Modulating glioblastoma chemotherapy response: Evaluating long non-coding RNA effects on DNA damage response, glioma stem cell function, and hypoxic processes

Edith Yuan, Kristie Liu, Justin Lee, Kathleen Tsung, Frances Chow, and Frank J. Attenello

Keck School of Medicine, University of Southern California, Los Angeles, CA, USA (E.Y., K.L.); Department of Neurological Surgery, Keck School of Medicine, University of Southern California, Los Angeles, CA, USA (J.L., K.T., F.C., F.J.A.)

Corresponding Author: Edith Yuan, BA, Keck School of Medicine, University of Southern California, 1200 North State St. Suite 3300, Los Angeles, CA 90033, USA (edith.yuan@usc.edu)

Abstract

Glioblastoma (GBM) is the most common and aggressive primary adult brain tumor, with an estimated annual incidence of 17 000 new cases in the United States. Current treatments for GBM include chemotherapy, surgical resection, radiation therapy, and antiangiogenic therapy. However, despite the various therapeutic options, the 5-year survival rate remains at a dismal 5%. Temozolomide (TMZ) is the first-line chemotherapy drug for GBM; however, poor TMZ response is one of the main contributors to the dismal prognosis. Long non-coding RNAs (lncRNAs) are nonprotein coding transcripts greater than 200 nucleotides that have been implicated to mediate various GBM pathologies, including chemoresistance. In this review, we aim to frame the TMZ response in GBM via exploration of the lncRNAs mediating three major mechanisms of TMZ resistance: (1) regulation of the DNA damage response, (2) maintenance of glioma stem cell identity, and (3) exploitation of hypoxia-associated responses.

Keywords

chemo-response | glioblastoma | long non-coding RNAs | Temozolomide.

Glioblastoma (GBM) is the most common primary and aggressive adult brain tumor, with an estimated annual incidence of 17 000 new cases in the United States.[1] Therapies targeting different pathological mechanisms, such as aberrant signaling pathways and angiogenesis, have been developed over the years. Despite these efforts, limited effective therapeutic options are available. The 5-year survival rate remains at a dismal 5%, with a median survival period of 14.6 months. Currently, the standard treatment entails maximal surgical resection followed by chemotherapy and radiation.[2]

Temozolomide (TMZ) is a first-line chemotherapy drug for GBM. TMZ exerts an antitumor effect via methylation of DNA at the O^6 position of guanine, forming O^6-methylguanine that incorrectly pairs with thymine. This base mispairing induces continuous activation of the mismatch repair (MMR) pathway and DNA double strand breaks (DSBs), which eventually results in cell cycle arrest and apoptosis (Figure 1A).[3] Concomitant treatment with TMZ and radiation for GBM has been shown to increase the median survival rate from 12.1 to 14.6 months when compared to radiation alone.[4]

Despite the survival advantage of TMZ, a large percentage of GBMs exhibits resistance to the chemotherapy. TMZ resistance may result from either intrinsic or acquired mutations and is thought to represent one of the main causes of treatment failure and tumor recurrence. Studies have previously focused on well-recognized contributors to TMZ resistance including O^6-methylguanine-DNA methyltransferase (MGMT) DNA damage response (DDR), MMR defects, as well as presence of cancer stem cells, and tumor hypoxia.[5–8] However, it has been increasingly recognized that long non-coding RNAs (lncRNAs) play an extensive role in mediating various aspects of GBM pathology, including the chemoresistance mechanisms described here.

LncRNAs are nonprotein coding transcripts longer than 200 nucleotides that have emerged as critical regulators of gene expression under normal physiology as well as disease states.[9] Studies have implicated lncRNAs in contributing to GBM pathogenesis by regulating gene expression at the epigenetic, transcriptional, and posttranscriptional levels.[10] For instance, *TUG1* is one of many lncRNAs that interacts with chromatin-modifying proteins responsible for histone methylations of genes critical for GBM progression.[11] At the posttranscriptional level, many lncRNAs behave as competing endogenous RNAs that act as molecular sponges for micro-RNAs (miRNAs). These interactions disinhibit the miRNA's downstream pathways and proteins, such as the Notch pathway and the VEGFA axis that promote glioma cell proliferation and angiogenesis, respectively.[12,13]

In the past few years, studies have explored the association between lncRNAs and TMZ response in GBM. The rising number of lncRNAs discovered may offer novel insights into GBM resistance to TMZ and represent potential therapeutic targets. Notably, while other reviews have discussed chemo-response in the context of lncRNAs, most prior reviews have focused on collections of well-known lncRNAs associated with chemo-sensitivity. Furthermore, prior reviews have also primarily concentrated on common lncRNAs noted to have chemoresistance roles without extensive exploration of chemosensitivity of specific lncRNAs.[14] In contrast, this review aims to frame the TMZ response in GBM via exploration of the lncRNAs mediating the three major mechanisms of TMZ resistance: (1) regulation of the DDR, (2) maintenance of glioma stem cell (GSC) identity, and (3) exploitation of hypoxia-associated responses (Table 1).

LncRNAs and the DDR

The DDR is a network of cellular processes that identifies and repairs DNA damage to prevent mutations and other genetic aberrations that threaten cell viability. Direct DNA-lesion reversal, MMR, base excision repair (BER), DNA DSB, and single-strand breaks (SSBs) repair are all components of the DDR. Dysregulated DDR pathways lead to the accumulation of DNA damage, evasion of cell cycle arrest, and chemoresistance. DSBs have an important role in mediating TMZ-induced apoptosis by triggering DNA damage kinase, ATM serine/threonine kinase, which then activates the p53-driven apoptosis pathway.[39] In addition to O^6-methylguanine, TMZ also forms N^7-methylguanine and N^3-methyladenine that are recognized and repaired by the BER pathway, specifically by N-methylpurine DNA glycosylase and DNA polymerase β. The abasic sites generated from the DNA glycosylase in the BER pathway are inherently unstable and readily convert into DNA SSBs; unrepaired SSBs can collapse the DNA replication pathway resulting in cell death (Figure 1B).[40]

LncRNAs have been identified to utilize the DDR pathways as a method of modulating treatment resistance. *SBF2-AS1*, which is upregulated in TMZ-resistant GBM cells, functions as a ceRNA against *miR-151a-3p* to enhance X-ray repair cross-complementing 4 (XRCC4) protein levels, augmenting DSB repair in gliomas.[20]

Radiation-induced DNA damage can similarly activate lncRNAs. *HMMR-AS1* was found to be elevated after irradiation along with increased expression of DDR proteins ATM, RAD51, and BMI1, ultimately promoting glioma cell growth.[41] Below, we will describe in further detail on how lncRNAs regulate TMZ resistance through direct DNA-lesion reversal and MMR.

LncRNA and MGMT

MGMT is a DNA methyltransferase that augments TMZ resistance by removing TMZ-induced alkyl groups and preventing the mispairing between O^6-methylguanine and thymine (Figure 1B). As part of the direct DNA-lesion reversal process, MGMT expression is induced with alkylating agents and serves as an important prognostic marker.[42] Clinical trials have demonstrated that MGMT promoter CpG methylation is associated with significantly improved overall survival in primary and recurrent GBM patients.[5,43]

However, even though clinical data on direct MGMT inactivators, such as O^6-benzylguanine and O^6-(4-bromothenyl) guanine (O^6-BG), showed improved survival in patients simultaneously treated with TMZ, TMZ was associated with increased hematopoietic toxicity due to nonspecific targeting, indicating that additional, more precise therapies targeting MGMT are required.[44] Because lncRNAs are highly cell type-specific, targeting lncRNAs as a method of regulating MGMT expressions may represent promising alternatives.[10] Here, we summarize the data on lncRNA-mediated epigenetic regulation of MGMT and the effect on TMZ resistance in GBM.

Lnc-TALC.—TMZ-associated lncRNA in GBM recurrence (*lnc-TALC*) is a newly identified lncRNA highly expressed in patient-derived TMZ-resistant GBM tissues. Clinically, GBM patients with lower *lnc-TALC* expression exhibit a significantly better response to TMZ with a lower likelihood of tumor recurrence.[15] To determine the association between *lnc-TAC* and TMZ-resistance, Wu et al. conducted clustered regularly interspaced short palindromic repeats (CRISPR)-induced *lnc-TAC* knockdown in TMZ-resistant human GBM cell lines, LN229R and U251R, and patient-derived GBM cells. The cells with decreased *lnc-TALC* had reduced MGMT mRNA and protein levels and increased TMZ-induced apoptosis. LN229R cells with *lnc-TALC* knockdown were then implanted in TMZ-treated mice; these mice showed significantly longer survival and smaller tumors when compared to mice with LN229R xenografts. This suggested that decreased *lnc-TALC* was associated with restored TMZ sensitivity in GBM cells. Additional in vitro experiments were conducted to delineate the mechanism linking higher *lnc-TALC* expression with TMZ resistance. A luciferase bioluminescent reporter assay confirmed that *lnc-TALC* functions as a ceRNA against *miR-20b-3p* to indirectly promote MGMT expression. Through sequestration of *miR-20b-3p*, *lnc-TALC* upregulates c-MET and its downstream target Stat3. Protein coimmunoprecipitation analysis subsequently demonstrated that Stat3 binds to a histone acetyltransferase to increase the acetyltransferase affinity to the *MGMT* promoter, effectively increasing MGMT expression.[15]
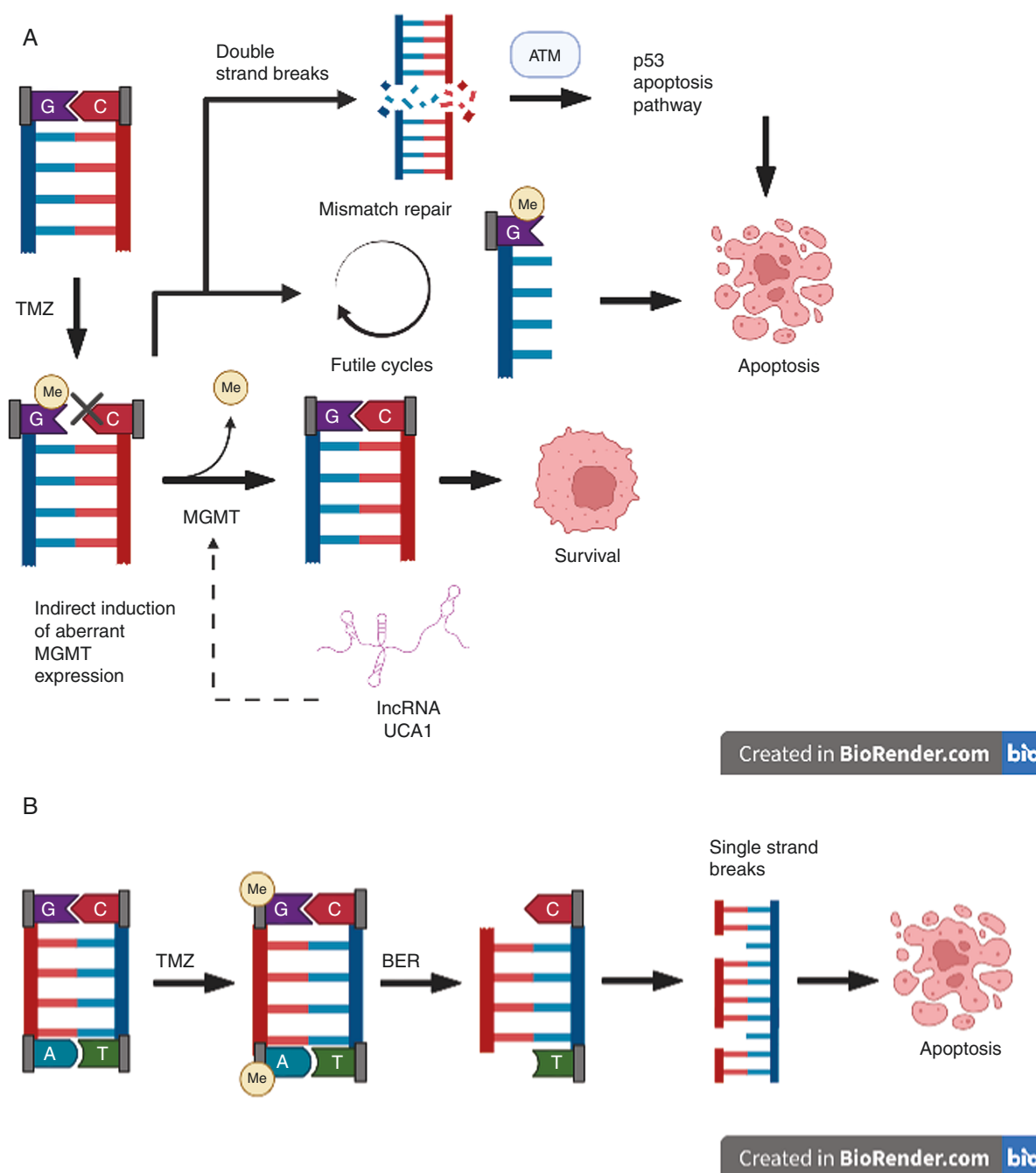
Figure 1. (A) Schematic diagram of TMZ and its relationship to the MMR, DSB, and MGMT pathways. TMZ adds a methyl group to guanine at the O^6 position, creating O^6-methylguanine that incorrectly pairs with thymine. The mispairing continuously activates the MMR pathway, resulting in DSBs and apoptosis. MGMT removes the methyl group from O^6-methylguanine and prevents the activation of the MMR pathway. LncRNA *UCA1* is shown to indirectly increase MGMT expression level. (B) Schematic diagram of TMZ and the BER and SSB pathways. TMZ adds a methyl group to guanine and adenine, forming N^7-methylguanine and N^3-methyladenine that are recognized by the BER pathway. When the bases are excised, SSBs result, creating instability that results in cell death. Me, methyl; G, guanine; C, cytosine; T, thymine.

FOXD2-AS1.—FoxD2 adjacent opposite strand RNA 1 (*FOXD2-AS1*) is a lncRNA highly expressed in recurrent GBM tumors and is associated with decreased patient survival.[45] A study showed that suppression of *FOXD2-AS1* via a small interfering RNA (siRNA) is associated with decreased GBM cell viability in TMZ.[16] While the specific mechanism is unclear, the study reported that *FOXD2-AS1* may oppose TMZ cytotoxicity through MGMT upregulation. siRNA-induced suppression of *FOXD2-AS1* (si-*FOXD2-AS1*) in human GBM cell lines, U251 and A172, is associated with hypermethylation of the MGMT promoter, resulting in decreased MGMT mRNA and protein levels. Furthermore,

Table 1. Summary of Mechanisms on LncRNA and TMZ Response

Mechanism	LncRNA	Expression Level in TMZ Resistant GBM	Sample Used	Summary of Mechanism on TMZ Response	ceRNA	References
I. DNA damage response						
MGMT	Lnc-TALC	Upregulated	Patient samples and LN229, U251	Increases MGMT expression through regulating c-met and stat3	miR-20b-3p	15
	FOXD2-AS1	Upregulated	U251 and A172	Increases MGMT expression	N/a	16
		Upregulated	U87, U251, LN229, A172	IncreasesCPEB4 (unrelated to MGMT)	miR-98-5p	17
	UCA1	Upregulated	Patient samples and A172,T98G, SHG44	Increases MGMT Expression	miR-182-5p	18
MMR	XIST	Upregulated	Patient samples and U251, U373, LN229, U118, LN229	Increases MSH6 indirectly through SP1, leading to abnormal MMR activity	miR-29c	19
DSB	SBF2-AS1	Upregulated	U87, LN229, A172, T98, U251	Enhances DSB repair by increasing XRCC4	miR-151a-3p	20
II. Glioma stem cells	TP73-AS1	Upregulated	G26 and G7 glioma stem cell lines	Highly upregulated in GSC and may mediate its effect through regulating *ALDH1A1*	N/A	21
	SOX2OT	Upregulated	Patient samples and U87, U251	Knockdown decreases SOX2 expression through methylating its promoter	N/A	22
	NEAT1	Upregulated	Patient samples and U87, U251	Highly upregulated in GSCs and activates MAP3K1	let7g-5p	23
		Upregulated	Patient samples	Activated by HMGB1 as part of the Wnt Pathway		24
	Linc00174	Upregulated	Patient samples and LN229, SHG44, U118, U251, U87	Maintains SOX9 expressions to enhance GSC-like behavior	miR-138-5p	25
	BC200	Upregulated	Patient samples,T98G, U87	Overexpression promoted GSC behaviors and TMZ resistance	miR218-5p	26
III. Hypoxia						
EMT	MALAT1	Upregulated	U251, U87	Knockdown decreases ZEB1	N/A	27
		Upregulated	U251	Increases GSK3ß, MGMT (unrelated to EMT)	miR-101	28
		Upregulated	Patient samples, U87, U251	Increases thymidylate synthase (unrelated to EMT)	miR-203	29
	RP11-838N2.4	Downregulated	Patient samples and U87, U251	Knockdown is associated with downregulation of EphA8	miR-10a	30
	H19	Upregulated	U251, M059J	Stimulates the Wnt/beta-catenin pathway	N/A	31
		Upregulated	U251, LN229	Stimulates the NF-kB pathway	N/A	32
Metabolism	HOTAIR	Upregulated	Patient samples and U87, A172	Knockdown suppresses HK2 and increases TMZ-induced apoptosis	miR-125	33
			Patient samples and serum	Increases EVA1 expression indirectly by inhibiting *miR-256b-3p*	miR-526b-3p	34

Table 1. Continued

Mechanism	LncRNA	Expression Level in TMZ Resistant GBM	Sample Used	Summary of Mechanism on TMZ Response	ceRNA	References
IV. Miscellaneous	AC003092.1	Downregulated	Patient sample and U87	Overexpression increases TFPI-2 and TMZ-induced apoptosis	miR-195	35
	CASC2	Downregulated	Patient samples, U251, U373, SNB19, U118, LN229	Knockdown leads to decreased PTEN and diminished TMZ response	miR-181a	36
	TUSC7	Downregulated	U87	Overexpression suppresses expression of multidrug protein 1	miR-10a	37
	ADAMTS9-AS2	Upregulated	Patient samples, T98G, U118	Overexpression upregulates the FUS/MDM2 Ubiquitination axis	N/A	38

Abbreviations: CPEB4, cytoplasmic polyadenylation element binding protein 4; GSK3ß, glycogen synthase kinase 3 Beta; EVA1, epithelial v-like antigen 1; TFPI-2, tissue factor pathway inhibitor-2; CASC2, cancer susceptibility candidate 2; PTEN, phosphatase and tensin homologs; TUSC 7, tumor suppressor candidate 7; FUS, fused in sarcoma; MDM2, murine double minute 2.

the si-*FOXD2-AS1* GBM cells showed decreased proliferation and increased cell death when treated with TMZ. This short and straightforward study indicated that *FOXD2-AS1* downregulation promotes TMZ sensitivity in GBM cell lines by decreasing MGMT expression.[16]

FOXD2-AS1 also drives TMZ resistance as part of the *FOXD2-AS1/miR-98-5p/*CPEB4 axis unrelated to MGMT regulation. A separate study found that *FOXD2-AS1* knockdown inhibited cell proliferation and promoted TMZ-induced apoptosis. RNA pull-down assay indicated that *FOXD2-AS1* serves as a ceRNA to adsorb *miR-98-5p*. Computational analysis showed that *miR-98-5p* and cytoplasmic polyadenylation element binding (CPEB4) shared binding sites. An oncogenic RNA binding protein, CPEB4 is associated with glioma migration, growth, and vascularization. The current study showed that expression of CPEB4 was inhibited by knockdown of *FOXD2-AS1* or upregulation of *miR-98-5p*, leading to decreased apoptosis and increased survival in vitro.[17]

UCA1.—A recently published paper by Cheng et al. demonstrated that the urothelial cancer-associated 1 (*UCA1*) lncRNA modulates TMZ response through MGMT. Identified as an oncogene, *UCA1* expression was directly correlated to glioma grade and MGMT expression. Transfection of GBM cells, A172 and SHG44, with si-*UCA1* showed decreased glioma cell viability with increased cell apoptosis. The si-*UCA1* cells also exhibited decreased MGMT protein level. *In vivo* experiments showed that the tumors with smallest volume and weight were from TMZ-treated mice implanted with si-*UCA1* GBM cells. The authors also identified *miR-182-5p* as a target of *UCA1*, forming the *UCA1/miR-182-5p*/MGMT axis that modulates MGMT expression and TMZ sensitivity.[18]

LncRNA and MMR

MMR is an important postreplication mechanism that recognizes and repairs nucleotide mismatches. In TMZ-treated cells, MMR is activated to excise the mismatched thymine. Because O^6-methylguanine remains on the template strand, this leads to futile cycles of thymine mispairing and excising that eventually results in DNA DSBs and cell death.[46]

Deficiencies in MMR render tumors resistant to TMZ, regardless of the MGMT methylation status.[7] Aberrations in MMR proteins, such as melanocyte-stimulating hormone 2, MSH6, and mutL homolog 1, or defective DSB repairs can lead to suppressed apoptotic response and increased survival.[46–49] Furthermore, while MSH6 mutations were not observed in pre-TMZ treated GBM tissues, 26% of recurrent tumors post-TMZ treatment had decreased MSH6 expression, suggesting that loss of MSH6 function is associated with tumor recurrence during TMZ treatment.[46] Currently, the relationship between lncRNAs and MMR is considerably understudied and deserves additional extensive investigations. Thus far, only the lncRNA X-inactive specific transcripts (*XIST*) has been identified to alter the TMZ response in GBM through modulating the MMR.

XIST. — *XIST* is a well-characterized lncRNA in various cancers, including GBM.[50] Paired analysis between patient-derived GBM and peritumoral brain tissues has revealed *XIST* to be significantly higher in the former. More importantly, *XIST* is directly associated with larger tumor size and overall shorter survival period.[50] Human GBM cells transfected with a siRNA targeting *XIST* displayed diminished proliferation rate and increased sensitivity to TMZ as demonstrated by the BrdU and viability assay, respectively.[51] RNA immunoprecipitation further showed that *XIST* affects the TMZ response by directly binding *miR-29c* and inhibiting its expression. This interaction results in upregulation of specificity protein 1 (SP1), a transcription factor that causes aberrant expression of the MMR-associated protein, MSH6.[19] Taken together, the result suggests GBM cells with elevated *XIST* may exhibit decreased TMZ sensitivity due to the abnormal MSH6 expression.

LncRNAs and GSCs

GBM harbors a subset of self-renewing population of cells termed GSCs that have been hypothesized to play a key role in GBM pathogenesis.[6] As TMZ preferentially targets proliferating glioma cells, the relatively quiescent GSCs are favored to survive and increase the likelihood of GBM recurrence. Notably, xenograft mouse models of human GBM cells have demonstrated that post-TMZ administration, the majority of residual, treatment-resistant tumor cells were fluorescently labeled GSCs with the ability to reinitiate GBM.[6] In addition, CD133+ GSCs exhibit higher levels of MGMT mRNA and lower expressions of autophagy- and apoptotic-related proteins that allow them to evade conventional therapies (Figure 2).[52–54]

Previous literature has illustrated that irregular expression of specific lncRNAs are closely related to the malignant phenotypes of GSCs. For instance, lncRNAs *XIST*, *H19*, and *MIAT* interact with transcription factors that promote and maintain GSCs in vitro.[55] High throughput microarray profiling studies in GSCs have further identified differential expressions of specific lncRNAs to be associated with GSC behaviors, such as neurosphere formation and therapy resistance.[56,57] GSC-associated lncRNAs represent an innovative strategy for targeting GSCs. One study has identified 1545 unique lncRNAs found to regulate GSCs' differentiation status; the lncRNAs identified are highly specific, with the ability to promote differentiation in GSCs and decrease the tumor cells' tumorigenicity.[58] Studies described below have investigated how different lncRNAs affect the TMZ response through regulating GSC-like behaviors.
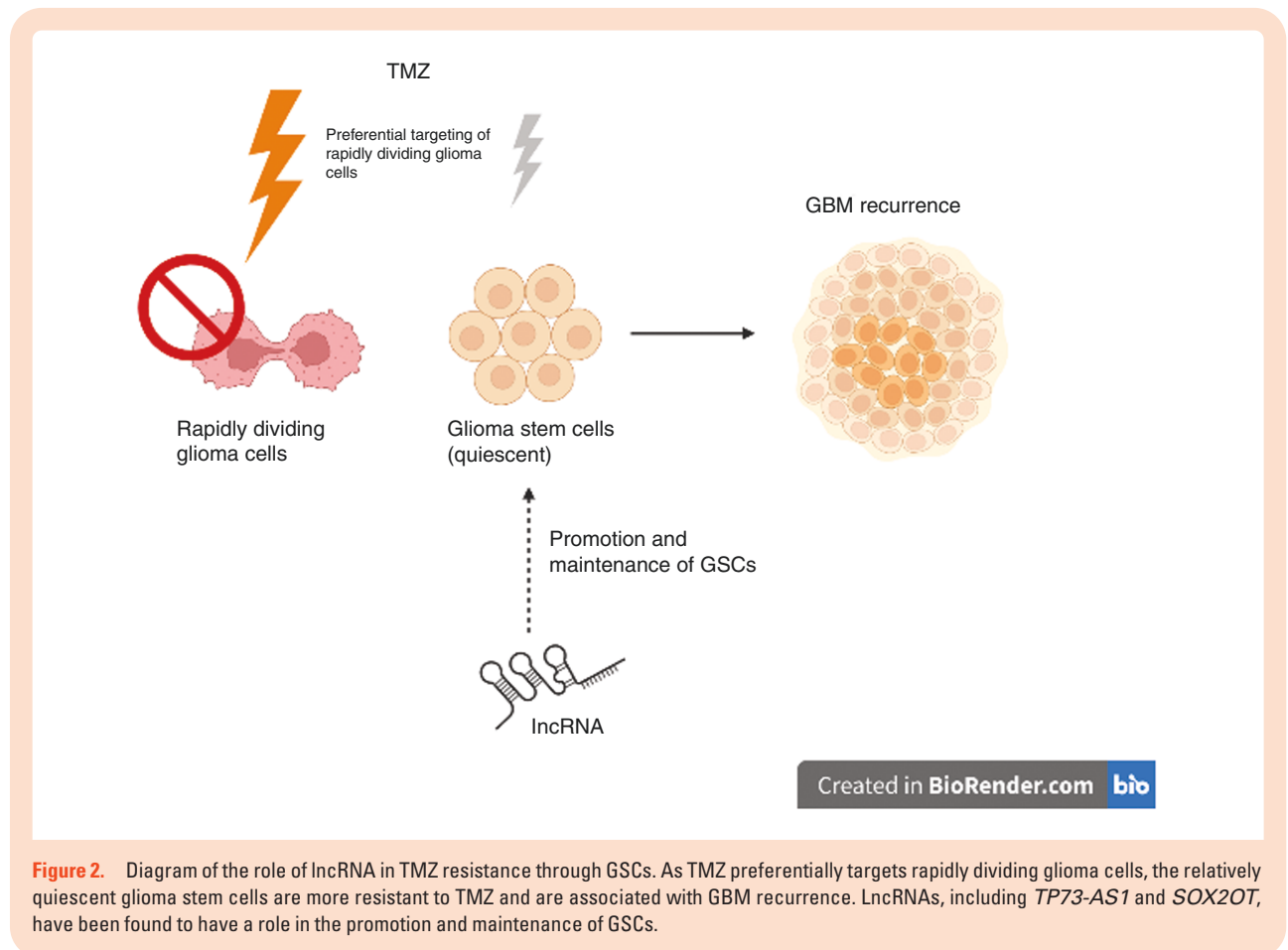

Figure 2. Diagram of the role of lncRNA in TMZ resistance through GSCs. As TMZ preferentially targets rapidly dividing glioma cells, the relatively quiescent glioma stem cells are more resistant to TMZ and are associated with GBM recurrence. LncRNAs, including *TP73-AS1* and *SOX2OT*, have been found to have a role in the promotion and maintenance of GSCs.

TP73-AS1

In GBM, lncRNA P73 antisense RNA 1T (*TP73-AS1*) expression level is elevated in patient-derived GSCs and correlates directly with early mortality.[21] Mazor et al. used CRISPR to downregulate *TP73-AS1* in two established human GSC lines and noted that the cells exhibited decreased viability under TMZ treatment. Further elucidating the mechanism underlying *TP73-AS1*-associated TMZ resistance, transcriptome analysis of the *TP73-AS1*-attenuated GSCs revealed perturbations of neuronal differentiation and metabolic reprogramming pathways. One of the transcripts found to be particularly downregulated was aldehyde dehydrogenase 1 family member A1 (*ALDH1A1*).[21] ALDH1A1 is recognized as a marker of GSCs and a promoter of clonogenicity; its expression is also elevated in recurrent, TMZ-resistant GBMs.[59] Moreover, treating GSCs with a pharmacological ALDH1A1 inhibitor significantly increased their sensitivity to TMZ, suggesting that *TP73-AS1* may mediate its effects on TMZ resistance through regulating *ALDH1A1*.[21]

SOX2OT

LncRNA SOX2 overlapping transcript (*SOX2OT)* is mapped to the human chromosome 3q26.3 and harbors the SRY-Box Transcription Factor 2 (*SOX2)* gene in its intronic region.[60] Analysis of patient-derived GBM samples has shown *SOX2OT* level to positively correlate with both advanced glioma grade and reduced median survival time.[61] In vitro studies showed that GBM cells U87 and U251 transfected with *SOX2OT* siRNA lentiviral vectors (si-*SOX2OT)* displayed increased TMZ-mediated apoptosis. As lncRNAs can exert cellular functions through regulating proximal genes, the authors predicted *SOX2OT* modulates the TMZ response in GBM through upregulating SOX2. This was confirmed in si-*SOX2OT* U87 and U251 cells demonstrating significantly decreased SOX2 mRNA and protein levels.[22] By de-differentiating tumor cells to GSCs, SOX2 is one of the four core transcription factors essential for GBM propagation.[62] Downregulation of SOX2 interferes with GSCs' ability to self-renew and consequently, to form gliomas.[63,64] Importantly, SOX2 has previously been established to take part in mediating TMZ resistance in GBM.[65] Mechanistically, *SOX2OT* maintains SOX2 expression by interacting directly with α-ketoglutarate-dependent dioxygenase alkB homolog 5, an RNA demethylase that binds to the SOX2 promoter and is similarly upregulated in TMZ-resistant GBMs.[22]

NEAT1

The lncRNA nuclear paraspeckle assembly transcript 1 (*NEAT1*) is overexpressed in GBM tissues and is a marker of poor survival in primary GBM patients.[66] *NEAT1* is upregulated in GSCs and plays an important role in augmenting their proliferation rate and resistance to chemotherapy. Induced by high-mobility group box 1 in GBM cells treated with TMZ, *NEAT1* has been shown to promote GSC formation through the Wnt/β-catenin pathway.[24] In addition, a recent study showed that GSCs transfected with short hairpin RNA (shRNA) against *NEAT1* (sh-*NEAT1*) are associated with significantly reduced viability and proliferation when exposed to TMZ in vitro. Using a bioluminescence assay, the authors identified *let7g-5p* as a downstream target of *NEAT1*. A prior study had demonstrated *let7g-5p* as a tumor-suppressive miRNA associated with impaired GSC behavior.[67] Bioinformatic analysis predicted that *let7g-5p* shares a binding site with mitogen-activated protein kinase 1 (MAP3K1), a protein previously shown to correlate directly with shortened patient survival and increased TMZ resistance in GBM cells.[68] Subsequent mRNA results showed that MAP3K1 expression was significantly increased in GBM cells transfected with *let7g-5p* shRNA. Through transcriptional and protein analyses, the authors concluded that *NEAT1* suppresses *let7g-5p* to activate MAP3K1 and ultimately inhibit the cytotoxic effects of TMZ.[23]

LINC00174

As a relatively newly discovered lncRNA, *linc00174* is markedly upregulated in human GBM tissues and its expression level is inversely correlated with patient survival rate.[25] *Linc00174* has been reported to strengthen TMZ resistance in GBM cells by directly targeting *miR-138-5p*, a tumor suppressive miRNA shown to arrest GBM cell cycle.[69] By inhibiting *miR-138-5p*, *linc00174* increases SRY-Box Transcription Factor 9 (SOX9), a downstream target of *miR-138-5p*. In gliomas, SOX9 has been shown to maintain the self-renewal ability of GSCs.[70] GBM cells transfected with shRNA-mediated *linc00174* knockdown are associated with increased *miR-138-5p* and decreased SOX9 levels. In vitro, the transfected cells showed enriched apoptotic markers in addition to reduced viability and colony formation under TMZ exposure. The results indicated that inhibiting *linc00174* improves TMZ-induced apoptosis by discouraging stem cell-like behaviors through regulating *miR-138-5p* and SOX9.[25]

BC200

In GBM tissues, Brain Cytoplasmic 200 (*BC200*) expression is significantly higher than in normal tissues. In addition, *BC200* was positively correlated with p53-mutation, indicating *BC200* association with poor prognosis in patients. Knockdown of *BC200* via siRNA suppressed common stem cell markers, including SOX2, and the self-renewal capacity of GBM cells. In contrast, GBM cells transfected with *BC200* overexpression vector demonstrated increased cell wound healing migration, invasiveness, and colony-forming ability. The cells also exhibited decreased TMZ resistance via viability assay. Western blot analysis showed increased MGMT, multidrug resistance protein, and ABC transporter. To further assess the effect of *BC200* on miRNA expression, bioinformatic analysis, and online database identified *miR-218-5p* level to be negatively associated with *BC200*. GBM cells with *miR-218-5p* inhibited showed higher TMZ resistance, stem cell markers, and colony-forming abilities. *In vivo* studies supported the *in vitro* experiments, demonstrating TMZ-treated mice

implanted with si*BC200* GBM cells had significantly reduced tumor size and prolonged overall survival.[26]

LncRNAs and Hypoxic Processes

The brain tumor microenvironment is composed of a heterogenous population of cancerous as well as nonneoplastic cells, including the surrounding tissue stroma and associated vascular supply. Tumor stromal cells secrete various angiogenesis-promoting factors where vascular endothelial growth factor is the most potent and its high expression level indicates poor outcome.[71] As a result of poorly functional blood vessels, tumors exhibit extensive hypoxia and limited response to systemic chemotherapeutics. The presence of hypoxia upregulates gene expressions that promote cell survival and chemoresistance.[72] Furthermore, the hypoxic niche hosts GSCs that stimulate endothelial cell growth and proliferation associated with neo-angiogenesis (Figure 3).[73]

Composed of several subunits (ie, HIF-1α, HIF-2α), hypoxia-inducible factor (HIF) is the main transcriptional regulator of cellular response to hypoxia. HIF overexpression promotes cancer angiogenesis, drug resistance, and certain lncRNAs.[8] LncRNA *AWPPH* level correlates directly with HIF-1α and is associated with metastatic GBM patients. Silencing HIF-1α attenuated the enhanced glioma invasion and migration seen with elevated *AWPPH*.[74] Consequently, the hypoxic microenvironment mediates two important processes implicated in the GBM TMZ resistance, the epithelial to mesenchymal transition (EMT) and metabolic alterations.

LncRNA and EMT

The EMT is a process that transforms tumor cells into a more mesenchymal phenotype with enhanced migratory and invasive properties. The hypoxic environment promotes EMT through triggering the HIF1α/ZEB1 signaling axis.[75] Cells undergoing EMT display decreased expression level of epithelial genes (ie, E-cadherin, ZO-1) and increased expression of mesenchymal genes (ie, N-cadherin and vimentin). These alterations lead to changes in cell morphology and promote stem cell-like features.[76] Though EMT is most often associated with GBM invasion, it has been increasingly recognized for its contribution to chemoresistance.

Several studies have shown that the relationship between specific EMT proteins and lncRNAs regulates the chemotherapy response in different cancers. For example,
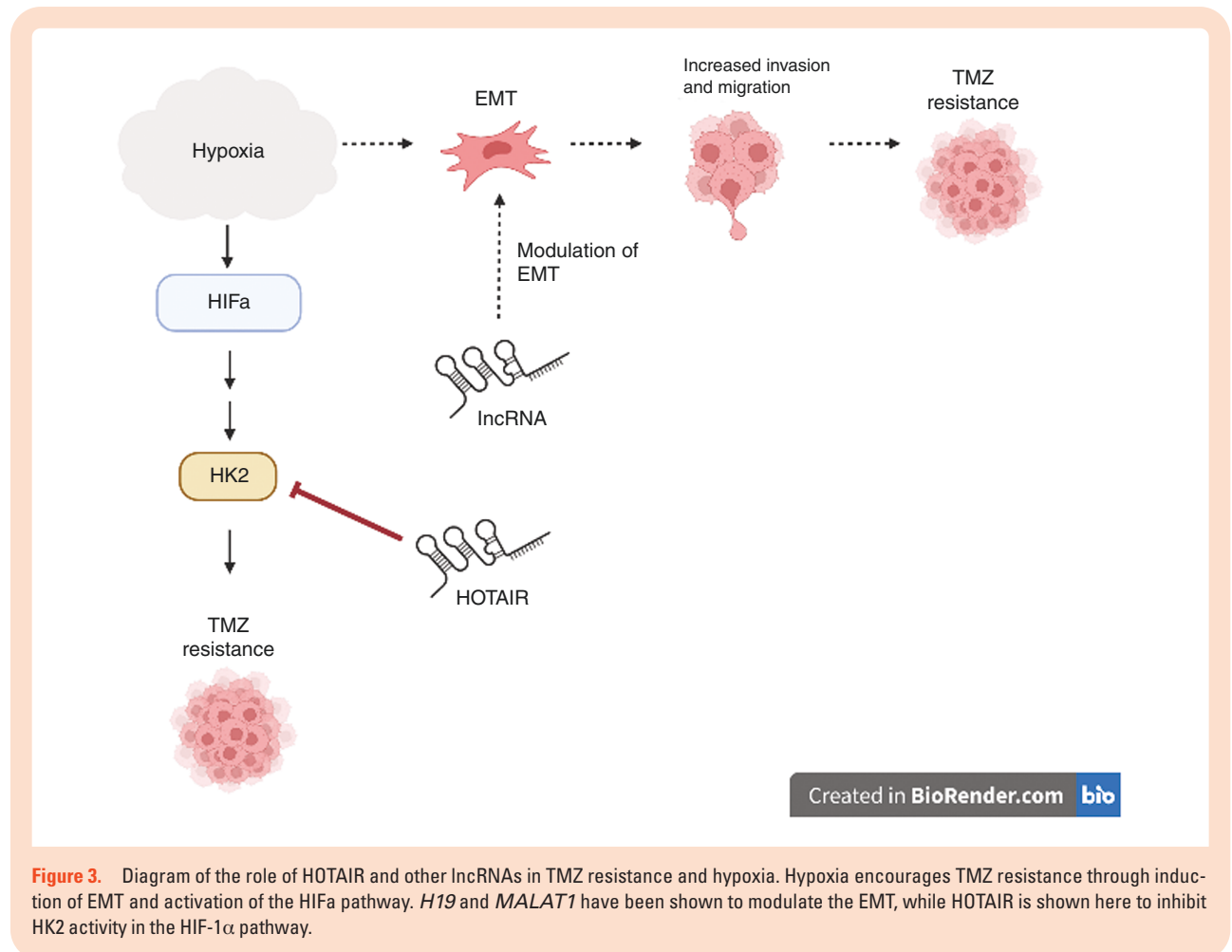

Figure 3. Diagram of the role of HOTAIR and other lncRNAs in TMZ resistance and hypoxia. Hypoxia encourages TMZ resistance through induction of EMT and activation of the HIFa pathway. *H19* and *MALAT1* have been shown to modulate the EMT, while HOTAIR is shown here to inhibit HK2 activity in the HIF-1α pathway.

in colorectal cancer, lncRNA *SLC25A25-AS1* increases the chemo-response through regulating EMT-associated proteins.[77] Similarly, *lncRNA-ATB* inhibits the EMT proteins, ZEB1 and ZEB2, to increase trastuzumab resistance in breast cancer cells.[78] Although the association between EMT and chemo-response necessitates more extensive investigation, understanding their relation to specific lncRNAs provides additional therapeutic avenues for patients with GBM.

MALAT1.—In GBM, lncRNA metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*) is associated with poor TMZ response and survival in GBM patients. *MALAT1* is a well-studied lncRNA known to be upregulated following TMZ-induced alkylation damage and recognized to increase chemoresistance through various mechanisms, including upregulating GSK3ß, MGMT, and thymidylate synthase.[28,29,79] A recent study described the direct association between *MALAT1*-mediated TMZ resistance and EMT. The investigators first transfected U87 and U251 GBM cells with *MALAT1* siRNA (si-*MALAT1*) to generate *MALAT1*-knockdown cells. After being treated with TMZ, the si-*MALAT1* cells showed reduced viability compared to the non-transfected cells. In addition, downregulation of ZEB1 was confirmed in the si-*MALAT1* cells at the mRNA and protein levels. Furthermore, *MALAT1*-associated TMZ resistance was reversed in U87 and U251 with siRNA-induced ZEB1 knockdown, suggesting that *MALAT1* enhances TMZ resistance via regulation of ZEB1.[27] This data complements other studies that have indicated ZEB1 to facilitate TMZ resistance in GBM cells by elevating MGMT expression and accelerating double-stranded break repairs.[80] Lastly, in mice implanted with patient-derived GBM cells, concurrent treatment of TMZ and a nanocomplex delivering si-*MALAT1* has been shown to significantly prolong survival, solidifying *MALAT1* as a lncRNA with promising therapeutic potential.[79]

RP11-838N2.4.—*RP11-838N2.4* is a largely understudied lncRNA recognized for modulating the TMZ response in GBM. Reports of *RP11-838N2.4* and primary GBM tissues have revealed lower expression of *RP11-838N2.4* is associated with an increased risk of tumor recurrence and early mortality.[30] Contrasting most other lncRNAs mentioned previously, *RP11-838N2.4* is inversely correlated with TMZ resistance. This is demonstrated when transfection of TMZ-resistant U87 (U87R) and U251 (U251R) GBM cells with a *RP11-838N2.4*-overexpressed plasmid exhibited increased TMZ-mediated cell death. Furthermore, the authors determined that *RP11-838N2.4* level is inversely associated with *miR-10a*, which negatively regulates its downstream target, ephrine tyrosine kinase receptor A8 (EphA8).[30] Prior data has shown enhanced EphA8 reverses EMT and transforms glioma cells to the more cobble-stoned appearance of epithelial cells. In contrast, loss of EphA8 is associated with shorter survival time, and diminished TMZ response in GBM patients.[81] Further mRNA and protein analysis indicated that *RP11-838N2.4* reduces the expression of *miR-10a* and relieves its inhibitory effect on EphA8, resulting in enhanced TMZ cytotoxicity by decreasing the EMT process.

H19.—LncRNA *H19* is upregulated in human TMZ-resistant GBM tissues and is directly associated with decreased patient survival rate. Reports have shown *H19* to support TMZ resistance in GBM through different mechanisms, including catalyzation of EMT-related pathways.[31,32,82] Studies have delineated the relationship between *H19*-associated TMZ resistance and the Wnt/β-catenin and NF-κβ processes, two signaling cascades known to induce both TMZ inefficiency and EMT.[31,32,83] TMZ-resistant GBM cells transfected with *H19* shRNA had significantly lower half maximal inhibitory concentration (IC_{50}) value for TMZ when compared to the control group. The *H19*-silenced cells also exhibited weaker signals of EMT-related and Wnt/β-catenin proteins. However, treatment with the Wnt/β-catenin activator, Licl, reversed the protein expressions and restored TMZ resistance in the *H19*-silenced cells.[31] Similarly, *H19* has also been implicated to promote TMZ resistance in GBM through the NF-κβ pathway. U251 and LN229 GBM cells cotransfected with *H19* siRNA (si-*H19*) and a NF-κβ luciferase reporter showed decreased bioluminescence and mRNA levels of NF-κβ downstream targets, suggesting an association between *H19* and the NF-κβ pathway proteins. The si-*H19* cells also underwent apoptosis at a lower TMZ concentration. Lastly, exposure to a NF-κβ inhibitor significantly impaired TMZ resistance in GBM cells transfected with a *H19*-overexpressing plasmid.[32]

LncRNA and Metabolism

Hypoxia is one of the key characteristics of GBM and glioma cells shift to aerobic glycolysis as a main source of ATP, a phenomenon referred to as the Warburg effect.[84] As one of the chief glycolytic enzymes and upregulated by HIF-1α, HK2 is a compelling metabolic target because of its strong presence in gliomas and its role in maintaining high glycolytic rates. Elevated expression of HK2 is also associated with TMZ-resistant GBM tissues and poor survival in patients. Importantly, HK2 inhibition has been shown to restore oxidative glucose metabolism and decrease the overall tumorigenesis in GBM cell lines.[33,85] Currently, HOX antisense intergenic RNA (*HOTAIR*) is the only lncRNA found to mediate its TMZ effects through HK2 in GBM.

HOTAIR.— *HOTAIR* is highly expressed in GBM tissues and serum-derived extracellular vesicles, serving as a negative prognostic factor.[34,86] Moreover, *HOTAIR* was found to be upregulated in TMZ-resistant GBM tissues and U87 and A172 GBM cells. A recent investigation described increased TMZ sensitivity in U87 and A172 GBM cells transfected with shRNA targeting *HOTAIR* (sh-*HOTAIR*). Zhang et al. treated sh-*HOTAIR* U87 and A172 cells with TMZ and analyzed the gene expressions. They found that the cells not only exhibited upregulated *miR-125* and decreased *HK2* levels, but also had increased apoptotic response to TMZ. Lastly, depletion of *HOTAIR* also enhanced the therapeutic effects of TMZ in vivo. Mouse xenograft models of sh-*HOTAIR* U87 cells were more sensitive to TMZ, leading to significantly smaller tumor and longer survival. Furthermore, the sh-*HOTAIR* U87 cells had suppressed HK2 and enhanced apoptotic protein levels. Collectively, these

results indicate that *HOTAIR* regulates GBM TMZ response through the expression of *miR-125* and HK2.[33]

Targeting LncRNA

Therapeutic regulation of lncRNAs represents an attractive approach for GBMs as they are highly tissue- and cell-type specific.[10] To target non-coding RNAs, multiple approaches including siRNA, antisense oligonucleotides (ASOs), and the CRISPR/Cas9 system have been investigated.[87]

siRNAs are short double-stranded RNAs between 19 and 30 nucleotides. They induce gene silencing by recruiting the RNA-induced silencing complex to degrade the target gene posttranscriptionally.[88] Although siRNAs can effectively target lncRNAs regardless of their intracellular locations, they are susceptible to nucleases and have lower bioavailability due to their large size and anionic charge. However, chemical modifications have improved their stability, specificity, and delivery.[88,89] siRNA drugs have been approved for diseases such as adult hereditary amyloidogenic transthyretin.[90]

ASOs are single-stranded ASOs that are taken up freely by cells and form heteroduplexes with the complementary lncRNA. ASOs can inhibit or alter gene expression via steric hinderance, splicing alterations, or cleavage induction by endogenous RNaseH.[91] ASOs have similarly undergone modifications of their backbone to improve their stability and delivery. Compared to siRNAs, ASOs are smaller, less immunogenic, and exhibit fewer off-target effects; the development of locked nucleic acids has also improved the potentcy significantly, though with increase hepatoxicity.[92,93] Moreover, certain ASOs have demonstrated lower RNAse H cleavage and can only enter the central nervous system via intrathecal injections.[94] Clinically, ASO-mediated therapies are currently used for treating diseases such as spinal muscular atrophy.[95]

The CRISPR/Cas9 system is composed of a single guide (sgRNA) and a Cas9 enzyme. The sgRNA guides the Cas9 nuclease to specific sites in the genome via complementary base pairing. The high efficiency and ease of programming makes CRISPR/Cas9 a robust tool to target lncRNAs.[96] Furthermore, CRISPR/Cas9 can be utilized to target splice sites to produce intron retention or exon deletion, which can be used for genome-wide screening for essential lncRNAs.[97] However, one major limitation is the risk of impacting adjacent genes, such as those that may overlap with the target lncRNA gene or those with DNA elements in the lncRNA locus that may regulate other genes.[98] Unlike siRNAs and ASOs, CRISPR/Cas9 drugs have not yet been approved for treating diseases.

Conclusion

TMZ resistance is a significant barrier to improving the dire prognosis of GBM. From the current aspects of recent research, lncRNAs are being increasingly recognized for their diverse roles in modulating various GBM pathologies. To the best of our knowledge, this review is the first to focus on and describe, in detail, the lncRNAs specifically involved in TMZ response in GBM. Furthermore, this review concurrently explores the mechanisms commonly associated with GBM chemo-resistance in the context of lncRNAs. We divided lncRNA-mediated TMZ resistance into three large categories: (1) regulation of the DDR, (2) maintenance of GSC identity, and (3) association with tumor hypoxia. Naturally, there are other existing mechanisms that do not fit in the above categories included in Table 1.

LncRNAs undoubtedly command a much more critical role in GBM pathogenesis than previously thought. The complex network between lncRNAs and TMZ resistance are only beginning to transpire and would require considerably more experiments to thoroughly understand and appreciate. Nevertheless, the highly-specific differential lncRNA expressions between normal and glioma cells, in conjunction with rapidly advancing sequence-based nucleic acid therapies emphasize lncRNAs as promising targets for novel GBM therapies.

Conflict of Interest

The authors have no conflicts of interest to disclose.

Authorship

Conceptualization of manuscript, literature search, writing and editing the draft: E.Y.; Editing and proofreading drafts: E.Y., K.L., J.L., K.T., F.C., F.J.A.; Revising manuscript: E.Y., K.L., F.C., F.J.A.

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