression post-IR (Brescia et al. 2013). There is three–fivefold enrichment of CD133+ cells in short-term culture of GBM xenografts and an increase from 2–3 to 6–10% in GBM tumor specimens after IR. Furthermore, the CD133+ cells form neurospheres and express stem cell markers Nestin and Sox2 and are highly tumorigenic. The colony-forming ability of these cells is increased irrespective of the presence or absence of growth factors. The CD133+ cells form heterogeneous mass as they retain multilineage differentiation. The tumor-forming capacity of $CD133^{+}$ cells after IR happens to be the same as that of untreated $CD133^{-}$ cells. In spite of having the same initial damage induction in CD133- and CD133+ cells as observed by alkaline comet assay, reduction of comet tail is 4–9 times faster in CD133+ cells, implying faster and more efficient repair. The faster repair efficiency of CD133+ cells is attributed to low ROS level maintenance. Mechanistically, it is shown that the CD133+ cells have heightened checkpoint kinase activation as observed by high expression of pATM, pATR, pChk1, pChk2, and pSer645Rad17, which provides a survival advantage to the GSCs. Thus, checkpoint kinase targeting is an efficient strategy to eliminate CD133+ GSCs mediated GBM resistance. Use of Chk1-Chk2 inhibitor, debromohymenialdisine (DBH), has shown increased sensitivity of CD133+ cells to irradiation. Checkpoint kinase inhibitors SCH 900776 (NCT00779584), SAR-020106, AZD7762 (NCT00413686), and AZD7762 have also been shown to potentiate radiosensitivity and chemosensitivity to the current treatment modalities of GBM (Bao et al. 2006; Alves et al. 2021). Hypoxia, predominant in brain tumors, also plays a significant role in radiation resistance as oxygen depletion causes decreased ROS generation. Stabilization of HIF1 α under hypoxic conditions leads to increased Cox4-2 regulatory subunit of electron transport chain, thus preventing aberrant electron transfers responsible for ROS generation. Altered mitochondrial metabolism leads to increased glycolysis resulting in lactate production and decreased oxidative phosphorylation in tumor cells. Metabolic symbiosis facilitates lactate transport from the hypoxic cells to the tumor stem cells, which harbor monocarboxylate transporter, MCT1. Oxidative phosphorylation, predominant in these stem cells, generates high levels of ATP, resulting in tumor repopulation. Furthermore, increased AMP/ATP ratio in hypoxic conditions induces protective autophagy, which helps the GBM cells to survive low oxygen. Thus, alleviation of hypoxia using various approaches like decreasing oxygen consumption rate by using inhibitors of mitochondrial respiration, anti-parasitic drugs like ivermectin and atovaquone, increased oxygen delivery to tumors using hyperbaric oxygen chambers, and use of compounds which mimic oxygen like nimorazole, hypoxia-activated prodrugs like transition metals, N-oxides, and quinones would facilitate radiation resistance reversal (Coates et al. 2019).

Temozolomide Resistance MGMT overexpression, along with deficiency or mutations of mismatch repair genes like MSH6, leads to TMZ resistance (Arora and Somasundaram 2019). Since majority of GSCs reside along hypoxic gradient, they

have high expression of HIF1-α and HIF2-α that are known to increase MGMT expression, thus maintaining the undifferentiated form of GSCs leading to TMZ resistance. Further, ROS-mediated Notch/Shh/RTK signaling, along with hypoxic conditions, also increases the expression of MGMT, leading to radioresistance. Additionally, these GSCs express high levels of active ABC transporters like ABCG2, which cause higher drug efflux and reduced drug uptake leading to chemoresistance. An essential mechanism of TMZ cytotoxicity in bulk tumor cells is via the generation of ROS, wherein superoxides cause DNA damage. However, GSCs express antioxidants like specific protein 1(Sp1), Nrf2, and glutathione reductase, which prevents ROS generation. In the case of ependymoma, TMZ administration shows anti-tumor effect both in vitro and in vivo only in MGMT negative stem cells by mediating G2/M phase arrest and inducing apoptosis (Meco et al. 2014).

Since brain tumor stem cell signaling pathways modulated by ROS are a significant cause of TMZ resistance, inhibition of these pathways helps in TMZ resistance reversal. For instance, cyclopamine that inhibits Sonic Hedgehog signaling has a synergistic effect and increases the cytotoxic effect of TMZ in GBM cells (U87MG and DBTRG-05MG). Additionally, since TMZ treatment induces autophagy, a combination of TMZ treatment with autophagy inhibitors like chloroquine and hydroxychloroquine causes sensitization of GBM cells to TMZ. Chloroquine and hydroxychloroquine are currently in phase III clinical trials, and their combination with TMZ shows better median survival than placebo (Yan et al. 2016). Further TMZ combination with inhibitors of mTOR (RAD001, TORK1 PP242, Torin 1), another important pathway that maintains stemness, induces cell death and decreases cell proliferation, invasiveness, and stemness by regulating β-catenin. In a high-risk cytogenetic group 3 and molecular group C ependymoma model (DKF2-EPINS) developed from cells of primary ependymoma patient samples with metastatic disease and stem cell features like neurosphere formation, increased in vivo tumorigenic potential is seen to be accompanied with transcriptomic plasticity. A shift from neural stem cell to ependymoma tumor stem cell has been seen upon transplantation which is sensitive to TMZ, vincristine, and cisplatin. Interestingly, neuronal differentiation and loss of stem cell properties have been observed upon administration of HDAC inhibitor, Vorinostat, with the restoration of TMZ sensitivity (Milde et al. 2011).

Stem Cells for Brain Tumor Therapy Although still in its infancy, the field of stem cells therapy for brain tumor has enormous potential as effective therapy against this incurable disease. The conventional chemo and radiotherapies, due to their non-specific effect, usually damage the normal highly proliferating cells. Stem cells can be used in the treatment of such injured cells and tissues due to their specificity and directed homing. Various factors influence the success rate of stem cell therapy. The foremost being the stem cell type, wherein, between HSCs and NSCs transduced with adenovirus in glioma model, virus release from NSCs were more than that of HSCs. Post intracranial administration, the median survival of

NSCs was 68.5% as compared to 44% of MSCs in orthotopic GBM. Carrier trafficking is better in NSCs due to the similar origin of stem cells and tumors. HSCs, which help in hematopoiesis, have been successfully used in haematological and non-haematological tumor treatment after chemotherapy to reconstitute the bone marrow; similarly, engraftment of hepatocytes derived from iPSCs had also been successful (Renga et al. 2003).

ROS Modulation in Brain Tumor Stem Cells for Therapeutic Intervention

Natural Compounds Of all the mechanisms adopted by the brain tumor stem cells to evade therapeutic response, the most prominent is the maintenance of low ROS levels due to high levels of antioxidant ROS scavengers and high DNA damage repair as shown in Fig. 2 (Diehn et al. 2009). Thus, administration of compounds that increase ROS levels would favor brain tumor stem cell elimination by apoptosis. Indeed, studies in astrocytoma 1321 N1 cells showed that administration of triterpenic diols, erythrodiol, and uvaol increases ROS levels leading to an increase in TNF/TNFR proteins, activation of c-Jun N-terminal Kinase, decrease in adherence proteins, loss of mitochondrial membrane potential, F-actin cytoskeletal network disruption, and finally apoptosis. Similarly, curcumin administration in patient-derived glioblastoma stem cells leads to increased mitochondrial ROS by MAPK activation and downregulation of STAT3 and its targets, causing loss of GSCs self-renewal capacity. Following tumor resection, the addition of curcumin decreases cell viability in a dose-dependent manner by decreasing GSC proliferation, sphere-forming, and colony-forming abilities. Antioxidant N-acetyl cysteine can reverse the effect of ROS increase caused by curcumin (Gersey et al. 2017).

Selective modulation of ROS levels in GSCs has also been shown using a non-psychoactive, non-toxic cannabinoid, cannabidiol (CBD) (Singer et al. 2015). Simultaneous treatment of GSCs with CBD and inhibitors of antioxidants abrogate GBM growth. CBD treatment leads to decreased frequency of GSCs and self-renewal in GSC cell lines (3832 and 387) in vitro and in intracranial GSC xenografts. The effect has been rescued by antioxidant Vitamin E treatment, implying that ROS levels modulate the self-renewal properties of GSCs. An increase in antioxidants like SLC7A11 and NRF2 and a decrease in proliferation markers and stemness markers were observed by microarray after CBD treatment implying regulation of the Nrf2 pathway by the compound. In GBM, there is an increased expression of a sodium independent, electroneutral transporter, system Xc-, which helps in protection against ROS. SLC7A11, the catalytic subunit of Xc-, imports cystine after getting converted to cysteine and increases antioxidant and "Reduced" glutathione (GSH) pool. In glioblastoma cell line U251, RNA sequencing upon SLC knockdown shows decreased expression of proliferation-related genes involved in mitosis, nuclear division, and chromosome organization

and increase in the invasion. At the same time, SLC overexpression leads to a decrease in adhesion and migration and increase in tumorosphere formation and stem cell markers like Nanog, Musashi-1, Sox2, and Nestin, thus establishing SLC7A11 as an important molecular target for selective elimination of GSCs. The combination of SLC7A11 knockdown and inhibition by sulfasalazine (SAS) along with CBD treatment also show an additive effect. These results imply combinatorial therapies mediated ROS increase may favor better elimination of GSCs (Singer et al. 2015). However, in the case of orthotopic ependymoma and medulloblastoma transplant models, there has been no significant survival benefit upon Δ9-tetrahydrocannabinol (THC) and cannabidiol (CBD) administration in spite of observed in vitro cytotoxicity. Thus, therapeutic benefit from the use of these cannabinoids in the case of ependymoma and medulloblastoma remains to be further investigated (Andradas et al. 2021).

Furthermore, administration of anti-cancer, chemopreventive, antioxidant compound resveratrol induces apoptosis by high ROS production and mitochondrial damage. Resveratrol treatment decreases the viability of U251 GBM cells in a dose-dependent manner with the formation of apoptotic bodies and chromatin condensation. Additionally, it leads to a decrease in the levels of SOD and catalase. However, the presence of resveratrol metabolizing enzymes like SULT1A1 and SULT1C2 and therefore lowered oxidative stress prevents this effect, implying that resveratrol mediates its effect by modulating the redox state of GBM cells (Song et al. 2019).

Stem Cell Marker Targeting Since CD133 is a highly expressed surface marker of GSCs, targeting this protein may help in their specific elimination. Genetic depletion of CD133 decreases neurosphere formation in GBM (Brescia et al. 2013). Interestingly, conjugation of the monoclonal antibodies against CD133 with carbon nanotubes selectively kills CD133+ cells post irradiation by photothermolysis. In greater than 50% glioma cases, EGFRVIII coexpresses with CD133, and thus their co-targeting by bispecific antibody can cause a further reduction in tumorigenicity. Cell adhesion molecule (LICAM, CD171) expressed on CD133+ cells when targeted with LICAM shRNA decrease sphere formation ability of only CD133+ cells and increase apoptosis through upregulation of p21 WAF1/cip1 tumor suppressor and downregulation of bHLH. Since CD133 may not be present in all brain tumor stem cells, thus, devising alternative therapeutic strategies simultaneously targeting other stem cell markers are required (Bao et al. 2006).

Stem Cell Signaling Pathway Inhibitors The ROS modulated key stem cell signaling pathways like Wnt, Notch, and Hedgehog are controlled by Casein Kinase 2. Therefore, the use of CX-4945, a selective Casein Kinase 2 inhibitor, sensitizes glioma stem cells through MGMT downregulation (Agarwal et al. 2013). In addition, Hippo signaling involved in maintaining tissue homeostasis, proliferation, and differentiation is also shown to be involved in GSC homeostasis. Yes Associated

Protein (YAP) binding to TAZ coactivator activates Hippo signaling. YAP increase is associated with chemoresistance, invasion, migration, and EMT. Thus, the use of YAP pharmacological inhibitor, Verteporfin, induces ROS production mostly in hypoxic GBM cells as observed using DCFDA fluorescence by binding to iron and may be used in sensitizing the resistant GSCs residing in hypoxic niches. Additionally, in GSCs, low expression of MKP1, a negative regulator of p38 MAPK and Erk1/2, is maintained. The use of HDAC inhibitors increases the sensitivity of resistant GBM by upregulating MKP1. A patient study has shown the association of high MKP1 with improved prognosis and survival rate. Use of a small molecule inhibitor, KHS1, which inhibits HSPD1 (Heat Shock Protein Family Member D), leads to metabolic exhaustion and loss of stem cell features by causing protein aggregation in patient-derived tumor xenografts. Furthermore, in medulloblastoma, inhibition of Hes1 involved in Notch Signalling by ɣ-secretase inhibitors and miR-199b-5p has helped in specific targeting of brain tumor initiating cells. In addition, the combination of hypoxia induction along with Notch inhibition leads to apoptosis and induction of differentiation in these stem cells (Manoranjan et al. 2012).

Future Perspective

The current treatment modalities of surgical resection followed by radiotherapy and temozolomide treatment in high-grade gliomas have an abysmal 5-year survival rate. Patients develop resistance to the therapeutic interventions and eventually come back with a relapse (Behin et al. 2003). Both IR and TMZ exert their effects by generating copious amounts of ROS to induce DNA damage which eliminates majority of cells of the tumor mass. However, a subpopulation of therapy-resistant brain tumor stem cells that are capable of repopulating the tumor escapes their toxicity due to their quiescent nature, efficient DNA damage repair, and adept oxidative stress management (Abou-Antoun et al. 2017). Thus, there is an unmet need to understand the redox balance maintenance in these tumor-initiating stem cells to devise strategies for specific elimination of these cells with self-renewal properties. A key strategy taken by tumor stem cells is the fine-tuning of the redox homeostasis by upregulation of antioxidants. The downregulation of ROS maintains the quiescent state of the brain tumor stem cells to evade therapy. This conundrum can be solved by devising strategies to increase ROS levels either directly by extracellular compound administration or by modulating the regulators and effectors of the signaling cascades involving ROS. Recently, the use of nanomedicine devices that involve encapsulation, adsorption, and covalent linkage of polymer or micellar drug conjugates with improved permeability, pharmacokinetic property, and half-life has been shown to facilitate efficient drug delivery to specific tumor sites (Shi et al. 2014). Since ROS accumulation can improve the targeting of GSCs, nanoparticles designed to release their cargos by ROS gradient specifically can also prove to be beneficial. In

addition, the normal stem cells modified with agents like N-acetylcysteine inhibitors to modulate ROS levels can be specifically made to target the brain tumor stem cells based on cell surface markers like CD133 and promote their elimination. However, despite successful clinical and pre-clinical trials, there are many challenges associated with stem cell therapy in cancer in the context of safety and efficacy (Volarevic et al. 2018). Thus, thorough in vitro and in vivo experimentation to study the mechanism for optimizing dose, delivery route, and the timing of administration needs to be done before their application in clinical and pre-clinical trials. Since reactive oxygen species play a major role in glioma initiation as well as progression mostly by regulating factors involved in maintaining the stemness of cells, use of natural compounds to increase ROS levels in brain tumor stem cells and use of normal stem cells to selectively target the metabolic pathways associated with ROS production in these quiescent tumor stem cells for their elimination also show a promising future.

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