Identification and Characterization of Long Non-Coding RNAs (lncRNAs) Involved in Transforming Growth Factor-Beta (TGF-β) Pathway in Glioblastoma Multiforme (GBM)

THESIS

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by

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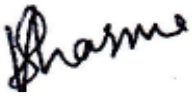
CERTIFICATE

This is to certify that the thesis titled **Identification and Characterization of Long Non-Coding RNAs (lncRNAs) Involved in Transforming Growth Factor-Beta (TGF-β) Pathway in Glioblastoma Multiforme (GBM)** submitted by **Bakhya Shree GB** ID. No **2017PHXF0001H**, for the award of Ph.D. of the Institute, embodies original work done by her under my supervision.

Signature of the Supervisor: 

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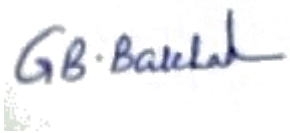
DECLARATION

This is to certify that the thesis titled "**Identification and Characterization of Long Non-Coding RNAs (lncRNAs) Involved in Transforming Growth Factor-Beta (TGF-β) Pathway in Glioblastoma Multiforme (GBM)**" is based on my own research work and has been carried out under the guidance and supervision of Prof. Vivek Sharma, Associate Professor, Dept. of Biological Sciences, BITS Pilani, Hyderabad Campus, Hyderabad, India.

The data and information which I have used from various sources have been duly acknowledged. I declare that this work has not been previously submitted by me to any other university/institute for the award of any other degree or diploma.



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Abstract

Glioblastoma multiforme (GBM) is the central nervous system's most aggressive and malignant tumor. Transforming growth factor-beta (TGF-β) expression is elevated in GBM and promotes invasion and EMT. TGF-β regulates the expression of several long-non-coding RNAs (lncRNAs), which promote glioma pathogenesis. TGF-β-regulated lncRNAs modulate several aspects of tumor development, such as proliferation, invasion, metastasis, epithelial to mesenchyme transition (EMT), and drug resistance in various cancers, including GBM. Using a genome-wide microarray screen, we identified several novel differentially expressed lncRNAs upon TGF-β treatment in T98G glioma cells. Among these differentially expressed lncRNAs, we further characterized the role of lncRNA-MUF and LINC01711 in GBM pathogenesis. TGF-β induces lncRNA-MUF and LINC01711 expression in glioma cells, and their expression is significantly upregulated in glioma tissues and is associated with poor overall survival of GBM patients. The SMAD2/3 transcription factors mediate the upregulation of LncRNA-MUF and LINC01711 during TGF-β treatment. Knockdown of lncRNA-MUF or LINC01711 reduces proliferation, migration, and invasion in glioma cells. In addition, depletion of LncRNA-MUF or LINC01711 sensitizes them to Temozolomide (TMZ) and Cisplatin-induced apoptosis. In line with their role in regulating invasion, lncRNA-MUF and LINC01711 function as competing endogenous RNA (ceRNA) for miR-34a and promotes SNAIL1 and ZEB-1 expression. Our findings suggest lncRNA-MUF/LINC01711 and miR-34a axis as attractive therapeutic targets for GBM.

Keywords: Glioblastoma multiforme, lncRNA, lncRNA-MUF, LINC01711, SNAIL, ZEB1, miR-34a, and TGF-β.

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List of abbreviations

1. AAAIR - average annual age-adjusted incidence rates
2. ADAMs – A disintegrin and metalloproteinases
3. AMH – anti-mullerian hormone
4. ASO – antisense oligonucleotide
5. BBB – blood-brain barrier
6. BMP – bone morphogenetic protein
7. CBTRUS - Central Brain Tumor Registry of the United States
8. CD133 – cluster of differentiation 133
9. CDB – ChIP dilution buffer
10. CDK4 – cyclin-dependent kinase 4
11. CDK6 – cyclin-dependent kinase 6
12. CDKN2A – cyclin-dependent kinase inhibitor 2A
13. ceRNA - competing endogenous RNA
14. CGGA – Chinese glioma genome atlas
15. CNS – central nervous system
16. CTGF - Connective tissue growth factor
17. CTL-4 – Cytotoxic T-lymphocyte-associated protein 4
18. CTLs – cytotoxic T lymphocytes
19. CV – crystal violet
20. D-2-HG – D-2-hydroxyglutarate
21. DCL – decrosslinking
22. DCs – dendritic cells
23. DEGs – differentially expressed genes
24. DGCR8 – DiGeorge syndrome critical region 8
25. DLL3 – delta-like canonical Notch ligand 3
26. ECM – extracellular matrix
27. EDTA – ethylene diamine tetra acetic acid
28. EGF – epidermal growth factor
29. EGFR– epidermal growth factor receptor
30. EMT – epithelial to mesenchyme transition
31. ERK – Extracellular signal-regulated kinase
32. ESM-1 – endothelial cell-specific molecule 1

33. FA – fatty acid
34. FDA – food and drug administration
35. FGF – fibroblast growth factor
36. GBM – Glioblastoma multiforme
37. GBM NOS - GBM not otherwise specified
38. GDF – growth and differentiation factors
39. GEPIA – gene expression profiling interactive analysis
40. GICs – glioma-initiating cells
41. GSCs - Glioma stem cells
42. HCC – hepatocellular carcinoma
43. HIF - hypoxia-inducible factor
44. HIF – hypoxia-inducible factor
45. HK2 – hexokinase 2
46. HMGA2 – high-mobility group AT-hook 2
47. HOTAIRM1 – HOXA transcript antisense RNA, myeloid-specific 1
48. HOXD-AS2 – HOXD cluster antisense RNA 2
49. HULC – Highly upregulated in liver cancer
50. ICAM – intercellular adhesion molecule
51. IDH1 - Isocitrate dehydrogenase 1
52. IGFBP7 – Insulin-like growth factor-binding protein 7
53. IL-10 – Interleukin-10
54. JAK-STAT – Janus kinase - signal transducer and activator of transcription
55. LGG – low-grade gliomas
56. LIF – leukemia inhibitory factor
57. lincRNAs – large intergenic non-coding RNAs
58. LINK-A – lncRNA for kinase activation
59. LncRNA – long non-coding RNA
60. MAPK – mitogen-activated protein kinases
61. MDM2 – Mouse double minute 2 homolog
62. MDSCs – Myeloid-derived suppressor cells
63. MEF2C – Myocyte enhancer factor 2C
64. MET – mesenchymal-epithelial transition factor
65. MGMT – methylguanine-DNA-methyltransferase
66. MH1 – Mad homolog 1

67. MH2 – Mad homolog 2
68. mIDH – mutated IDH
69. miRNA – microRNA
70. MMPs – Matrix metalloproteases
71. NaCl – Sodium chloride
72. NADP+ - Nicotinamide adenine dinucleotide phosphate
73. NCAM – neuronal cell adhesion molecule
74. NCGs – non-protein-coding genes
75. ncRNA – non-coding RNA
76. NEAT1 – Nuclear enriched abundant transcript 1
77. NF-κB – Nuclear factor-κB
78. OLIG2 – Oligodendrocyte Transcription Factor 2
79. PARIS – psoralen analysis of RNA interactions and structures
80. PCGs - protein-coding genes
81. PCGs – protein-coding genes
82. PD1 – programmed death-1
83. PDGF – Platelet-derived growth factor
84. PDGFRA – platelet-derived growth factor receptor alpha
85. PFA – paraformaldehyde
86. PI – protease inhibitor
87. PI3K – phosphatidylinositide 3-kinase
88. PI3K/PTEN/Akt – Phosphatidylinositol4,5-Bisphosphate 3-Kinase/Phosphatase and Tensin Homolog/serine-threonine kinase Akt
89. PIK3CA – phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
90. PINK1 – PTEN-induced kinase 1
91. PLK1 – polo-like kinase 1
92. pSMAD2 – Phospho-SMAD2
93. pSMAD3 – Phospho-SMAD3
94. pSMAD7 – Phospho-SMAD7
95. *PTEN* – Phosphatase And Tensin Homolog
96. RAS – rat sarcoma
97. RB – retinoblastoma
98. R-SMADs – receptor-regulated SMADs
99. RTK – Receptor tyrosine kinase

100. SBE – SMAD binding elements
101. SCNA – somatic copy number alterations
102. Smad – small mothers against decapentaplegic
103. SOX – SRY-related HMG-box
104. SVZ - subventricular zone
105. TAF15 – TATA-box binding protein associated factor 15
106. TERT - telomerase reverse transcriptase
107. tFBS – transformed fibroblasts
108. TGF-β – transforming growth factor - beta
109. TGFβRI - Transforming growth factor – β receptor I
110. TIMPs – tissue inhibitors of MMPs
111. TMZ – temozolomide
112. TRAF4/6 – TNF receptor-associated factor 4/6
113. Tris HCl – tris hydrochloric acid
114. uPA – urokinase-type plasminogen activator
115. UTR – Untranslated region
116. VEGF - vascular endothelial growth factor
117. ZEB1 - zinc finger e-box binding homeobox 1
118. ZFAS1 – Zinc finger antisense 1

List of symbols

1.	µl	Microlitre
2.	β	Beta
3.	µM	Micromolar
4.	nM	Nanomolar
5.	ng	Nanogram
6.	µg	Microgram
7.	~	approximate
8.	^{0}C	degree celsius
9.	ng/ml	nanogram per mili litre
10.	%	percentage
11.	ml	mili litre
12.	h	hour
13.	sec	second
14.	min	minutes
15.	±	plus/minus
16.	≥	greater than or equal to
17.	<	lesser than
18.	=	equal to

Chapter 1

Introduction and review of literature

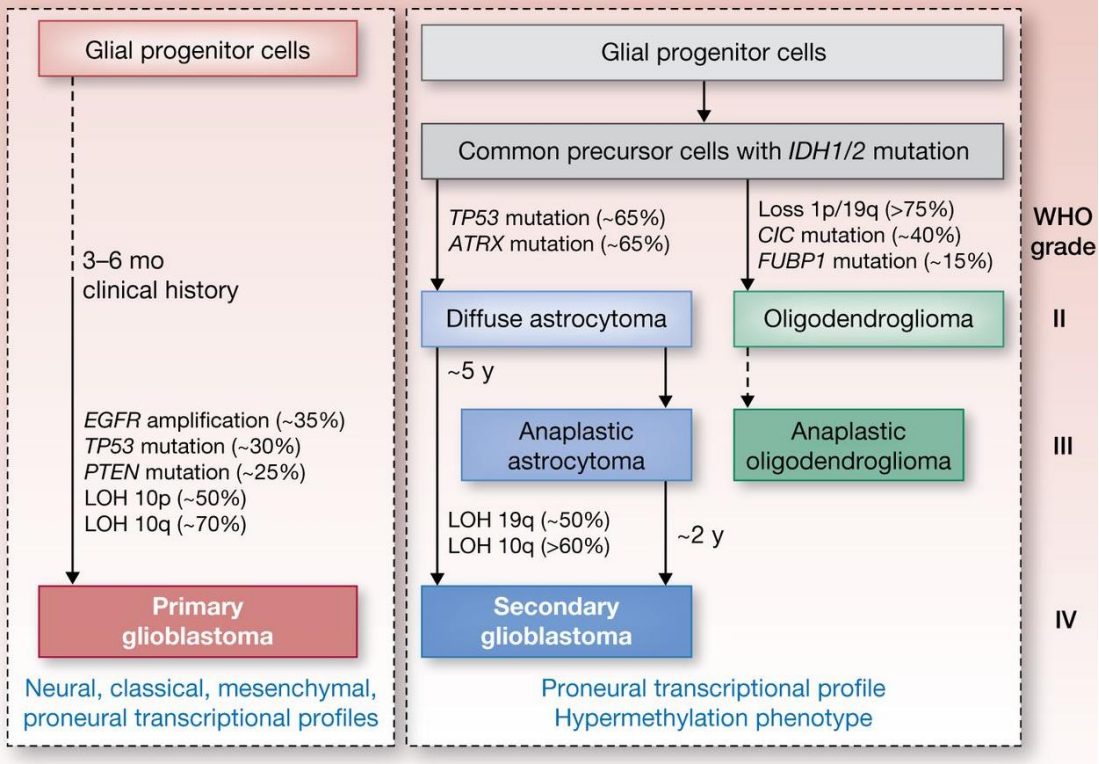
1.1 Glioblastoma multiforme (GBM)

Research in oncology has vastly increased our understanding of various cancers. However, the mortality rate of most cancers is still growing. Malignant brain tumors are among the primary causes of cancer mortality worldwide, with few therapeutic options (1). The Global Cancer Statistics 2020 (GLOBOCAN 2020) reported 0.3 million new brain tumor cases worldwide (2). The number of deaths estimated for brain tumors in 2020 globally is 0.25 million (2). Gliomas are the group of most aggressive forms of brain tumors, clinically divided into four types from grade I to grade IV. GBM accounts for 15-20% of all intracranial neoplasms in adults, classified as grade IV astrocytoma (3–5). GBM is highly aggressive, with a dismal prognosis of <21 months after diagnosis and a 5-year survival rate of 5% (1,6). The annual incidence of glioblastoma is approximately six cases per 100,000 individuals worldwide (4). According to the Central Brain Tumor Registry of the United States (CBTRUS), among all the central nervous system (CNS) malignant tumors, the average annual age-adjusted incidence rates (AAAIR) were highest for glioblastoma (3.22 per 100,000 population) (7). CBTRUS also reported that men are 1.6-fold more likely to diagnose gliomas than women (7). GBM significantly impacts patients' quality of life due to its aggressiveness, having very little progress in its management since the 1970s (3). The etiology of glioblastoma is complex and involves mutation or overexpression of multiple genes. All genetic aberrations identified in GBM are acquired somatic genetic alterations, and they do not have any inheritable mutations or predisposed tumor risk.

GBM is thought to arise from astrocytes; however, it is challenging to track the cell of origin in GBM as the tumor exhibits various levels of heterogeneity (3). Glioblastoma mainly occurs in the supratentorial brain (frontal, temporal, parietal, and occipital lobes), with a rare occurrence in the cerebellum, the brain stem, and the spinal cord (3). Recent evidence reports that the neural stem cells in the subventricular zone (SVZ) are the cells of origin for glioblastoma (8). Glioma stem cells (GSCs) are a subpopulation of tumor cells and express stemness markers, such as CD133 (8). GSCs differentiate into multiple tumor cell types contributing to intratumor heterogeneity in GBM (9), essential for resistance to therapies and tumor recurrence (10). GSCs contribute to tumor initiation, therapeutic resistance, and recurrence.

GBM can occur as primary glioblastoma as denovo from normal glial cells without evidence of a lower-grade precursor. They can also occur as secondary tumors from

lower-grade lesions (11). Primary and secondary GBM have distinct molecular features and genetic abnormalities (Fig 1). Primary GBMs are characterized by relatively high frequencies of epidermal growth factor receptor (EGFR) amplification, Phosphatase, tensin homolog (PTEN) deletion, and cyclin-dependent kinase inhibitor 2A (CDKN2A) (p16) loss. In contrast, secondary GBMs often contain TP53 mutations (Fig 1), especially those involving codons 248 and 273 or G:C→A: T mutations at the CpG site (12,13). GBM does not metastasize outside the CNS but is invasive, one of the fundamental features of malignant gliomas. This results in the inability of surgery to cure patients even when these lesions arise in areas where wide surgical resection is possible (14).



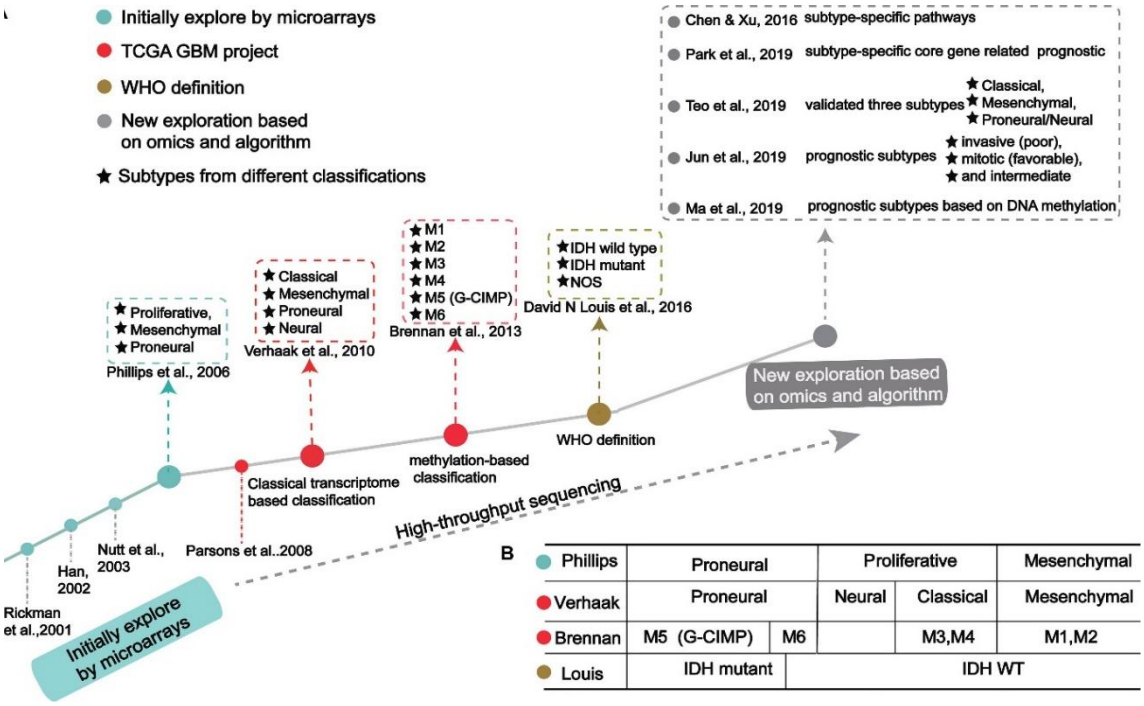
(Adapted from Ohgaki and Kleihues 2012)

Fig 1: Primary and secondary GBM: *GBM can occur as a primary tumor due to mutations and genetic alterations from glial progenitor cells or as a secondary tumor from low-grade gliomas. Primary and secondary GBMs have distinct genetic mutations and pathways.*

1.2 Tumor heterogeneity and molecular classification of GBM

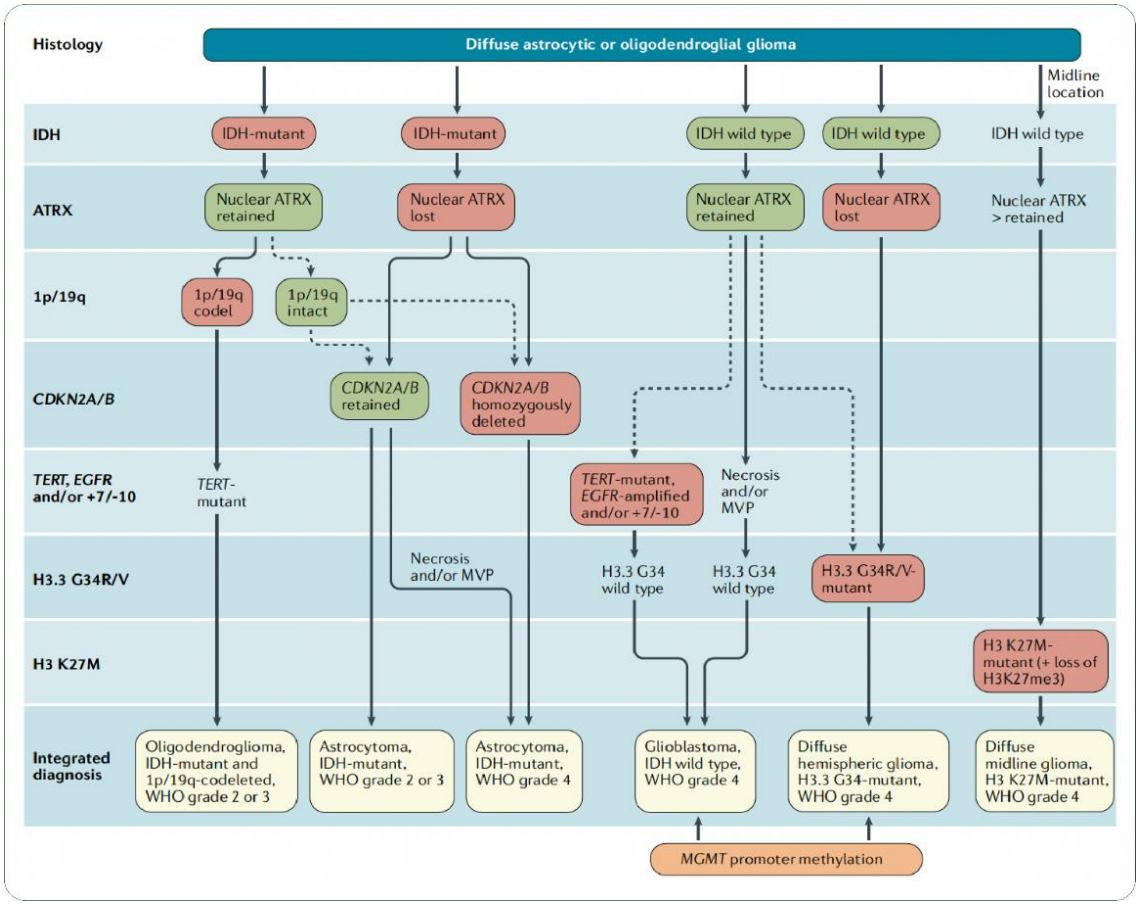
Histologically and genetically, GBM shows inter and intratumor heterogeneity with distinct gene expression, mutations, and epigenetic states, reflecting on the diagnosis, therapy choices, and clinical outcomes. Hence molecular classification of the tumor becomes inevitable (15). In line with these insights, two independent studies in the past have contributed majorly to the classification of GBM. Philips et al., using DNA microarrays of GBM tissues, identified specific signaling pathways in GBM (16). The study classified GBM tissue samples based on those gene signatures as three transcriptional subtypes—proneural, mesenchymal, and proliferative (16). Followed by this study, Verhaak et al. using clustering analysis, classified 200 glioblastoma cases into four subtypes—proneural, mesenchymal, classical, and neural (17). Both studies identified specific genes in each subtype. For example, proneural genes, including delta-like canonical Notch ligand 3 (DLL3) and Oligodendrocyte Transcription Factor 2 (OLIG2), and mesenchymal genes, such as CD40, are highly expressed in the respective subtypes of GBM tissues. They also reported that the expression of these genes correlated with poor patient survival (16,17). This unique expression of gene sets across subtypes explains that intertumoral heterogeneity is an essential factor to consider for molecular classification and therapeutics of GBM. Followed by these studies, Brennan et al. used large-scale methylated sequencing data and classified GBM based on the O^6 - methylguanine-DNA-methyltransferase (MGMT) promoter methylation as cluster M1 to cluster M6 (18). MGMT promoter methylation is a prognostic factor for glioblastoma patients and significantly correlates with worse patient survival. Glioblastoma displays diversity across tumors at DNA levels, apart from variation at gene expression levels. Several studies have documented commonly amplified genes such as EGFR, mesenchymal-epithelial transition factor (MET), platelet-derived growth factor receptor alpha (PDGFRA), Mouse double minute 2 homolog (MDM2), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA), cyclin-dependent kinase 4 (CDK4), cyclin-dependent kinase 6 (CDK6), and typically deleted genes, including CDKN2A/B, PTEN, and RB1 across GBM patients (19). Also, the existence and importance of single nucleotide mutations and somatic copy number alterations (SCNAs) in GBM are well documented in the cancer genome atlas (TCGA) database (20). Recurrent mutations are reported in PTEN (31%), TP53 (29%), EGFR (26%), PIK3R1 (11%), PIK3CA (11%), and Isocitrate dehydrogenase 1 (IDH1) (5%) (18). The classification of gliomas has undergone

significant changes (21) (Fig 2), as represented in the fifth edition of the WHO Classification of Tumors of the Central Nervous System, 2021 (22). Based on the molecular characteristics of GBM, WHO classifies it into three types: GBM IDH wild type, GBM IDH mutant, and GBM not otherwise specified (NOS) (22,23). Reclassification of GBM types based on molecular features has more predictive value than general histopathological classification. The European Association of Neuro-Oncology (EANO) has provided a seven layers structure of GBM diagnosis combining the histological and molecular characteristics (Fig 3).



(Adapted from Zhang et al. 2020)

Fig 2: The process of GBM classification and the overlap between GBM subtypes from different classification methods.



(Adapted from Weller et al., 2020)

Fig 3: Classification of GBM – WHO 2021

Mutations in the IDH group of genes represent the most critical genetic alterations in GBM, which plays a vital role in therapeutic responses (24). IDH enzymes catalyze the oxidative decarboxylation of isocitrate in Kreb's cycle (25). IDH mutations are often observed in low-grade gliomas (LGGs) and secondary GBMs (26). There are three forms of IDH – IDH1 in the cytoplasm and peroxisomes, IDH2, and IDH3, located in the mitochondrial matrix (27). All three forms of IDH converts isocitrate into a-ketoglutarate, an intermediate in the citric acid cycle. IDH1 and IDH2 use NADP+ as a cofactor to produce Nicotinamide adenine dinucleotide phosphate (NADPH), while IDH3 uses NAD+. Substrate binding to the IDH enzyme leads to a change from an open to a closed enzyme conformation. This conformational change and enzyme homodimerization is essential for enzyme activity (27). The most commonly mutated site in IDH enzymes is at the arginine 132 (R132) active site residue which is critical for substrate recognition (28). Missense mutations at this position result in the replacement of arginine with lower polarity amino acids such as histidine (H), lysine (K), or cysteine (C), resulting in decreased affinity for isocitrate. However, in most

tumors, there is a heterozygous mutation of IDH, where one copy of the gene is mutated. In heterozygous mutations, the IDH1 wild-type dimer component converts isocitrate into α-KG to produce NADPH.

In contrast, the mutant part of the dimer exhibits neomorphic activity, converting α-KG into D-2-hydroxyglutarate (D-2-HG) in an NADPH-dependent manner (29,30). Such neomorphic activity of IDH mutation has several consequences on cancer cell metabolism and oncogenesis, which are reviewed elsewhere in more detail (25). Due to its important implications in tumor cells, several studies have attempted to use inhibitors against the mutated IDH (mIDH) forms as therapeutic options for cancers, including glioma and acute myeloid leukemia (AML) (31). Inhibitors of mIDH1 – AGI-5198 and mIDH2 – AGI-6780 were published in 2013 to be effective *in vitro* and *in vivo* against the proliferation of glioma and leukemia cells, respectively (32,33). The food and drug administration (FDA) approved a few similar inhibitors, AG-221 and AG-120, for AML treatment (34). However, in the case of GBM, several further studies on mIDH inhibitors revealed controversial results, such as the inability of the inhibitors to reverse the global hypermethylation caused by mutant IDH and the inhibitors' inability to attenuate the proliferation of glioma cells (26). Also, it is reported that IDH1 mutation is most often acquired in the later stages of glioma, rendering its conversion from a driver to a passenger mutation (35). This favors the glioma cells to proliferate in a mutant-IDH-independent manner (35). Despite these conflicting results, research is still ongoing in employing mutant IDH inhibitors for glioma therapy. The unique dependencies caused by mutant IDH in glioma cells pave the way for the following therapeutic approaches: targeting metabolic deficiencies caused by mutant IDH, altered DNA repair pathways, distinct epigenetic profiles of IDH mutant gliomas, and immunotherapeutic options targeting mutant $IDH1^{R132H}$ (26).

1.3 Signaling pathways involved in GBM pathogenesis

The molecular events and aberrations which occur in GBM are heterogeneous and complex. Several molecular signaling pathways are found to be altered, contributing to GBM pathogenesis: 1) EGFR signaling is activated due to the overexpression of EGFR ligand or due to mutation in the EGFR receptor and amplified receptor activation contributing to GBM cells proliferation (especially EGFRvIII mutations) (36); 2) Aberrant Ras signaling pathway activation is observed in most of the GBM cases (37); 3) Phosphatidylinositol4,5-Bisphosphate 3-Kinase/Phosphatase and Tensin Homolog/serine-threonine kinase Akt (PI3K/PTEN/Akt) pathway is mediated by

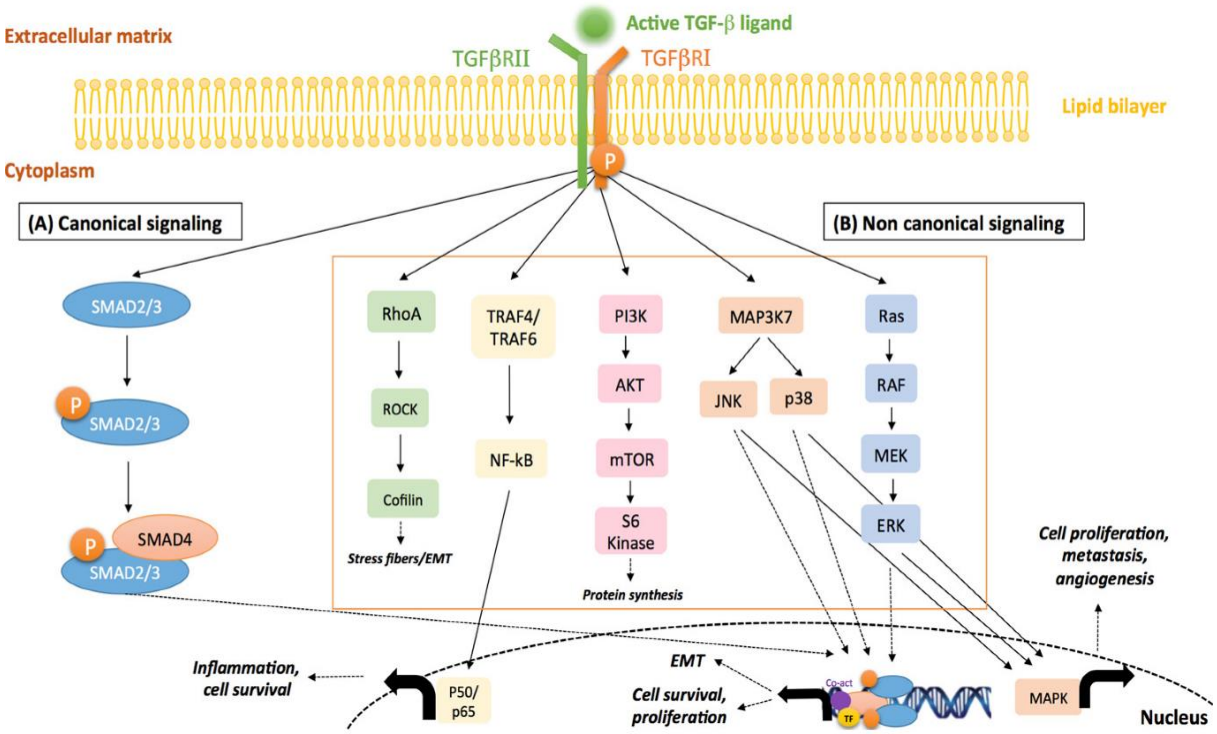
receptor – growth factor interactions (38). PI3K activates Akt leading to a proliferative advantage of GBM cells. PTEN, in turn, suppresses the activation of PI3K under normal conditions. However, malignant GBM cells have a lethal mutation of PTEN, leading to its inactivation and constitutive activation of PI3K; 4) Signalling pathways involved in cell cycle regulation, such as Rb and TP53 pathways, are also affected in GBM. These signaling pathways are partly regulated by cell cycle-regulating genes like cyclin-dependent kinase inhibitor proteins (CDKN) belonging to the INK family (CDKN2A-p16INK4^{ARF}) (39). In GBM cases, inactivating deletion mutations are observed in this gene locus, leading to the dysregulation of Rb and TP53 pathways, further facilitating GBM pathogenesis in a cell cycle-dependent manner. Transcription factors such as Nuclear factor kappa B (NF-κB) promote the expression of proinflammatory genes, stemness factors, and epithelial to mesenchyme transition (EMT) markers in glioma (40). Overexpression of various growth factors such as transforming growth factor-beta (TGF-β), Platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), and vascular endothelial growth factor (VEGF) are contributing to the pathophysiology of GBM (41).

1.4 Transforming growth factor-beta (TGF-β) pathway

TGF-β is a pleiotropic cytokine that regulates cell proliferation, differentiation, tissue homeostasis, motility and invasion, extracellular matrix (ECM) production, angiogenesis, and immune response (42,43). TGF-β superfamily consists of a large spectrum of ligands, including TGF-β1, TGF-β2, TGF-β3, activin, nodal, bone morphogenetic protein (BMP), growth and differentiation factors (GDF), and anti-mullerian hormone (AMH) (44). Seven types I and five types II transmembrane serine/threonine-protein kinase receptors exist for the TGF-β superfamily in the mammalian genome (45). TGF-β signaling is initiated by the binding of ligand to TGF-β receptor II (TGFβRII), which gets phosphorylated, alters its conformation, and in turn phosphorylates TGF-β receptor I (TGFβRI). The signal through TGFβR1 is transduced downstream either through small mothers against decapentaplegic (SMAD) proteins - in the canonical TGF-β pathway or through other effectors like mitogen-activated protein kinases (MAPK), extracellular signal-regulated kinase (ERK), and JUN kinase - in the non-canonical TGF-β pathway (44). SMAD proteins have a globular N-terminal Mad homolog 1 (MH1) domain that binds to DNA and a C-terminal Mad homolog 2 (MH2) domain that interacts with other SMADs (46). There are three different classes

of SMAD proteins, namely: (i) receptor-regulated SMADs (R-SMADs) (SMAD1, SMAD2, SMAD3, SMAD5, SMAD8), which interact with the TGF-β receptors; (ii) co-SMAD (SMAD4) which binds to activated R-SMADs forming heterotrimeric transcriptional complexes; and (iii) inhibitory SMAD (SMAD6, SMAD7) which suppress receptor and SMAD signaling functions (46). SMAD2 and SMAD3 are substrates for TGF-β subfamily receptors, while SMAD1, SMAD5, and SMAD8 are substrates for BMP signaling. In the canonical pathway, phosphorylated TGFβRI phosphorylates and activates SMAD2/3 proteins. The phosphorylated SMAD2/3 complex is then translocated into the nucleus by SMAD4. TGF-β target genes consist of evolutionarily conserved putative SMAD binding elements (SBEs) in their promoter regions. Nuclear translocation of the activated SMAD2/3 complex and binding of the translocated SMAD2/3 complex to the SBEs leads to the activation or repression of hundreds of TGF-β target genes (Fig 4) (46). Non-canonical or non-SMAD pathways include various branches of MAP kinase pathways, Rho-like GTPase signaling pathways, and phosphatidylinositol-3-kinase/AKT pathways (Fig 4). For example, in the ERK-mediated signal transduction, the activated TGFβRI phosphorylates and activates the ShcA protein, forming the ShcA/Grb2/SOS complex, followed by the sequential activation of c-Raf, MEK, ERK (47). Also, TGF-β can rapidly activate JNK through MKK4 and p38 MAPK through MKK3/6 (48). The detailed mechanism of non-canonical TGF-β signaling has been extensively reviewed elsewhere (48).

Dysregulation of TGF-β signaling promotes cancer progression by promoting invasion, EMT, and chemoresistance (Fig 4) (43,46,49). In normal cells and the early stages of cancer, TGF-β restrains cell proliferation whereas, in advanced stages of cancer, due to accumulation of mutations in the TGF-β pathway components or selective impairment of its tumor-suppressive function, it turns out to be oncogenic (50–52).

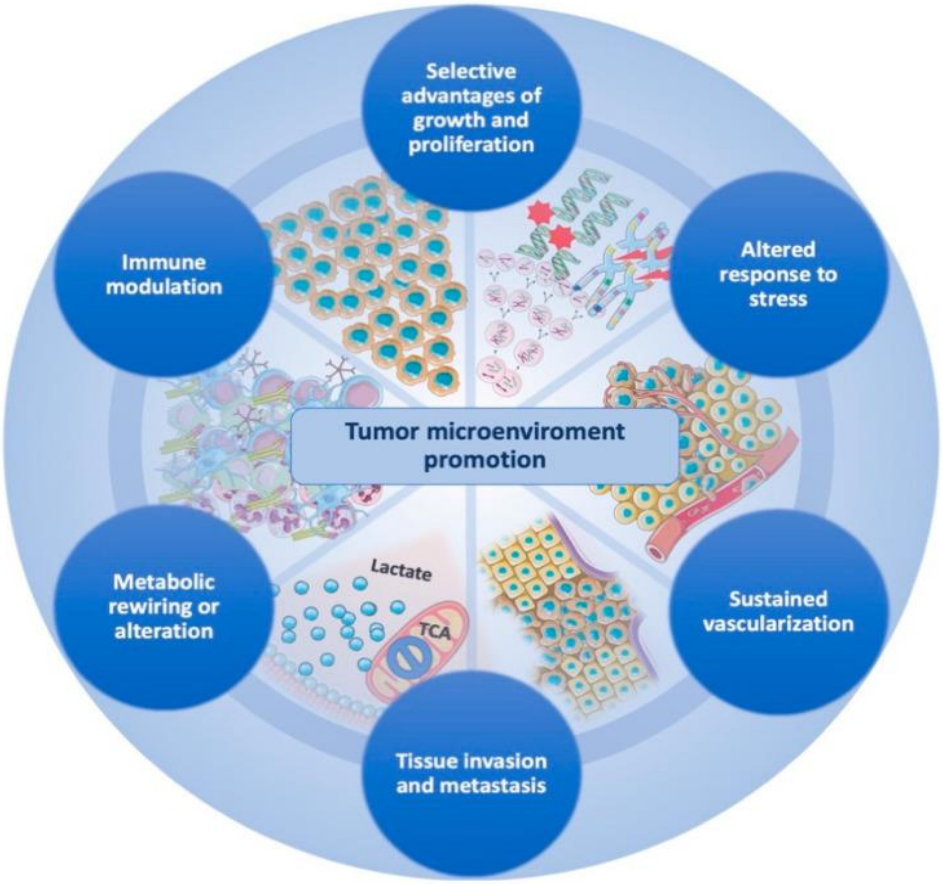


(Adapted from Costanza et al., 2017)

Fig 4: TGF-β pathway overview: *TGF-β signal transduction occurs through the canonical or non-canonical pathway. In the canonical pathway, activated R-SMADs form a heterotrimeric complex with SMAD4 (co-SMAD) and are translocated into the nucleus. The translocated complex binds to the SBEs of the target genes promoter and regulates gene expression. In the non-canonical pathway, the signal is transmitted downstream of TGF-β receptors through mitogen-activated protein kinases (MAPKs), phosphatidylinositide 3-kinase (PI3K), TNF receptor-associated factor 4/6 (TRAF4/6) and Rho family of small GTPases. TGF-β activation of the TRAF proteins can initiate nuclear factor-κB (NF-κB) signaling activity. TGF-β also activates PI3K and AKT by inducing a physical interaction between the PI3K p85 subunit and the receptor complex leading to translational responses via mTOR/S6kinase activation.*

1.5 Hallmarks of GBM

The hallmarks of GBM include selective advantages of growth and proliferation, altered response to stress, sustained vascularization, tissue invasion and metastasis, metabolic rewiring or alteration, and immune modulation (Fig 5) (53).



(Adapted from Torrisi et al. 2022)

Fig 5: Hallmarks of GBM. *GBM cells have various hallmarks, as mentioned in the above figure, which aid them in maintaining a favorable tumor microenvironment that supports the survival and progression of GBM.*

1.5.1 Selective advantages of growth and proliferation

Alterations in receptor tyrosine kinase (RTK)/ rat sarcoma (RAS)/PI3K, VEGF, PDGFR, c-Met, EGFR, and its mutated form EGFRvIII, SRC family of protein tyrosine kinases, Notch, NF-κB, TGF-β signaling, are most prominent in promoting GBM cell proliferation, and growth (53). Additionally, the Rb signaling pathway is also altered in GBM. The p16(INK4a)-CDK4/6-Rb axis deregulation correlates with GBM proliferation (54). Loss of heterozygosity at the RB gene, homozygous deletion of the CDKN2A gene, and CDK4 amplification are found in GBM patients (54,55). Also, it is previously reported that the double knockout mice model of p16Ink4a/p19ARF leads to neural stem cell self-renewal through upregulation of Bmi1, which is noteworthy in the context of GSCs (56). Furthermore, mutation of p53 and dysregulation of the p53-ARF-MDM2 axis are reported in 85% of GBM patients and 90% of GBM cell lines;

several of the above-mentioned gene targets are studied for targeted therapy against GBM, either in isolation or in combination with TMZ treatment. For example, antibody-drug conjugate Depatuxizumab Mafodotin to inhibit EGFR (57); Everolimus and Temsirolimus were tested against mTOR (58); Buparlisib was tested in patients with recurrent GBM to inhibit PI3K (59). GBM cells also show replicative immortality due to the mutated TERT gene (18). 51% of GBM cases have overexpression of telomerase reverse transcriptase (TERT) that causes increased telomere lengths (11). GBM cells also resist apoptotic signals due to the loss of TP53 and RB genes. The abundant survival signals from PI3K, AKT, and mTOR pathways also block apoptosis in GBM (11).

1.5.2 Tissue invasion and metastasis

GBM is characterized by its local invasion of the surrounding parenchyma and not outside the CNS, mainly due to i) the relatively short survival of the patients and ii) physical barriers of the CNS – the blood-brain barrier (BBB) (60,61). Various transcription factors and proteases are involved in the invasion and metastasis of GBM cells. Myeloid cells recruited at the inflammatory hypoxic tumor microenvironment (TME) trigger essential growth factors such as TGF-β, PDGF, EGF, and FGF2, which leads to the upregulation of master transcription factors such as ZEB1 and Snail1 (62). This led to a loss of cell-to-cell adhesion and increased cell motility. Precisely, several events occur during the GBM invasion process. Detachment of tumor cells from the surrounding tumor tissue is the primary event in GBM invasion. Downregulation of neuronal cell adhesion molecule (NCAM) and upregulation of N-cadherins promote the detachment of glioma cells (63). Also, radiation in GBM patients enhances the expression of intercellular adhesion molecules (ICAM), facilitating GBM cell migration (64,65). Also, specific integrins such as a6b1 are involved in the invasive phenotype of GBM cells *in vitro* (U87-MG) and *in vivo* (66). Matrix metalloproteases (MMPs) play an important role in degrading the surrounding ECM of glioma cells. MMP-2, MMP-9, A disintegrin and metalloproteinases (ADAMs), and urokinase-type plasminogen activator (uPA) are commonly found MMPs in ECM degradation (67). Glioma cells become loosely bound to the ECM by interacting with focal adhesion molecules and attaining spindle shape. Master transcription factors that promote mesenchymal transition and invasion, such as ZEB1/2, Snail1, Snail2 (Slug), and Twist, get induced (68–71). These transcription factors, in turn, simultaneously upregulate

mesenchymal markers (N-cadherin, vimentin, fibronectin) and downregulate epithelial markers (E-cadherin, claudins, occludins, and cytokeratins) which further promotes invasion (72). The role of ZEB1, Snail, and Slug in promoting GBM invasion is well documented (68,70).

1.5.3 Sustained vascularization

GBM maintains sustained vascularisation despite necrotic and hypoxic areas (73). Specific essential genes involved in this process are VEGF, erythropoietin, thrombospondin 1, platelet/endothelial-cell-adhesion molecule, matrix metalloproteinases, and inhibitors of metallopeptidase (74). Bevacizumab was used against vascularization in GBM (75). However, it was ineffective due to its insignificant improvement in GBM patients' survival (75). Also, tumor cells develop alternative mechanisms through metabolic reprogramming during anti-angiogenic therapy, establishing Warburg effect-mediated resistance (76).

1.5.4 Reprogramming cellular energetics

The metabolic reshaping facilitates the ability of cancer cells to invade a nutrient and oxygen-deprived microenvironment in its TME (53). Reprogramming cellular energetics aids GBM cell invasion and promotes immunosuppression (53). Besides glycolysis, lipid, amino-acid, and nucleotide metabolism promote GBM invasion (77). For example, the synthesis of fatty acid (FA) promotes its FA-uptake channel CD36 upregulation, associated with a pro-invasive phenotype (77). In the hypoxic microenvironment, microglia adopt aerobic glycolysis, increase lactate production, M2-polarization, and hypoxia-inducible factor (HIF) stabilization (77). HIF promotes GBM tumorigenesis by inducing stemness and metastasis in GBM cells (78). Mutated IDH genes also play a vital role in reshaping metabolism in GBM. The oncometabolite 2-HG derived from the mutated IDH1/2 reduces the inflammatory responses in GBM by inhibiting microglia activation through AMPK/mTOR/NF-κB pathway (79). Ivosidenib, an IDH inhibitor, is tested in a Phase I clinical trial to target the metabolic reprogramming of GBM cells (80). Other essential pathways/genes which are involved in the metabolic reprogramming of GBM cells include JAK/STAT pathway, PTEN-induced kinase 1 (PINK1)243, and hexokinase 2 (HK2) (81,82).

1.5.5 Immune Modulation

Under inflammatory conditions in GBM, the endothelial cells in the tight junctions are weakened, leading to variable BBB permeability and selectivity (83). Myeloid-derived suppressor cells (MDSCs), bone-marrow-derived macrophages, microglia, dendritic cells (DCs), and neutrophils are the primary immune cells in GBM (84). Secretion of immune suppressive factors, TGF-β derived from GBM cells, interleukin-10 (IL-10), prostaglandin E-2, and immune checkpoint molecules such as Cytotoxic T-lymphocyte associated protein 4 (CTL-4), programmed death-1 (PD1), impedes antitumoral immune response in GBM by suppressing the cytotoxic T-cells, and DCs (84). CTL-4 and PD1 inhibitors in combination are now under clinical trials for GBM therapy (85). Also, dendritic cells vaccine or peptide vaccines are under trial to prime the immune system to identify and destroy cancer cells (85).

1.6 Role of TGF-β in hallmarks of GBM

TGF-β contributes to almost all of the hallmarks of GBM discussed above: sustained proliferation, invasion, drug resistance, angiogenesis, immunosuppression, and maintenance of stemness of GSCs (86). GBM brain tissues have elevated levels of TGF-β (especially TGF-β1 and 2) compared to normal brain tissues suggesting an oncogenic role of TGF-β in GBM (87). Bryukhovetskiy et al. reported changes in proteins related to the cell cycle, integrins, fibronectin, modulation in EMT markers expression, DNA replication proteins, and DNA repair proteins upon TGF-β treatment in U87-MG GBM cells (88). Infiltration of microglia in the later stages of GBM constitutes an essential factor for a constant secretion of TGF-β, which promotes various malignant behavior of the tumor (89).

TGF-β plays an essential role in GBM cell proliferation. Due to its pleiotropic nature, during the early stages of the tumor, TGF-β restrains the proliferation of normal astrocytes and the early stages of GBM. However, in the later stages of the tumor, it promotes the expression of essential genes such as PDGFB and promotes tumor cell proliferation (90).

TGF-β induces the expression of several genes involved in tumor invasion and migration: MMP2, MMP9, vimentin, fibronectin, ZEB1, TWIST1, and SNAIL1 (91,92). TGF-β signaling also downregulates genes, such as tissue inhibitors of MMPs (TIMPs), facilitating the ECM's degradation and cellular invasion (91). TGF-β also

induces the expression of genes belonging to the ADAM group, namely: ADAM17, and ADAMTS-1, aiding in the degradation of extracellular matrix proteoglycans (93,94).

TGF-β contributes to TMZ resistance by activating connective tissue growth factor (CTGF) through Smad and ERK1/2 signaling (95). The control of angiogenesis in GBM by TGF-β is at multiple levels, directly inducing vessel formation or indirectly modulating target gene expression (96). TGF-β induces microtube formation by enhancing the expression of thrombospondin 1 (97). It also promotes the expression of VEGF and promotes angiogenesis (98). Also, VEGF expression is controlled combinatorially by TGF-β and HIF signaling (99). Also, targeting the crosstalk between these two signaling pathways is an excellent therapeutic option for GBM (99). Other studies reveal that TGF-β induces insulin-like growth factor-binding protein 7 (IGFBP7). Human brain endothelial cells cultured with U87-MG conditioned media secret significantly higher levels of TGF-β with a concomitant increase in levels of IGFBP7, which is downregulated upon treatment with SMAD inhibitor SB431542 (100).

Studies on glioma stem cells prove an essential role of autocrine TGF-β signaling in promoting glioma progression (101). SRY-related HMG-box family genes (SOX) - Sox2, Sox4, and Sox9 are direct targets of TGF-β, which are upregulated by TGF-β signaling, leading to stemness in GBM (101). The TGF-β pathway induces leukemia inhibitory factor (LIF) transcription, activates Janus kinase - signal transducer and activator of transcription (JAK-STAT) signaling, and promotes GSC self-renewal (102). High CD44 and ID1 levels in GSCs lead to poor prognosis in GBM patients (103). GSCs enriched population expresses high levels of CD44 and ID1 ($CD44^{high}/ID1^{high}$), which is downregulated upon treatment with TGF-β inhibitors (103). Another critical study by Ventura et al. demonstrates the role of TGF-β-activated ERK in the self-renewal of glioma-initiating cells (GICs) (104). Several proprotein convertases perform the activation of pro-TGF-c into the active form. One such convertase, Furin, activates TGF-β1 and TGF-β2 from their proprotein form in T-325, T-269, ZH-161, S-24, and ZH-305 GICs (104). The activated TGF-β2, through the ERK signaling pathway, induces Furin in a positive feedback loop which is essential for the self-renewal of GICs (104).

TGF-β contributes to GBM progression by aiding glioma cells to escape from the host immunity (105). TGF-β blocks the anti-tumor immune response in GBM by inhibiting cytotoxic T lymphocytes (CTLs), cytotoxic T cell exclusion, repression of $CD4^+$ cells, and inhibiting MHC class II expression (106,107). Also, the downregulation of activating receptor NKG2D on the surface of NK cells and $CD8^+$ T cells is observed in glioma patients. This further leads to immune suppression, partially reversed upon the knockdown of TGF-β1 and TGF-β2 using siRNAs (108). A recent study conducted by Irshad et al. demonstrated the positive correlation of TGF-β1/2 with the FAT atypical cadherin 1 (FAT1) gene in resected human glioma, primary glioma cultures, and GBM cell line (U87-MG) (109). FAT1 enables an immune suppressive microenvironment in GBM via TGF-β1/2 (109).

1.7 Current therapeutic strategies for GBM

Current therapeutic strategies for GBM include maximum surgical resection, followed by radio- and chemotherapy with temozolomide (TMZ) (110). TMZ is an oral alkylating agent that alkylates DNA bases. This causes mismatch during DNA replication, leading to futile rounds of DNA repair, DNA double-strand breaks, and apoptosis (111). However, MGMT can resolve some TMZ-induced alterations and thus mediate the survival of GBM cells (112). MGMT inhibitors are considered beneficial for improving the action of TMZ in GBM patients (113,114). Localized application of pseudo-substrates or tumor-specific delivery of blocking peptides against MGMT increases TMZ efficiency (112). Overall, TMZ treatment extends the survival of GBM patients from 12.1 to 14.6 months (110), while tumor recurrence in GBM patients is inevitable. Several GBM cell lines, such as T98G and LN229, are known to be resistant to TMZ (111). However, it is noteworthy that the MGMT gene is frequently downregulated in 45% of GBM patients since the promoter of this gene is often hypermethylated (114).

The benefit of using antagonisms of the TGF-β pathway for GBM treatment is demonstrated by several *in vitro* studies, rodent glioma models, and clinical studies (115,116). Moreover, the local application of TGFβ2 antisense oligonucleotides was also evaluated in a randomized clinical trial for recurrent malignant glioma (117). Several anti-TGF-β antibodies and inhibitors against components of the TGF-β pathway are in the pre-clinical and clinical trial stages as treatment regimens for GBM. Studies have reported using antisense oligonucleotides, small-molecule inhibitors of the

kinase activity of the TGF-β receptor complex, and antibodies against the components of the signaling pathway to treat GBM (86). Bogdahn et al., in a phase IIb clinical study, evaluated the efficacy of antisense oligonucleotide, trabedersen (AP 12009) administered intratumorally in recurrent GBM patients with chemotherapy (115). An improved survival response in patients with standard treatment with Temozolomide was observed with the combination of AP 12009 (115). Rodon et al. performed a phase 1 - in-human dose study to evaluate the effect of transforming growth factor-β receptor I kinase inhibitor galunisertib (LY2157299 monohydrate) in patients with advanced GBM (116). The study displayed that the intermittent administration of galunisertib is safe and improves clinical outcomes of GBM patients (116). Hollander et al. conducted a Phase II study of the human analog of the 1D11 antibody, GC1008 (118). Although direct clinical outcomes were not evident in this study due to the limited study population, significant uptake of GC1008 antibody was observed in recurrent GBM patients (118).

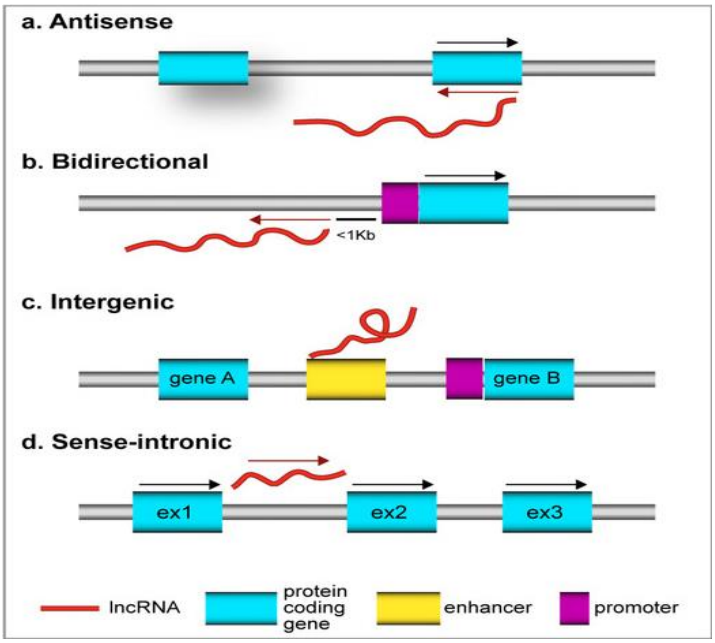
Given the multifaceted role of TGF-β signaling in various hallmarks of GBM, several small molecule inhibitors and antisense therapies were employed in the past as GBM therapy (86). Despite all these efforts, the success of these small molecule inhibitors as therapy for GBM is very negligible. Also, the systemic inhibition of TGF-β signaling might hamper several other normal physiological functions of the signaling pathway.

1.8 Long non-coding RNAs (lncRNAs) – discovery, function, and link to cancer

Less than 3% of the human genome codes for proteins, while the rest pervasively transcribes several non-coding transcripts (119–122). The non-coding category of the genome is highly heterogeneous, consisting of small ncRNA (non-coding RNAs) and long ncRNAs (lncRNAs). Among these, lncRNA transcripts are the most abundant and are loosely defined as transcripts longer than 200 bps with no ability to code for proteins (119–123). The advent of DNA microarrays and the complete sequencing of the human genome provided the first insight into the pervasive transcription of lncRNAs (124,125). Two major studies by Kapranov et al. and Rinn et al. provided evidence of non-coding genes in the human genome (126,127). These studies used tiling microarrays and identified that there might be as many lncRNA genes as protein-coding genes (126,127). The discovery of widespread active chromatin marks such as H3K4me3 and H3K36me3 in the human genome gave a clue that most of the human genome is transcribed despite having a significantly less proportion of proteins coded

from the transcribed RNA genes (128). Most active chromatin marks were identified in regions between the protein-coding genes. Hence these RNAs were named large intergenic non-coding RNAs (lincRNAs) (124).

The sequence conservation of lncRNAs and syntenic lincRNAs across species provides evidence of their functionalities (129). Genetic and biochemical studies discovered the function of lncRNAs, namely, *X-inactive specific transcript (XIST) and H19*, in critical cellular processes such as genomic imprinting (130). About one-third of identified lncRNAs are associated with chromatin-modifying complexes and are determined to modulate critical cellular processes (131). RNA sequencing and loss of function studies by RNA interference allow the identification and functional characterization of lncRNAs in cellular processes. Guilt by association is used for predicting the function of lncRNAs (124). This method uses gene expression analysis to identify the pathways and protein-coding genes correlated with a given lncRNA. Hence, the hypothesis of the functionality of the lncRNA is framed based on the known function of the protein-coding genes (124). Based on the coding genes, lncRNAs can be classified into four major categories: (i) antisense lncRNAs, when overlapping one or more exons of another transcript on the opposite strand; (ii) bidirectional lncRNAs, when its expression and the expression of the neighboring coding transcript on the opposite strand are initiated in close proximity; (iii) intergenic lncRNAs, when produced from an independent transcription unit in between two protein-coding genes; and (iv) sense intronic lncRNAs, when transcribed from the sense strand of an intronic region with no overlap of an exonic sequence of a protein-coding gene (132) (Fig 6).



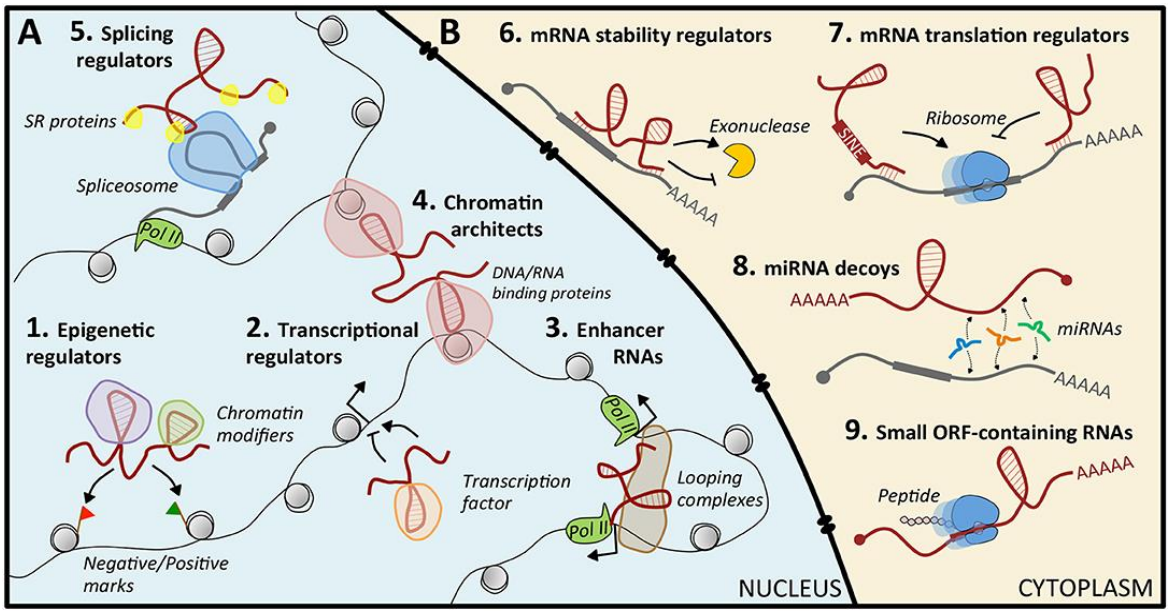
(Adopted from Lanzafame et al. 2018)

Fig 6: Classification of lncRNAs. *Based on the position of protein-coding genes, lncRNAs are classified as antisense, bidirectional, intergenic, and sense-intronic lncRNAs.*

LncRNAs play crucial roles in regulating cellular processes like cell cycle, differentiation, metabolism, and diseases, including cancer (133–138). Although lncRNAs do not code for proteins, they modulate gene expression in *cis* and *trans* via transcriptional regulation, epigenetic modifications, protein/RNA stability regulation, translation, and post-translational modifications by interacting with DNA, RNAs, and proteins (Fig 7) (139). LncRNAs also directly interact with signaling receptors to control gene expression (140). Like the mRNAs, most lncRNA species are transcribed by RNA polymerase II, but the expression of lncRNAs is lower than mRNAs, and they exhibit robust tissue-specificity compared to mRNAs (121,141–143). However, recent studies have revealed distinct transcription, processing, export, and turnover of lncRNAs, are closely linked with their cellular fates and functions (123).

LncRNAs interact with proteins, other coding/non-coding RNAs, and DNA to regulate gene expression in *cis* and *trans* (119,122,144–146). Nuclear lncRNAs are involved in transcriptional regulation, chromatin remodeling, RNA processing, nuclear architecture, DNA repair, etc. (Fig 7) (147,148).

LncRNAs perform transcriptional regulation in several ways, such as i) recruitment of a regulatory protein to a gene or an entire chromosome in *cis* or *trans*; ii) inhibition of binding of a transcriptional regulatory factor by acting as a decoy for the protein; iii) in some cases, transcription of a lncRNA has a direct effect on the transcription of its nearby genes, either positively or negatively regulating its nearby genes; iv) LncRNAs are also involved in genome organization and nuclear architecture (149). For example, Nuclear enriched abundant transcript 1 (*NEAT1)* is a nuclear-paraspeckle-associated lncRNA, which is essential for the organization and integrity of nuclear paraspeckles (147).



(Adapted from Laneve et al., 2019)

Fig 7: Mechanism of action of lncRNAs. *LncRNAs have distinct functions in the nucleus and cytoplasm. In the nucleus, lncRNAs act as a guide for i) epigenetic factors, ii) transcription factors to specific loci iii) they act as enhancer RNAs. iv) they modulate the structure of the three-dimensional conformation of chromatin. v) they aid in recruiting proteins such as splicing factors. In the cytoplasm, lncRNAs vi) modulate mRNA stability. vii) they act as translational regulators of mRNA. viii) they act as competing endogenous RNAs (ceRNAs) for miRNAs. ix) lncRNAs also code for small peptides with functional potential.*

LncRNAs are known regulators of hallmarks of cancer, such as proliferation, metastasis, and drug resistance in several types of cancers, including GBM (150–152). Various lncRNAs such as MALAT1, NORAD, and HOTAIR promote tumor

progression in lung, breast, ovarian, and hepatocellular carcinoma (HCC) and GBM (153). LncRNAs in the cytoplasm contribute to functions such as regulating mRNA turnover, translation, protein stability, sponging cytosolic factors, and modulation of signaling pathways (Fig 7) (154). The vast majority of cytoplasmically localized lncRNAs function as ceRNAs for miRNAs and stabilize the mRNA target of those miRNAs.

1.8.1. LncRNAs and EMT

EMT is essential during development, wound healing, and tissue remodeling. However, it is also observed in pathological conditions like cancer (62). EMT has roles in embryogenesis and tumorigenesis, and it is triggered by different signaling pathways, such as TGF-β, WNT/β-catenin, notch, and receptor tyrosine signaling pathways (155). Although glial cells vary from epithelial cells, specific invasive mechanisms overlap between cancers of the CNS and other cancer types (156). Critical genes which cause EMT phenotype in GBM include Snail, Slug, ZEB1, Twist, CD44, Notch, and WNT/β-catenin pathway genes. LncRNAs activate or repress essential genes involved in EMT. For example, lncRNA FOXD2-AS1 promotes EMT in glioma cells by activating various genes, such as MMPs, Vimentin, and N-cadherin, by sponging miR-506-5p (157). Yang et al. analyzed the TCGA dataset of glioma tissues and normal tissues and reported top seven lncRNAs (AC012615.1, H19, LINC00609, LINC00634, POM121L9P, SNHG11, and USP32P3) which have a potential role in promoting EMT (158). LncRNAs, such as HOTTIP, DANCR, H19, and SNHG18, promote EMT gene expression in glioma cells (152,159–162). Glioma cells with EMT characteristics also display drug resistance, e.g., MALAT1 is upregulated in glioma cells (163). It promotes the expression of various EMT-related genes, such as ZEB1, SNAIL, and Slug, and sensitizes glioma cells with TMZ-mediated apoptosis (163). LncRNAs also repress EMT gene expression, such as MEG3 and Gas5 (164,165).

1.9 Oncogenic lncRNAs in GBM

Several lncRNAs are differentially expressed in GBM, and the study of lncRNA-mediated regulation of cancer pathogenesis is gaining importance. Hu et al. identified that lncRNA AB073614 is upregulated in 65 glioma tissue samples compared to normal brain tissues (166). The study also reported that elevated lncRNA AB073614 positively correlated with the glioma grades, with the highest expression in grade IV GBM. Also,

higher levels of the lncRNA correlate with poor survival of GBM patients, indicating the prognostic value of lncRNAs in GBM (166). Followed by this study, Li et al., with the help of siRNA-mediated knockdown of lncRNA AB073614, established that the lncRNA facilitates GBM cells invasion, migration, and upregulation of EMT markers (fibronectin and vimentin) (167).

Yu et al. observed upregulation of LINC00152 in glioma tissues and GSCs (168), promoting proliferation, invasion, and resistance to apoptosis of GSCs (168). LINC00152 induced the expression of forebrain embryonic zinc finger protein 1 (FEZF1) by competing with miR-103a-3p, which in turn activates oncogenic gene-cell division cycle 25A (CDC25A) (168). Upregulated CDC25A activates the PI3K/AKT pathway, which induces the malignant behavior of GSCs (168).

LncRNA Colon cancer-associated transcript 1 (CCAT1) is upregulated in glioma tissues and cell lines (169). Loss of function assays demonstrated that CCAT1 promotes GBM cell proliferation and colony formation and attenuates cancer cell apoptosis (169). The study also revealed that CCAT1 downregulated a tumor suppressor miRNA – miR-410 in GBM cells (169). Further, Cui et al. characterized the role of CCAT1 in GBM. In concordance with the previous study, CCAT1 has an oncogenic role in GBM, promoting glioma cells proliferation, migration, invasion, and inhibition of apoptosis (170). Mechanistically, CCAT1 competes with miR-181b leading to the de-repression of its endogenous targets FGFR3 and PDGFRα, thereby promoting GBM pathogenesis (170).

Wang et al. studied the expression of lncRNAs in malignantly transformed fibroblasts (tFBs) from tumor xenografts induced through GSCs (171). They identified that lncRNA HOXA transcript antisense RNA, myeloid-specific 1 (HOTAIRM1), is highly expressed in tFBs and high-grade gliomas (171). Loss of function and gain of function studies indicate that HOTAIRM1 promotes the malignant phenotype of tFBs (171). Mechanistically, HOTAIRM1 could bind to and compete with miR-133b-3p to stabilize TGF-β expression. LncRNA-HOTAIRM1 thus promotes the malignant transformation of fibroblasts remodeled by glioma stem-like cells through the miR-133b – TGF-β axis (171).

Lulli et al. analyzed GBM patient samples and patient-derived GSCs and identified that miR-370-3p is downregulated in GBM (172). The proliferation, migratory capacities of GSCs, and tumor growth in mouse xenografts *in vivo* were significantly impaired upon over-expression of miR-370-3p (172). To further study the mechanism of action of

miR-370-3p, the research group conducted a microarray experiment for GSCs with miR-370-3p overexpression and control GSCs. Microarray results demonstrated that pathways related to EMT and hypoxia were significantly associated with the miRNA, specifically high-mobility group AT-hook 2 (HMGA2), the master transcriptional regulator of HIF1A (172). Also, over-expression of miR-370-3p significantly impaired lncRNA NEAT1 levels, an oncogenic lncRNA in GBM, and several other cancers (172). This study depicts the tumor suppressor function of miR-370 and the oncogenic function of lncRNA NEAT1 by targeting HMGA2 in GBM (172). The functional roles of lncRNA and their potential mechanisms in GSCs have been reviewed by Wang et. (173).

Zhu et al., using qRT-PCR, identified that lncRNA Highly upregulated in liver cancer (HULC) is upregulated in GBM cell lines (174). Also, a positive correlation between HULC and VEGF, endothelial cell-specific molecule 1 (ESM-1), was identified (174). Knockdown of HULC reduced glioma cell proliferation, induced anoikis, and blocked the cell cycle at the G1/S phase via the PI3K/AKT/mTOR signaling pathway (174). These physiologies were reversed in HULC-depleted cells upon ESM-1 overexpression, indicating the role of HULC in promoting angiogenesis in GBM through the PI3K/Akt/mTOR signaling pathway (174). Concordance to this study, Yan et al. reported elevated levels of HULC in GBM patients' tissue samples, glioma cell lines, and poor survival of glioma cells with high HULC levels (175).

LncRNA ANRIL and mRNA SOX9 were significantly upregulated in glioma patients' samples compared to control samples (176). Knockdown of ANRIL and SOX9 attenuated glioma cells' proliferation, migration, and invasion (176). High ANRIL and SOX9 levels indicate an unfavorable prognosis for glioma patients (176).

Zhang et al. reported using the TCGA database that lncRNA ASB16-AS1 is significantly upregulated in glioma tissues compared to normal tissues (177). ASB16-AS1 promotes glioma cell proliferation, migration, and invasion (177). Bioinformatics analysis identified several possible ceRNA networks in glioma progression (177).

Liu et al. demonstrated that lncRNA- CASC9 is significantly upregulated in glioma tissues and cell lines (178). The study also reported that CASC9 promotes glioma progression by inducing glioma cell proliferation, invasion, and metastasis (178). ChIP experiments identified that the expression of CASC9 was induced by the STAT3 transcription factor (178). The study further identified that CASC9 facilitates the de-repression of STAT3 by competing with tumor suppressor miR-519d (178). The

positive feedback loop – STAT3/CASC9/miR-519d/STAT3 axis promotes glioma pathogenesis (178).

LncRNA CCND2-AS1 is significantly upregulated in malignant glioma tissues and cell lines (179). Also, CCND2-AS1 promotes glioma cell proliferation (179). Knockdown of CCND2-AS1 impaired the activation of Wnt/β-catenin and reduced the level of nuclear b-catenin signaling (179). This study reveals the oncogenic role of CCND2-AS1 by promoting glioma cell proliferation through the Wnt/β-catenin pathway (179).

Hu et al. identified the elevated levels of DLGAP1-AS1 in glioma tissues and cell lines and a positive correlation of its expression with increasing glioma grades and shorter survival time of glioma patients (180). DLGAP1-AS1 promoted glioma cell proliferation, migration, and invasion, while miR-628-5p established the opposite effect (180). The study also identified that DLGAP1-AS1 acts as a molecular sponge for miR-628-5p and promotes glioma progression through DLGAP1-AS1/miR-628-5p/DDX59 axis (180).

Using microarray analysis of glioma brain tissues and normal brain tissues, Zhang et al. identified 22 differentially lncRNAs, among which ENST00000413528 was the highest expression with a 3.58-fold change (181). The knockdown of ENST00000413528 inhibited cell proliferation, induced apoptosis, and cell cycle arrest *in vitro*, and reduced tumor volume and weight *in vivo* (181). An inverse correlation between ENST00000413528 and miR-593 was observed in glioma tissues (181). Also, miR-593 targeted ENST0000413528, polo-like kinase 1 (PLK1), and PLK1 is upregulated in glioma tissues (181). ENST0000413528 promotes PLK1 expression and glioma proliferation by targeting miR-593 (181).

Using RNA sequencing of GBM and normal tissue samples, Wu et al. identified lncRNA-HOXA-AS3 to be significantly upregulated in GBM (182). Gene set enrichment analysis revealed that genes involved in cell cycle progression and E2F targets were correlated with HOXA-AS3 levels (182). HOXA-AS3 promoted GBM proliferation and invasion *in vitro* and *in vivo* in xenograft models indicating the prognostic value of lncRNA HOXA-AS3 in glioma (182).

The role of lncRNA Myocardial Infarction Associated Transcript (MIAT) in glioblastoma pathogenesis was studied by Bountali et al. using RNA sequencing of MIAT knockdown cells and control 1321N1 glioma cells (183). Pathway analysis of the RNA sequencing data revealed genes involved in cancer-related processes, such as cell growth and survival, apoptosis, reactive oxygen species (ROS) production, and

migration, are enriched in the MIAT knockdown group (183). Further characterization of the lncRNA in GBM pathogenesis is yet to be explored.

The expression of lncRNA plasmacytoma variant translocation 1 (PVT1) and EZH2 transcript is upregulated in glioma tissues and cell lines (184). PVT1 promotes GBM cell proliferation and invasion and prevents apoptosis, and the levels of PVT1 positively correlate with the levels of EZH2 (184). Exogenous expression of PVT1 increased EZH2 levels in glioma cells, hinting at a possible role of PVT1 in GBM progression through EZH2 (184).

Another study by Ma et al. reported the overexpression of PVT1 in human cerebral microvascular endothelial cells cultured with glioma-conditioned media (185). Functional assays indicate that PVT1 induces glioma cell proliferation, invasion, and angiogenesis (185). Also, PVT1 induces protective autophagy in glioma cells, characterized by increased expression of Atg7 and BeclinI genes (185). Mechanistically, PVT1 competes with miR-186, which targets Atg7 and BeclinI, prevents degradation, and promotes protective autophagy and glioma pathogenesis (185). In concordance with these two studies, Xue et al. reported the upregulation of PVT1 in glioma tissues and cell lines (186). The study also reported that PVT1 induces the malignant behavior of GBM (186). On the other hand, miR-190a-5p and miR-488-3p are downregulated in GBM, and their overexpression reversed the malignant phenotype induced by PVT1 (186). miR-190a-5p and miR-488-3p directly target Myocyte enhancer factor 2C (MEF2C), an oncogene in GBM (186). MEF2C, in turn, binds to the promoter of JAGGED1 and upregulates JAGGED1 expression. PVT1-miR-190a-5p/miR-488-3p-MEF2C-JAGGED1 axis is involved in the proliferation and progression of glioma, indicating PVT1 as an important target of glioma (186). Zou et al., using gene expression profiling, identified that lncRNAs PVT1 and CYTOR are upregulated, and lncRNAs HAR1A and MIAT are down-regulated glioma tissues (187). The study also revealed that high expression of PVT1 and low expression of HAR1A lead to poor patient survival who received chemo and radiotherapy (187).

Using a comprehensive analysis of TCGA data, Reon et al. identified that LINC00152 is upregulated in GBM patients compared to normal brain tissues (188). LINC00152 is not upregulated in LGGs except for the IDH wild type (IDHwt) LGG, which explains its role in glioma aggressiveness (188). Overexpression of the lncRNA conferred poor survival of GBM and IDHwt LGG (188). Loss of function assays in U87 GBM cells shows that LINC00152 promotes the expression of mesenchymal markers and GBM

invasion (188). Further, using the psoralen analysis of RNA interactions and structures (PARIS) technique, the study identified secondary stem-loop structures in LINC00152 in regions 285 to 373 of the 496 nucleotides long lncRNA (188). The study also reported a possible binding of RNA binding proteins (RBPs) in the secondary structure of the lncRNA by analyzing the publicly available Riboseq data (188). Also, mutations in the secondary structure region of lncRNA impaired the invasion of GBM cells, indicating that the part containing the secondary structure is essential for the oncogenic function of LINC00152 (188).

Furthermore, the study reported lncRNA MIR4435-2HG as a homolog of LINC00152, present in the same chromosome with nearly identical sequences (with only six base mismatches). MIR4435-2HG expression was similar to LINC00152, and it conferred poor survival of GBM patients similar to LINC00152. MIR4435-2HG also contains a stem-loop structure from nucleotides 382 to 478. siRNA-mediated knockdown of LINC00152 also was found to downregulate MIR4435-2HG (188). The study reveals that LINC00152 and its homolog MIR4435-2HG associate with aggressive tumors and promote cellular invasion through a mechanism that requires the structural integrity of a hairpin structure.

Linc-OIP5 is overexpressed in glioma patients' samples compared to the adjacent normal brain tissues (189). Linc-OIP5 expression correlated with glioma grade, and knockdown of the lncRNA reduced glioma cells proliferation and migration *in vitro* and suppressed tumor growth *in vivo* (189). Knockdown of the lncRNA also reduced the protein levels of the YAP and Notch signaling pathway (YAP, Jag-1, Notch-1, Hes-1), indicating an oncogenic role of linc-OIP5 in glioma through the YAP/Notch pathway (189).

Li et al. observed that LINC00511 overexpression in GBM tissues positively correlated with poor patients' survival (190). Also, epigenetically, the lncRNA was induced by the SP-1 transcription factor in GBM (190). LINC00511 is essential in promoting GBM cell proliferation, invasion invitro, and tumor growth *in vivo* (190). Downstream, the lncRNA competes with miR-124-3p to stabilize CCND2 expression to promote GBM pathogenesis (190). The SP-1/LINC00511/miR-124-3p/CCND2 axis accelerates GBM pathogenesis (190).

Gao et al. studied the role of oncogenic lncRNA LINC02308 in GBM pathogenesis (191). LINC02308 overexpression correlated with poor GBM patient prognosis and promoted glioma cell proliferation, invasion *in vitro*, and tumor growth in a xenograft

mouse model (191). LINC02308 promotes glioma progression by sponging miR-30e-3p to stabilize TM4SF1 and activating AKT/mTOR signaling pathway downstream (191).

Using an RNA-seq screen, Zhang et al. identified lncRNA LPP-AS2 is significantly upregulated in glioma tissues compared to normal brain tissues (192). Physiological assays indicated that lncRNA LPP-AS2 promotes GBM cell proliferation and invasion, inhibits apoptosis, and promotes glioma growth *in vivo* (192). Also, LPP-AS2 stabilizes EGFR, phospho PI3K, and phospho AKT levels by sponging miR-7-5p (192). The study also identified using ChIP assays that c-Myc interacts with the promoter of LPP-AS1 and induces its expression, and in turn, LPP-AS1 increases the levels of c-Myc downstream of EGFR (192). This study establishes an oncogenic positive feedback loop of c-MYC/LPP-AS1/miR-7-5p/EGFR/PI3K/AKT/c-MYC in promoting gliomagenesis (192).

LncRNA Zinc finger antisense 1 (ZFAS1) is upregulated in glioma tissue and GBM cells (U251, T98G, A172, LN229, and HS683 cells) (193). It promotes GBM cell proliferation, migration, invasion, restricted apoptosis, and tumor growth *in vivo* (193). The oncogenic effects of ZFAS1 were inverted upon overexpression of miR-1271-5p in GBM cells (193). In addition, miR-1271-5p targeted and downregulated the oncogenic gene HK2, which was rescued by ZFAS1 by competing with miR-1271-5p (193).

Hua et al. studied the role of lncRNA for kinase activation (LINK-A) in GBM (194). The study reported elevated levels of LINK-A in the serum of GBM patients with a high level of survivin (194). Knockdown of LINK-A promoted apoptosis of GBM cells, which is rescued by overexpression of survivin (194). Also, overexpression of LINK-A induced survivin expression, indicating that LINK-A inhibits glioma cell apoptosis through survivin (194).

P73 antisense RNA 1T (non-protein coding), or TP73-AS1 or PDAM, is a lncRNA involved in apoptosis in several cancers. Zhang et al. identified that TP73-AS1 is upregulated in glioma tissues and cell lines (U251, SNB119, U118, LN229, SHG44), and it is associated with poor patient prognosis (195). Knockdown studies revealed that TP73-AS1 promotes GBM cell proliferation and invasion (195). miR-142 negatively correlated with TP73-AS1. TP73-AS1 competes with miR-142 to prevent the degradation of HMGB1 and RAGE, thereby inducing GBM pathogenesis (195).

LncRNAs present in exosomes are involved in cancer pathogenesis. Ma et al. identified that LINC00470 is upregulated in exosomes extracted from GBM patients (GBM-Exo) (196). U251 cells cultured with GBM-Exo or with LINC00470 overexpression significantly reduced autophagosome as indicated by reduced LC3II/LC3I and Beclin expression, while proliferative ability was increased (196). A similar effect was observed upon Wee1 overexpression, which was abolished by miR-580-3p (196). Also, LINC00470 promotes PI3K/Akt/mTOR pathways, indicated by decreased phosphorylation of PI3K, AKT, and mTOR upon lncRNA knockdown (196). LINC00470 promotes GBM proliferation by acting as a ceRNA for Wee1 by competing with miR-580-3p and promoting PI3K/AKT/mTOR signaling (196).

Zheng et al. identified that lncRNA DDX11-AS1 levels are higher in GBM tissues and GBM cell lines (A172, LN229, U87, U251). Loss of function assays demonstrated that the lncRNA promotes GBM cell proliferation and invasion and prevents their apoptosis (197). Mechanistically, DDX11-AS1 interacts with tumor suppressor miR-449b-5p and titrates its levels to elevate the expression of the oncogenic gene RWDD4, further promoting GBM pathogenesis (197).

Followed by this study, Xiang et al. characterized the role of DDX11-AS1 in GBM (198). Xiang et al. studied the expression of DDX11-AS1 in GBM tissues and cells and identified elevated expression of the lncRNA leads to poor patient prognosis (198). CHIRP-MS analysis depicted that DDX11-AS1 primarily interacts with protein HNRNPC (198). Also, the tumor-promoting phenotype of DDX11-AS1 in GBM cells was similar to those identified upon HNRNPC knockdown, indicating that both are oncogenic (198). Notably, DDX11-AS1 knockdown significantly reduced HNRNPC protein levels (198). Overexpression of DDX11-AS1 elevated the protein levels of β-catenin, pAKT, vimentin, and N-cadherin. In contrast, the knockdown of HNRNPC showed a reverse trend of these proteins, which was partially rescued upon DDX11-AS1 overexpression. Mechanistically, DDX11-AS1 interacts with protein HNRNPC to promote GBM pathogenesis through activation of WNT/ β-catenin and AKT pathway (198).

Xu et al. using a microarray screen, discovered that lncRNA AC003092.1 was markedly decreased in TMZ-resistant GBM cells (U87TR and U251TR) compared to their parental cells (U87 and U251) (199). Also, lncRNA AC003092.1 expression negatively correlated with glioma grade. Low levels of the lncRNA increased the risk of relapse, chemoresistance, and poor prognosis (199). Xu et al. also depicted a negative

correlation between AC003092.1 and miR-195 in GBM (199). The TFPI-2 gene was downregulated in TMZ-resistant GBM cells and positively correlated with AC003092.1 levels (199). Mechanistically, AC003092.1 promotes TMZ chemosensitivity in GBM by inhibiting miR-195-mediated degradation of TFPI-2 (199). Following this study, Guo et al. identified AC003092.1 as an immune-related eRNA from the TCGA database (200). Using gene set enrichment analysis, they also reported that genes regulating immune surveillance and anti-tumor immune response positively correlated with the lncRNA, indicating lncRNA's role in immune surveillance in GBM (200).

Wang et al., using the loss of function and over-expression studies, identified that lncRNA EPIC promotes GBM proliferation, TMZ resistance, and invasion (201). Further, EPIC induced the expression of Cdc20 to promote glioma progression (201).

H19 is a well-studied lncRNA in several cancers and other pathological conditions. Jiang et al. identified that H19 is upregulated in TMZ-resistant tumor samples and TMZ-resistant GBM cell lines, $U87^{TMZ}$ and $U251^{TMZ,}$ compared to normal cells (202). Knockdown of H19 in TMZ-resistant clones significantly reduced their IC_{50}, accompanied by increased PARP cleavage in resistant lines (202). Knockdown of H19 further reduced the expression of essential drug-resistance genes, such as MDR, MRP, and ABCG2, indicating that H19 is a crucial target for inducing chemosensitivity in GBM (202).

Oncogenic lncRNA, FOXD3-AS1, was upregulated in glioma tissues with increasing glioma grades (203). Elevated FOXD3-AS1 is an independent indicator of poor prognosis in glioma patients (203). FOXD3-AS1 promoted glioma cell proliferation, migration, and invasion. It also induces the expression of FOXD3, the oncogenic sense mRNA in GBM, to enhance glioma progression (203). Further, another study conducted by Ling et al. reported that FOXD3-AS1 participates in the tolerance of GBM cells to TMZ (204). TMZ-resistant cells exhibited higher FOXD3-AS1 expression compared to parental cells (204). Also, over-expression of FOXD3-AS1 in TMZ-sensitive cells rendered them TMZ-resistant (204). Mechanistically, FOXD3-AS1 competes with miR-128-3p to prevent the degradation of Wee1 and induces TMZ resistance in glioma cells through the miR-128-3p/Wee1 axis (204).

1.10 Tumor suppressor lncRNAs in GBM

LncRNAs also function as tumor suppressors in GBM. Han et al. characterized the tumor suppressor role of Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) in GBM (205). The study reported a significant downregulation of

MALAT1 in GBM brain tissues and cell lines (205). Also, over-expression of MALAT1 attenuated the tumor's malignant features, such as reduced cell proliferation, invasion, and metastasis *in vitro* and *in vivo* in xenograft models (205). Mechanistically the study reported that MALAT1 exhibits its tumor suppressor function by inhibiting extracellular signal-regulated kinase/ mitogen-activated protein kinase (ERK/MAPK) signaling activity and expression of matrix metalloproteinase 2 (MMP2) (205).

Ruan et al. using a microarray screen, studied lncRNAs regulated by TATA-box binding protein associated factor 15 (TAF15) (206). TAF15 is downregulated in glioma and negatively correlates with GBM patients' poor survival (206). It is involved in mRNA transcription, splicing, and transportation and shows a high translocation rate in various cancers (206). The microarray screen identified that LINC00665 is downregulated in U87 and U251 glioma cell lines compared to normal astrocytes (206). Further, co-expression of TAF15 and LINC00665 overexpression constructs reduced GBM cells proliferation, invasion, and migration and promoted GBM cells apoptosis, indicating tumor suppressor function of TAF15 and LINC00665 in GBM (206). RNA pulldown and RIP assays depict a direct interaction between TAF15 and LINC00665; TAF15 overexpression significantly increased LINC00665 half-life, indicating that TAF15 stabilizes the lncRNA and the duo exerts a tumor suppressor function in glioma (206). Further downstream, LINC00665 interacted with STAU1 protein and degraded MTF1 and YY2 oncogenes through STAU1-mediated mRNA decay (SMD) (206). mRNA degradation of MTF1 and YY2 leads to degradation of GTSE1, an oncogene in glioma (206). TAF15/LINC00665/MTF1(YY2)/GTSE1 is identified in this study to modulate GBM progression (206). Thus, this study explains the role of tumor suppressor lncRNA in GBM and the role of lncRNAs in regulating mRNA degradation by interacting with other proteins (206).

Xu et al., using a microarray screen of GBM tumor tissues and normal brain tissues, identified that lncRNA- UBE2R2-AS1 is significantly downregulated in GBM and inversely correlated with higher grades of glioma (207). Also, overexpression of UBE2R2-AS1 significantly decreased U87 and U373 glioma cells proliferation and migration but increased cell apoptosis (207). Furthermore, UBE2R2-AS1 overexpression increased phosphorylated ERK1/2 levels. Upregulation of the lncRNA increased the expression of toll-like receptor-related genes and apoptosis-related genes (TLR4, FADD, MYD88, CASPASE3, CASPASE8, and CASPASE9). Consequently,

UBE2R2-AS1 acts as a ceRNA for toll-like receptor 4 (TLR4) by competing with miR-877-3p and promoting GBM cell apoptosis (207).

Deng et al. identified that lnc-ZNF281 is downregulated in glioma tissues, and over-expression of lnc-ZNF281 attenuates proliferation, migration, and invasion of T98G and HS683 GBM cells (208). However, activating β-catenin reversed the tumor-suppressive effects of lnc-ZNF281 (208). In consensus with this, overexpression of lnc-ZNF281 inactivated the AKT/GSK-3β/β-catenin signaling, indicating the tumor suppressor function of lnc-ZNF281 in GBM by inactivation of the β-catenin pathway (208).

Xia et al. identified that lncRNA PTCSC3 is significantly downregulated in GBM cells (U87-MG, U251, SHG44, and SHG139) compared to normal astrocytes (209). Overexpression of PTCSC3 inhibited EMT, migration, and invasion of U87 and U251 GBM cells (209). Also, upregulated PTCSC3 reduced the levels of active β-catenin, LRP6, cyclinD1, and c-Myc and increased the levels of Axin1, indicating a tumor suppressor role of lncRNA PTCSC3 through the inactivation of Wnt/β-catenin pathway (209).

1.11 TGF-β regulated lncRNAs in GBM pathogenesis

TGF-β-regulated lncRNAs modulate essential aspects of tumor development, such as invasion, metastasis, and epithelial to mesenchymal transition of various cancer types (210–213). Several differentially expressed lncRNAs are reported in GBM, regulating multiple aspects of GBM pathology, which has been extensively reviewed elsewhere (214–230). In glioma, TGF-β regulated lncRNA-ATB, UCA1, LINC00645, LINC00115, lncRNA-MUF, and LINC01711 modulate proliferation, invasion, and glioma stem cell renewal (212,213,231–235). In addition, TGF-β-induced lncRNAs H19 and HOXD cluster antisense RNA 2 (HOXD-AS2) confer TMZ resistance in glioma by regulating miR-198 biogenesis and competing with KSRP (236). Also, a few lncRNAs, namely lncRNA TCONS_00020456, MIR4435-2 HG, RPSAP52, PVT1, and RP11-838N2.4, which TGF-β does not induce, modulate GBM pathogenesis by regulating the various components of the TGF-β pathway (237–240).

1.11.1 LncRNA-ATB

Ma et al. reported the upregulation of lncRNA-ATB in glioma tissues and GBM cell lines compared to normal brain tissues (231). The levels of lncRNA-ATB are higher in grade IV glioma than in lower grades. Concomitantly, increased expression of lncRNA-ATB correlated with poor survival of GBM patients (231). Further, loss of function

studies depicted a reduced proliferation, migration, and invasion of U251 and A172 GBM cells *in vitro* (231). The study also reported a negative correlation between the expression of lncRNA-ATB and miR-200a in GBM tissues. miR-200a is downregulated in GBM tissues and GBM cell lines, and the knockdown of lncRNA-ATB significantly increased miR-200a expression in GBM cells (231). Further, using the Targetscan database, the study identified the direct binding of lncRNA-ATB and miR-200a. Luciferase reporter assay and Ago2 pull-down assays validated the direct interaction of miR-200a with lncRNA-ATB and TGF-β2, indicating that lncRNA-ATB and TGF-β2 are direct targets of miR-200a (231). Also, the mRNA and protein levels of TGF-β2 were downregulated upon lncRNA-ATB knockdown and miR-200a overexpression (231). In contrast, miR-200 inhibition upregulated TGF-β2 (231). Physiological assays indicated that lncRNA-ATB mediated reduction in cell proliferation, colony formation, and invasion of U251 and A172 GBM cells were rescued upon a combination of lncRNA-ATB knockdown and miR-200a inhibition. Additionally, the knockdown of lncRNA-ATB significantly reduced the levels of TGF-β2 expression, which was rescued upon a combination of lncRNA-ATB knockdown and miR-200a inhibition. A positive correlation between lncRNA-ATB and TGF-β2 and a negative correlation between miR-200a and TGF-β2 was observed. The reduction in mRNA and protein levels of TGF-β2 upon lncRNA-ATB was further downregulated upon miR-200a overexpression. In contrast, TGF-β2 expression was rescued with the combination of lncRNA-ATB knockdown and miR-200a inhibition (231). Additionally, *in vivo*, nude mice model demonstrated a reduction in tumor volume, tumor weight, and reduced proliferation index indicated by Ki67 staining, supporting the oncogenic role of lncRNA-ATB in GBM. These results suggest that lncRNA-ATB could competitively bind miR-200a to stabilize TGF-β2 and promote TGF-β2-mediated GBM pathogenesis (231).

Tang et al. reported that lncRNA-ATB is upregulated by TGF-β treatment in LN18 and U251 GBM cells (213). The upregulation of lncRNA-ATB by TGF-β treatment was abrogated upon treatment with TGF-β inhibitor, SB505124, indicating the SMAD2/3 mediated regulation of lncRNA-ATB expression (213). The study also reported that lncRNA-ATB overexpression in combination with TGF-β treatment increases GBM cell invasion. Further, lncRNA-ATB overexpression in combination with TGF-β treatment significantly increased the phosphorylation of P65 and the nuclear translocation of P65, and the phosphorylation of P38 in GBM cells. These results

indicate the activation of the NF-κB and P38/MAPK pathways by TGF-β induced lncRNA-ATB, which are downstream targets of the non-canonical TGF-β pathway (213). Physiological assays indicated that the increase in invasion in LN18 and U251 GBM cells upon lncRNA-ATB overexpression and TGF-β treatment was significantly abolished upon treatment with inhibitors of NF-κB and P38/MAPK pathways. Hence this study demonstrates that the SMAD2/3 transcription factor induces TGF-β-regulated lncRNA-ATB in GBM, which is oncogenic. Mechanistically, lncRNA-ATB promotes TGF-β-mediated GBM cell invasion through the NF-κB and P38/MAPK pathways.

1.11.2 LncRNA-UCA1

Li et al. identified that TGF-β significantly upregulates lncRNA-UCA1 in U87 and U251 glioma cells (232). Further, the knockdown of lncRNA-UCA1 attenuated the invasion and stemness of glioma cells induced by TGF-β (232). Particularly, lncRNA-UCA1 knockdown downregulates Slug, ALDH1, and Nanog, which TGF-β upregulates. Luciferase reporter assay and Ago2 RIP depict direct binding of lncRNA-UCA1 and miR-1, and miR-203a. Also, miR-1 and miR-203a directly target Slug. The study also reported a positive correlation between lncRNA-UCA1 and Slug in GBM tissues. LncRNA-UCA1 promotes Slug expression by binding to and titrating miR-1 and miR-203a. Further, the downregulation of spheroid formation, downregulation of Slug, and reduction in ALDH1 activity upon lncRNA-UCA1 knockdown were partially rescued upon Slug overexpression. Hence, the study identified that lncRNA-UCA1 acts as a molecular sponge for miR-1 and miR-203a to promote Slug expression and Slug-mediated GBM cell stemness (232).

1.11.3 LINC00645

Li et al. identified LINC00645 as a differentially expressed lncRNA in GBM samples from TCGA and GSEA repositories (212). The research group further identified that TGF-β induces LINC00645 in GBM (212). Knockdown of this lncRNA attenuated the malignant behavior of GBM by decreasing glioma cell proliferation, invasion, migration, and EMT in T98G and U251 GBM cells. Notably, LINC00645 knockdown reduces ZEB1 levels, an essential target of miR-205-3p (212). miR-205-3p is downregulated in GBM tumor tissues and GBM cell lines compared to normal brain tissues (212). Low expression of miR-205-3p indicates poor survival in GBM patients (212). TGF-β treatment significantly downregulated miR-205-3p, and the study also

reported a negative correlation between LINC00645 and miR-205-3p in GBM patients' samples (212). Also, miR-205-3p overexpression significantly reduced the levels of LINC00645, while depletion of LINC00645 promoted miR-205-3p expression. Luciferase reporter assay and Ago2-RIP depicted the direct interaction between LINC00645 and miR-205-3p (212). Epithelial marker, E-cadherin was downregulated, and mesenchymal markers, vimentin, N-cadherin, SNAIL, and ZEB1 were upregulated upon TGF-β treatment and LINC00645 overexpression. However, TGF-β treatment followed by miR-205-3p reversed these effects (212). miR-205-3p is a tumor suppressor that directly targets and degrades ZEB1 (212). TGF-β treatment and LINC00645 overexpression also induced GBM cell invasion, and migration was also reversed upon miR-205-3p overexpression (212). Hence, LINC00645 sponges miR-205-3p to stabilize ZEB1 and promote GBM pathogenesis (212). In addition to its effect on GBM cell invasion, migration, and EMT, LINC00645 also induces stemness in GBM cells. Western blotting analysis upon LINC00645 depletion reduced the expression of stemness factors, Bmi-1, Oct-4, Sox-2, and Nanog (212). Sphere-forming assay indicated a decrease in sphere-forming ability on LINC00645 knockdown. In contrast, LINC00645 overexpression had the opposite effect (212). Also, LINC00645 depletion partly decreased Nestin expression and increased the GFAP expression in U251-GSC cells (212). Hence, LINC00645/miR-205-3p/ZEB1 axis is a critical player in the invasion, migration, and EMT in GBM, and LINC00645 also plays an essential role in promoting stemness in GSCs (212).

1.11.4 LINC00115

Tang et al. identified that LINC00115 is induced by TGF-β using RNA-sequencing of TGF-β treated GSCs (233). Also, LINC00115 expression is higher in GBM tumor samples than in normal tissues, and increased expression of LINC00115 leads to poor patient prognosis (233). LINC00115 knockdown inhibits GSC proliferation and neuro-like sphere formation *in vitro* and reduces tumor formation *in vivo* in the xenograft model (233). Further, using bioinformatics tools, a luciferase reporter assay, and an RNA pulldown assay, the study demonstrates that LINC00115 physically associates with miR-200b and miR-200c (233). Also, LINC00115 depletion reduced the expression of ZEB1 and ZNF596 and reduced invasion in GSCs (233). Reporter assays indicate that ZEB1 and ZNF596 are targets of miR-200. Downregulation of ZEB1 upon LINC00115 knockdown was reversed upon miR-200b overexpression, indicating that

LINC00115 competitively binds to miR-200b to promote ZEB1-mediated GBM invasion (233). The study also reported that LINC00115 and its target ZNG596 are co-expressed in clinical glioma samples. Concomitantly, exogenous expression of ZNF596 in LINC00115-depleted GSCs reversed the inhibition of cell proliferation caused by LINC00115 depletion, indicating that ZNF596 is the downstream effector of LINC00115-driven GBM tumorigenicity (233). Also, LINC00115 binds to miR-200 to promote the expression of ZNF596 (233). Further, the study identified through CRISPR knockout of ZNF596 that EZH2 is a direct target of ZNF596, and promoter analysis of EZH2 revealed that ZNF296 is a transcription factor in promoting the expression of EZH2 (233). LINC00115 depletion downregulates EZH2 levels, which is reversed upon ZNF596 overexpression, indicating that ZNF596 functions as a transcription factor for EZH2 downstream of LINC00115 (233). Consistent with this, LINC00115 further activates STAT3 downstream of EZH2 through ZNF596, indicating that LINC00115 activates EZH2/STAT3 signaling through ZNF596, thereby promoting GSC self-renewal and tumorigenicity (233). LINC00115 aids GSC's self-renewal by acting as a ceRNA for transcription factors ZEB1 and ZNF596 by sponging miR200 (233). It also promotes GSC's tumorigenicity through ZNF596/EZH2/STAT3 signal axis (233).

1.11.5 H19 and HOXD-AS2

Nie et al. identified eight differentially regulated lncRNAs upon TGF-β treatment (H19, HOXD-AS2, LINC00635, LINC00277, RP11-196G11.2, LINC00152, MALAT1, and LOC100506207) in D54, P-GBM2 cells (236). TGF-β induces LncRNAs H19 and HOXD-AS2 through Smad signaling (236). Further, RIP analysis of TGF-β treated GBM cells depicted enhanced binding of H19 and HOXD-AS2 with K-homology (KH) splicing regulatory protein (KSRP) (236). KSRP degrades follistatin-like 1 (FSTL1) and promotes the maturation of miR-198 in the nucleus (236). The study observed that TGF-β-induced H19 and HOXD-AS2 competitively bind to KSRP and thus prevent its nuclear translocation (236). miR-198 is a tumor suppressor miRNA that promotes temozolomide sensitivity in GBM cells by downregulating MGMT (236). Overexpression of KSRP reversed the H19 and HOXD-AS2-mediated upregulation of MGMT expression, which could reverse TGF-β-induced TMZ resistance (236). The study thus demonstrated that H19 and HOXD-AS2 confer TMZ resistance by regulating miR-198 biogenesis by competing with KSRP (236).

1.11.6 LncRNA TCONS_00020456

Tang et al. using a microarray screen, identified 1759 upregulated and 1932 downregulated lncRNAs in U251 GBM cells (237). Among these differentially expressed lncRNAs, the study further characterized the role of the most downregulated lncRNA – TCONS_00020456 in GBM pathogenesis (237). The expression of TCONS_00020456 decreased with increasing glioma grades, and the low expression of TCONS_00020456 indicated poor survival of GBM patients (237). Physiological assays demonstrated that the knockdown of TCONS_00020456 increased GBM cell migration, and invasion, while overexpression of TCONS_00020456 gave the opposite effect (237). Functional analysis of TCONS_00020456 in cbioportal revealed that various mRNAs with oncogenic function correlated with its expression (237). Among them, Smad2 and PKCα were the top hits (237). Further, western blotting analysis revealed that the knockdown of TCONS_00020456 increased the expression of SMAD2, PKCα, N-cadherin, vimentin, and downregulation of E-cadherin. Also, the phosphorylation of JNK and ERK was elevated upon TCONS_0002045 knockdown (237). The overexpression of TCONS_0002045 reversed these effects (237). These results indicate that TCONS_0002045 abrogates GBM cell invasion and migration by targeting SMAD2 and PKCα pathways (237). *In vivo,* nude mice model of GBM depicted a decrease in tumor size and weight in the TCONS_0002045 overexpression group compared to the TCONS_002045 knockdown group (237). Also, the immunohistochemical staining of tumor tissues from the nude mice indicated increased expression of SMAD2 and PKCα in the TCONS_002045 knockdown group compared to the TCONS_0002045 overexpression group (237). Computational analysis using the miRDB database revealed several miRNAs targeting TCONS_0002045, SMAD2, and PKCα (237). Among these miRNAs, miR-1279 was identified as the common miRNA target between the three. This study identified the differentially expressed lncRNAs in U251 GBM cells (237). Among these lncRNAs, lncRNA TCONS_0002045 abrogates GBM migration and invasion by targeting SMAD2 and PKCα (237). SMAD2 is an important downstream transcription factor in the TGF-β pathway and is a well-known inducer of GBM pathogenesis (237). However, the exact mechanism of downregulation of SMAD2 and PKCα by TCONS_0002045 and the role of miR-1279 is yet to be investigated.

1.11.7 MIR4435-2 Host gene (MIR4435-2 HG)

Xu et al. reported the high expression of MIR4435-2HG in GBM by analyzing the GEPIA database and GBM tissue samples (238). Loss-of-function studies in U251 and U87 GBM cells depicted decreased cell proliferation, colony formation, migration, and invasion upon MIR4435-2HG knockdown (238). *In vivo,* nude mice models also demonstrated reduced tumor volume and growth upon MIR4435-2HG depletion (238). However, overexpression of MIR4435-2HG had the opposite effect on GBM progression, indicating an oncogenic role of MIR4435-2HG in GBM (238). The starbase tool revealed that miR-1224-5p targets MIR4435-2HG (238). Also, it was observed that miR-1224-5p is downregulated in LN229, U87-MG, and U251 GBM cells compared to normal human astrocytes (NHA) (238). Luciferase reporter assay confirmed the direct binding of MIR4435-2HG and miR-1224-5p (238). Functional rescue experiments revealed that the increase in cell proliferation and colony formation induced by MIR4435-2HG overexpression was abrogated upon miR-1224-5p mimics, indicating that the MIR4435-2HG-miR-1224-5p axis promotes GBM pathogenesis (238). Further, the starbase tool and luciferase reporter assay demonstrated that TGFBR2, a critical oncogene is a direct target of miR-1224-5p (238). Further, MIR4435-2HG overexpression promoted the expression of TGFBR2 at mRNA and protein levels (238). Also, miR-1224-5p inhibition reduced GBM cell proliferation and colony formation ability, which was rescued upon TGFBR2 overexpression (238). Also, TGFBR2 knockdown antagonized MIR4435-2HG overexpression-induced GBM cell proliferation and colony formation, indicating that MIR4435-2HG promotes GBM proliferation by sponging miR-1224-5p and stabilizing TGFBR2 (238). The study revealed a positive correlation between MIR4435-2HG and TGFBR2 and a negative correlation between miR-1224-5p and MIR4435-2HG (238). Hence, this study identifies a critical oncogenic function of lncRNA MIR4435-2HG in GBM by targeting the miR-1224-5p/TGFBR2 axis (238).

1.11.8 LncRNA RPSAP52

Using the TCGA database and qRT-PCR analysis of GBM tumor samples with matched controls, Wang et al. identified that lncRNA RPSAP52 is overexpressed in GBM samples (239). Also, high expression of RPSAP52 conferred poor survival in GBM patients (239). The study also identified the increased expression of TGF-β1 in GBM tissues and a positive correlation between RPSAP52 and TGF-β1 (239). Further,

overexpression of RPSAP52 demonstrated increased TGF-β1 protein, and knockdown of RPSAP52 exhibited the opposite effect (239). GBM cell stemness was assessed by measuring the percentage of CD133+ cells upon overexpression of RPSAP52 and TGF-β1 (239). Overexpression of RPSAP52 and TGF-β1 individually increased the percentage of CD133+ cells. At the same time, the opposite effect was observed upon silencing RPSAP52 (239). Further, the overexpression of TGF-β1 rescued the reduction in the percentage of CD133+ cells observed upon RPSAP52 silencing (239). Hence, this study explores the role of lncRNA RPSAP52 in promoting stemness in GBM by promoting TGF-β1 (239).

1.11.9 LncRNA Plasmacytoma variant translocation-1 (PVT1)

Li et al. reported the overexpression of lncRNA PVT1 and downregulation of p53 in higher grades of glioma and GBM cell lines (HS683, T98G, U373, SHG44, A172, U251, and U87-MG) compared to normal brain cells (240). High expression of lncRNA PVT1 conferred poor survival in GBM patients (240). Also, a negative correlation was observed between lncRNA PVT1 and p53 in GBM tissues (240). The lncATLAS tool depicted the presence of putative p53 binding sites in the promoter of lncRNA PVT1 (240). Further, luciferase reporter assay revealed that p53 inhibits the transcription of lncRNA PVT1 (240). Loss-of-function studies demonstrated that the knockdown of lncRNA PVT1 decreased GBM cell viability, proliferation, migration, and invasion and induced cell cycle arrest at S and G2/M phases (240). Protein expression of mesenchymal markers, N-cadherin, MMP-9, and MMP-2 was reduced, and E-cadherin was upregulated upon lncRNA PVT1 knockdown. At the same time, the knockdown of p53 had a reverse effect. However, a combined knockdown of lncRNA PVT1 and p53 abolished the tumor suppressive effects of p53 in GBM pathogenesis, indicating that p53 suppresses GBM cell proliferation and promotes apoptosis by downregulating lncRNA PVT1 (240). Further, the study analyzed the expression of TGF-β and phosphorylation of SMAD2/3 in GBM tissues and cell lines. TGF-β was upregulated, and phosphorylation of SMAD2/3 was increased in GBM tissues and GBM cell lines. Also, luciferase reporter assay revealed that lncRNA-PVT1 promotes the expression of TGF-β. The elevated expression of TGF-β and increased phosphorylation of SMAD2/3 in GBM cells were reduced upon lncRNA-PVT1 knockdown. Whereas knockdown of p53 increased TGF-β expression and phosphorylation of SMAD2/3 (240). However, the combined knockdown of p53 and lncRNA-PVT1 abolished the suppressive effects

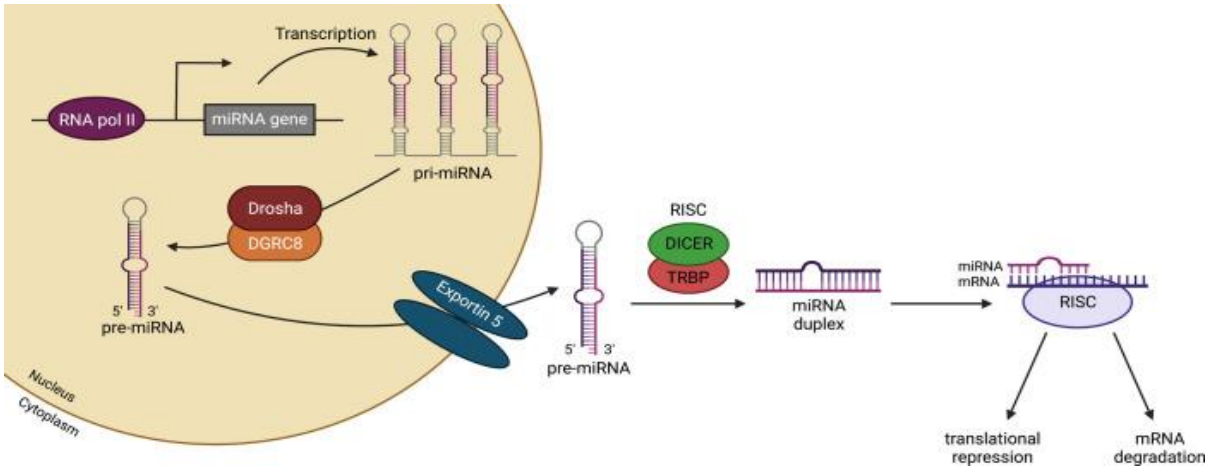
of p53 on the TGF-β signaling (240). *In vivo,* nude mice model revealed a reduction in tumor volume upon lncRNA-PVT1 knockdown, while the p53 knockdown had the opposite effect (240). The combined knockdown of lncRNA PVT1 and p53 abolished the impact of p53-mediated tumor growth suppression (240). Expression of TGF-β and lncRNA PVT1 and phosphorylation of SMAD2/3 were elevated in tumor tissues in the nude mice with p53 knockdown (240). An opposite effect was observed in the lncRNA PVT1 knockdown group (240). Also, the pro-apoptotic genes Caspase-3 and Bax were upregulated, and the anti-apoptotic Bcl-xL and Bcl-2 were downregulated on lncRNA PVT1 knockdown (240). An opposite effect was observed upon p53 knockdown. In contrast, the combined knockdown of lncRNA PVT1 and p53 abrogated the tumor suppressive effects of p53 (240). This study demonstrates that p53 potentially contributes to suppressing lncRNA PVT1 and thereby suppresses the activation of TGF-β by lncRNA PVT1 and lncRNA PVT1-TGF-β axis mediated GBM progression (240).

1.11.10 LncRNA RP11-838N2.4

Liu et al. identified that lncRNA RP11-838N2.4 is lower in TMZ-resistant GBM cells (U87TR, U251TR) compared to the parental non-resistant GBM cells (U87, U251) (241). The study also observed that decreased lncRNA RP11-838N2.4 conferred poor prognosis, high risk of GBM relapse, and shorter postoperative survival times (241). Also, overexpression of lncRNA RP11-838N2.4 enhances the cytotoxic effects of TMZ of GBM cells *in vitro* and *in vivo* (241). Consequently, TMZ-resistant U251TR cells with high lncRNA RP11-838N2.4 displayed low levels of miR-10a (241). The lncRNA acts as an endogenous sponge for EphA8 by competing with miR-10a and increasing the levels of EphA8, which promotes apoptosis in glioma cells (241). Notably, lncRNA RP11-838N2.4 overexpression hindered the TGF-β signaling pathway independent of miR-10a by reducing mRNA and protein levels of TGF-β1, TGFBR1, SMAD2, SMAD3, SMAD4 levels (241). LncRNA RP11-838N2.4 hinders GBM cell proliferation, promotes TMZ sensitivity, and TMZ-mediated GBM cell apoptosis by sponging miR-10a and stabilizing EphA8. Also, it downregulates the TGF-β pathway by reducing the expression of the signaling pathway's components and reducing GBM progression. However, the exact molecular mechanism of lncRNA RP11-838N2.4-mediated downregulation of the TGF-β pathway needs further investigation.

1.12 miRNA biogenesis and function

miRNAs are small non-coding RNAs of 21-23 nucleotides in length that generally perform post-transcriptional gene silencing of their mRNA targets (242). *Lin-4* was the first miRNA discovered in *C.elegans* (243). Ambros and Ruvkun's research group further identified that *lin-4* had several complementary binding sites at the 3' untranslated region (3' UTR) of the *lin-14* gene, which substantially reduced the mRNA and protein levels of the *lin-14* gene (244). Later *lin-4* was categorized into a set of genes called miRNA genes. Followed by this study, in 2000, *let-7* miRNA was discovered, which was conserved across species (245). To date, approximately 2000 miRNA genes have been found in the human genome, which accounts for roughly 1-5% of the predicted genes in the human genome (242). miRNAs have gene regulatory functions in developmental processes and normal physiological and disease conditions. The majority of miRNAs are transcribed by RNA polymerase II either as intergenic miRNAs (using their promoter) or intragenic miRNAs (derived from introns or a few exons of protein-coding genes) (246). About 30% of miRNAs are produced from introns of protein-coding genes (247). Initially, the miRNAs are transcribed as primary miRNAs (pri-miRNAs), which are capped, spliced, and polyadenylated (247). A single pri-miRNA can produce either a single mature miRNA or a cluster of two or more miRNAs processed from a common primary transcript (247). The processing of pri-miRNAs occurs either by canonical or non-canonical mechanisms, which are reviewed extensively elsewhere (242). In the canonical pathway, the long pri-miRNA is processed by the double-stranded RNase III enzyme, DROSHA, and its cofactor, double-stranded RNA binding protein, DiGeorge syndrome critical region 8 (DGCR8) (242,248) (Fig 8). The base of the secondary stem-loop structure is cleaved, giving precursor miRNA (pre-miRNA) containing 60-70 nucleotides stem-loop structure (242,247,248). The pre-miRNA is exported by Exportin 5/Ran GTP complex to the cytoplasm. In the cytoplasm, the pre-miRNA is cleaved by the DICER1 enzyme. Then the guide strand (5' or 3') is loaded onto the Argonaut (AGO) complex, giving rise to the mature miRNA, and the passenger strand is degraded (242,247,248) (Fig 8). The non-canonical pathway comprises Drosha/DGCR8 independent or Dicer independent mechanisms (242,248). The role of miRNAs in various aspects of GBM pathogenesis is studied elsewhere (249–258).



(Adapted from Cerqueira et al. 2022)

Fig 8: miRNA Biogenesis. *miRNAs are transcribed as primary miRNAs (pri miRNAs). Then, the RNase III enzyme, DROSHA, and RNA binding protein, DGCR8, bind and cleave the pri-miRNA into precursor miRNA (pre-miRNA), which is exported to the cytoplasm by exportin5. In the cytoplasm, the DICER/TRBP complex forms the mature miRNA.*

1.13 Competing endogenous RNA (ceRNA) hypothesis

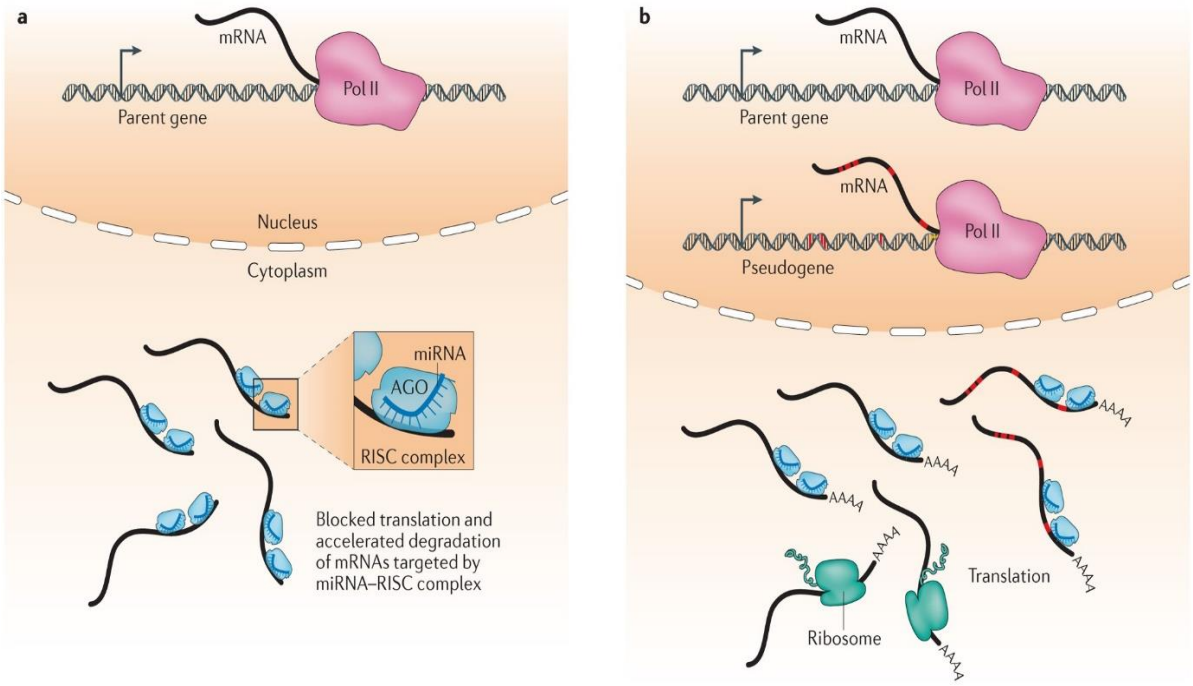
miRNAs function by modulating post-transcriptional gene regulation by primarily interacting with the miRNA response elements (MREs) present on the 3' UTR of a transcript. However, interaction with 5' UTR, coding sequences, and gene promoters are also observed (248,259). They bind to the target gene and either degrade the transcript or, more often, limit the target gene's translation (260). Degradation of the target gene occurs in an AGO2-dependent manner when there is complete mRNA:miRNA complementarity (261). At the same time, miRISC-mediated translational repression occurs with the help of GW182, PAN2-PAN3, and CCR4-NOT complex (246). While most miRNAs perform gene silencing in certain physiological conditions, such as amino acid starvation and quiescent oocytes, miRNAs perform translational activation (262–264).

Gene expression is regulated by both protein-coding and non–coding genes by acting as sponges of miRNAs, known as the ceRNA hypothesis (261). ceRNA hypothesis infers that a protein-coding or non-coding transcript that shares a particular MRE sequesters the respective miRNA, stabilizing and upregulating its mRNA target (265) (Fig 9). LncRNAs localized in the cytoplasm are proposed to function primarily through the ceRNA hypothesis due to the availability of the RISC machinery at the cytoplasm (139). This hypothesis is also extended to circular RNAs and pseudogenes (261,265).

Karreth et al. identified ZEB2 as a ceRNA decoy for *PTEN* by sequestering miR-181, miR-200b, miR-25, and miR-92a, thereby reducing melanoma progression (266).

The ability of lncRNAs to act as ceRNAs to protein-coding genes was first demonstrated in muscle differentiation (267). Muscle-specific lncRNA linc-MD1 promotes differentiation by competing with miR-133 and miR-135, which explicitly targets MAML1 and MEF2C protein-coding genes (267). This concept of the ceRNA hypothesis is now well-established and well-studied in various cancers, including GBM (268). Several factors determine the ceRNA balance, such as subcellular localization of the ceRNA components, miRNA/ceRNA affinity, RNA editing, and interaction with RNA binding proteins (RBPs) (268). Recent studies have established several ceRNA networks in GBM using GO and KEGG analysis (269).

More often, the ceRNA hypothesis at a cellular level does not happen as a simplified theoretical concept as one-one ceRNA event. Instead, it occurs as a multiple-multiple ceRNA interaction, making it much more complex (265,268). Also, the various mechanisms of miRNA-mediated target degradation should be considered an essential factor in studying the ceRNA hypothesis. The canonical miRNA silencing mechanism in animals involves a combination of translational repression and mRNA decay of target genes (246). When bound to the target transcript, miRNAs guide Argonaute protein to form the core miRISC consisting of GW182. This complex interacts with and recruits PAPB and deadenylase leading to deadenylation, decapping, and decay of the target transcript (246). Translational repression occurs by the interference of the miRISC complex with the assembly or activity of elF4F via eIF4E-T and DDX6 (246). Alternatively, (i) complete complementarity of miRNA and the target transcript leads to AGO-mediated target degradation; (ii) Target-mediated miRNA degradation is induced by interaction with targets through extensive pairing, in particular extending to the miRNA 3’ end; (iii) Recruitment of Argonaute in the absence of GW182 results in inhibition of translation without affecting mRNA stability (likely involving alternative co-factors) (246).



(Adapted from Thomson and Dinger, 2016)

Fig 9: The ceRNA hypothesis. *When a lncRNA or a pseudogene that acts as a ceRNA is transcriptionally inactive/silent, the miRNA targets its mRNA target genes, leading to degradation or translational repression of the target mRNA mediated by microRNA–guided RNA-induced silencing complex (miRNA–RISC) (left panel). When the ceRNA becomes transcriptionally active, it competes and binds to the miRNA, sequesters the miRNA, and prevents the degradation of the target mRNA (right panel).*

1.14 ceRNA hypothesis in GBM

Using microarray analysis, recent studies have identified the various differentially expressed lncRNAs, mRNAs, and their corresponding miRNA targets in glioma (270). Using GO and KEGG pathway analysis and statistical tools, these studies have attempted to construct the possible ceRNA networks between the candidate lncRNAs, mRNAs, and miRNAs in GBM. The correlation between these miRNAs and their target genes and the signaling pathways regulated by these genes are used to predict their role in GBM pathogenesis. Zhu et al. analyzed the expression profiles of lncRNAs and mRNAs in GBM samples from the GSE51446 dataset (270). They also examined the miRNA expression profiles in GBM samples from the GSE65626 dataset (270). Three thousand four hundred thirteen differentially expressed lncRNAs and mRNAs and 305 differentially expressed miRNAs were identified. Co-expression network analysis identified a potential ceRNA network containing three lncRNAs (RP11-268F1.3, RP11-547C13.1, RP11-60M5.4), five miRNAs (hsa-miR-381-3p, hsa-miR-132-3p,

has-miR-346, has-miR-584-3p, has-miR-874-3p), and 60 mRNA genes (270). This ceRNA network was also predicted to impact GBM patients' survival (259).

Zhang et al. identified 41 differentially expressed miRNAs, 398 differentially expressed lncRNAs, and 1995 differentially expressed mRNAs using microarray analysis in eight GBM and eight control tissue samples (271). Fifty-five lncRNAs acting as ceRNAs were identified based on the lncRNA-miRNA-mRNA network analysis performed using Pearson correlation and clustering analysis. Among these, 39 ceRNA networks were identified to regulate GBM cell apoptosis, proliferation, adhesion, angiogenesis, and metastasis (271).

Long et al. used the TCGA dataset of GBM patients and identified differentially expressed lncRNAs, miRNAs, and mRNAs (272). The differentially expressed genes were used to construct a ceRNA network using Cytoscape software (272). Functional enrichment analysis and KEGG analysis depicted that the predicted ceRNA network has an essential function in calcium signaling, Rap1 signaling pathway, cytoskeleton regulation, MAPK signaling pathway, PI3K-Akt signaling pathway, and T-cell receptor signaling pathway (272).

As discussed in the literature review, several aberrantly expressed lncRNAs control critical biological functions in gliomas, including cell proliferation, invasion, and apoptosis. Furthermore, the tumor-promoting role of the TGF-β pathway in glioma is well established, and several anti-TGF-β agents are in different stages of clinical trials. Given that lncRNA expression is de-regulated in glioma and aberrant activation of the TGF-β pathway in GBM, we suspect many lncRNAs are regulated by the TGF-β pathway in GBM. They function downstream of the TGF-β pathway to promote tumor initiation and progression. This study aims to identify and functionally characterize the role of TGF-β regulated lncRNAs and their mechanism of gene regulation in GBM pathogenesis.

1.15 Aim and Objectives

The aim of the proposed research is:

To identify and functionally characterize the role of lncRNAs regulated by TGF-β in GBM. The specific objectives are:

1. To do a genome-wide microarray screen to identify TGF-β regulated lncRNAs in T98G GBM cell line.
2. To identify the transcription machinery involved in the expression of TGF-β regulated lncRNAs in GBM.
3. To characterize the function of TGF-β regulated lncRNAs in gene regulation.
4. To evaluate the role of TGF-β regulated lncRNAs in GBM as therapeutic targets.

Chapter 2

Materials and methods

Materials and methods

2.1 Cell culture and treatments

T98G cells were purchased from the American Type Culture Collection (Manassas, VA). LN229, LN18, and U87-MG cells were purchased from (NCCS, Pune). The status of IDH, PTEN, and p53 mutations in all the cell lines are as follows:

Table 1

IDH, PTEN, and p53 mutation status in GBM cell lines

Cell line	IDH status	PTEN status	P53 status
T98G	Wildtype	Mutant	Mutant
LN229	Wildtype	Wildtype	Mutant
U87-MG	Wildtype	Mutant	Wildtype
LN18	Wildtype	Wildtype	Mutant

All cells were grown in a complete medium, DMEM (Invitrogen), containing 10% fetal bovine serum (FBS) supplemented with 1 mm l-glutamine and penicillin/streptomycin (Gibco). Cells were treated with TGF-β1 (10 ng/ml) Peprotech (# 100-21) in serum-free medium (SFM) for the concentration and duration indicated in the figures and legends. SB505124 (Tocris # 3263), an inhibitor of TGFβRI/smad2/3, was used at a concentration of 6μM for pre-treatment of GBM cells to inhibit TGF-β signaling wherever indicated.

2.2 Microarray analysis

Agilent SurePrint G3 Gene Expression Microarrays for Human (v3) for lncRNAs, containing 30,606 lncRNAs, and 37,756 RefSeq-coding transcripts, was used to interrogate lncRNA and mRNA changes in vehicle versus TGF-β1 (10 ng/ml)-treated T98G cells after 24 h. RNA was isolated using MN Nucleospin RNA plus isolation kit (Cat.No:740984.5). 10 μg of purified RNA samples were treated with recombinant DNAse I (Invitrogen Thermo Scientific - Cat.NO: EN0521) as per manufacturer's instructions, and the RNA samples were column purified using an MN Nucleospin column purification kit. Hybridization and analysis were performed at the Molecular Genomics Core at Genotypic Technology (Bangalore). Briefly, total RNA was end-labeled using Agilent Quick-Amp labeling Kit (p/n5190-0442) and hybridized to Agilent Human Gene Expression Microarray 8X60K. Fragmentation of labeled cRNA

and hybridization were done using the Gene Expression Hybridization kit (Agilent Technologies, In situ Hybridization kit, # 5190-0404). The hybridized slides were scanned using the Agilent Microarray Scanner (Agilent Technologies, Part Number G2600D). Data analysis was done by using GeneSpring GX software version 14.5. Gene expression in the test group (TGF-β) was compared with the control group (C) to identify DEGs upon TGF-β treatment. Differentially expressed genes (DEGs) were selected based on a log base 2 (Fold ≥ 0.6) and log base 2 (Fold ≤ -0.6) with a statistical significance of p-value < 0.05.

2.3 RNA isolation and Real-time PCR

Total RNA was extracted from glioma cells using MN Nucleospin RNA plus isolation kit (Cat.No:740984.5). 1 µg of NA was converted into cDNA using PrimeScript first-strand cDNA kit from Takara (# 6110A). Quantitative real-time PCR (qRT-PCR) was performed with the SYBR Green PCR kit (# RR820A, Takara) in Biorad CFX96 real-time qPCR system. All reactions were performed in triplicates and normalized with TBP as an internal control. Relative gene expression of each sample was calculated using the using 2^{-ddct} formula. For miRNA expression analysis, RNA was isolated using ZYMO Quick-RNA™ Miniprep Plus Kit (# R1057). miRNA cDNA was synthesized using mir-X miRNA 1st strand synthesis kit (# 638313, Takara). qRT-PCR of miRNA was carried out using a universal primer and the primer specific to miR-34a-5p (Appendix). U6 was used as a normalizing control. The gene primer sequences are shown in Appendix.

2.4 Bioinformatic analysis

LncRNA-MUF and LINC01711 expression values and associated prognostic information from 693 glioma cases were obtained from the Chinese Glioma Genome Atlas (http://www.cgga.org.cn). These 693 samples comprised 505 WHO III and IV tumors and 188 WHO II tumors. LncRNA expression was also analyzed using the Cancer genome atlas (TCGA) dataset using the gene expression profiling interactive analysis (GEPIA) database (273). The Kaplan-Meier estimation method was used for the overall survival analysis of patients based on lncRNA expression. The Kaplan-Meier survival analysis was performed using the atlas of non-coding RNA in cancer (TANRIC) database and CGGA database.

LncRNA promoter analysis was performed to identify functional Smad binding elements using the online tool – JASPAR (http://jaspar.genereg.net/). miRNA targets of lncRNA-MUF were predicted by the RNAInter database (http://rnainter.org/). The interaction between lncRNA-MUF and miR-34a was confirmed by RNAhybrid (https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid?id=rnahybrid_view_submission) and IntaRNA (https://www. rna-society.org/rnainter/IntaRNA.html). miRNA targets of LINC01711 were predicted by DIANA tools-lncbase v2 (https://diana.e-ce.uth.gr/lncbasev3/interactions), targetscan (https://www.targetscan.org/vert_71/). Interaction between LINC01711 and miR-34a was confirmed by RNAhybrid (https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid?id=rnahybrid_view_submission) and IntaRNA (https://www.rna-society.org/rnainter/IntaRNA.html). mRNA targets of miR-34a were identified using the miR-DB (http://mirdb.org/) database and TargetScan (http://www.targetscan.org/vert_72/) databases. Kaplan–Meier survival analysis of miR-34a was performed using data from the CGGA database.

2.5 Chromatin immunoprecipitation (ChIP)

Cross-linking

T98G cells (8 $X10^6$ cells) from 2-10 cm dishes were taken for control and TGF-β treatment conditions of the ChIP assay. Control or TGF-β (10 ng/ml) treated cells were fixed with 37% formaldehyde in 10ml culture media (final concentration of 1%-270µl for 10 ml culture media) for 8 min at room temperature with shaking. It was followed by quenching with 2.5M Glycine (final concentration - 0.125M-500 µl for 10 ml media) for 5 mins with shaking at room temperature. The growth media containing formaldehyde and glycine were emptied into chemical waste.

Sonication

Cells were washed twice with ice-cold PBS containing fresh PI, harvested by scraping, and pelleted in a 15 ml falcon by centrifugation at 1800 rpm for 5 min at 4^0C. The supernatant was aspirated, and the cell pellet was gently resuspended in 250 µl of ChIP lysis buffer containing fresh PI (50 mM Tris-HCl - pH 8.1, 0.9% SDS, 10 mM EDTA). Samples were incubated on ice for 1h. After 1h, samples were sonicated using bioruptor plus (Diagenode) with sonication conditions: 30 secs on, 30 secs off - for 20 cycles.

Chromatin estimation, pre-clearing of lysates, and immunoprecipitation

After sonication, samples were centrifuged at 14,000 rpm at 4^0C for 20 min. Supernatants were transferred carefully into pre-chilled 1.5 ml tubes. For chromatin

estimation, 15 µl of the sonicated chromatin was reverse cross-linked with 180 µl Decrosslinking buffer (DCL) (222mM NaCl, 50mM Tris, 10mM EDTA) and 5 µl proteinase K by incubating at 50^0C for 10 min, followed by 65^0C for 2.5 h. After reverse cross-linking, the chromatins were estimated using Nano-drop. The chromatin amounts were calculated using the following formula:

The value measured in nanodrop x dilution factor (13.3) x Volume of the sonicated sample (~ 230 µl).

Simultaneously, 40 µl of beads per IP was washed with ChIP dilution buffer (CDB) (167 mM NaCl, 16.7 mM Tris-HCl - pH 8.1, 1.2 mM EDTA, 1.1% Triton X100) containing fresh PI. Washed beads were used for pre-clearing lysates in rotation for 2h at 4^0C. From the estimated chromatins, 25 µg of each chromatin was taken for each IP condition and were diluted 5-fold in ChIP dilution buffer (167mM NaCl, 16.7mM Tris-HCl pH8.1, 1.2mM EDTA, 1.1% Triton X100, protease inhibitors). 5% of the sample was taken as input and stored at -80^0C until use. Samples were then incubated at 4^0C overnight with SMAD2/3 (1:200) (CST #8685S) or anti-rabbit IgG (CST #2729S) antibody.

Reverse cross-linking

IP samples were removed from the rotation the following day, and a short spin was performed at RT. Then, 50 µl of Dynabeads, protein G (Invitrogen #10004D) per IP, were added to the tubes and incubated for 2h at 4^0C. The beads were then washed once each with low salt buffer (0.1% SDS, 1% Triton x-100, 2mM EDTA, 20mM Tris HCl pH8.1, 150mM NaCl), high salt buffer (0.1% SDS, 1% Triton x-100, 2mM EDTA, 20mM Tris HCl pH8.1, 500mM NaCl), LiCl buffer (0.25M LiCl, 1% NP-40, 1% deoxycholate, 1mM EDTA, 10mM Tris-HCl pH 8.1), and TE buffer (10mMTris-HCl, 1mM EDTA pH 8.1). Washes were performed by incubating the samples containing respective buffers on a thermomixer at 4^0C for 5 min with shaking for each wash, and the supernatants were separated using a magnetic stand. Following this, samples and inputs were reverse cross-linked using decrosslinking buffer 2 (DCL2- 222mM NaCl, 50mM Tris, 10mM EDTA, 0.025% SDS), containing 5 µl proteinase K (NEB #P8107S – 800 U/ml) per sample with an overnight incubation at 65^0C.

Genomic DNA isolation and qRT-PCR analysis

After overnight incubation, genomic DNA was extracted with a DNA purification kit (Zymo kit cat.No: D3020), and lncRNA-MUF/LINC01711 in immunoprecipitated samples were measured using qRT-PCR. The following primers specific to the lncRNA-MUF promoter were used for qRT-PCR analysis:

Forward primer: 5' CTCAGTGCCTTCATGGTGGA 3'

reverse primer: 5' GAGGGGCTTACAGATGTGGC 3'.

The following primers specific to the LINC01711 promoter were used for qRT-PCR analysis: Forward primer: 5' GAGAAGGAGCTCCATGCCAA 3'

reverse primer: 5' CCAGAGGACCCCAGAAGAGA 3'.

2.6 Western blot analysis

Whole-cell lysates were isolated from T98G and U87-MG glioma cells with lysis buffer containing Triton X (1%), NaCl (150mM), Tris base (10mM), EDTA (1mM), EGTA (0.2 mM), IGEPAL (0.5%), PI (3µl/ml) and phosphatase inhibitors - $NaOVa_3$ (0.2M) and NaF (0.5M) 48h after transfection. Cell lysates were incubated on ice for 20-30 minutes, with intermittent vortexing, and centrifuged at 14000 rpm at 4^0C for 20 minutes. The supernatant was collected in fresh pre-chilled tubes, total protein was estimated using the BCA method, and extracts were frozen at −80°C until use. Western blotting was performed as described previously (28). Briefly, equal amounts of each sample protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a PVDF membrane, followed by blocking with 5% bovine serum albumin (A7906, Sigma) for 1.5 hr. After that, the membrane was incubated with respective antibodies overnight at 4°C. The following primary antibodies were used: p-SMAD2/3 (CST #8828) (1:2500), total SMAD (CST #8685) (1:2500), ZEB1 (CST #3396) (1:2000), Vimentin (CST #5741) (1:2000), N-cadherin (CST #13116) (1:2000) and Snail1 (CST #3895) (1:2000). Secondary antibodies – HRP conjugated anti-rabbit (Vector laboratories Cat.No: PI 2000-1) (1:20,000) or anti-mouse IgG (Invitrogen cat.No: A16072) (1:50,000) were incubated for two hours at room temperature. Immune blot bands were visualized with an ECL solution (Amersham ECL Prime Western Blotting Detection Reagent – GE healthcare (Cat.No: RPN2232)) and detected using VILBER Fusion Pulse ChemiDoc. Images were captured using Evolution capture software. The blots were stripped and reprobed with β-actin antibody (1:100,000; Sigma #A1978) to determine equivalent loading as described previously (274,275). For stripping, blots were incubated at 50^0C in stripping

buffer containing 10% SDS, 0.5M Tris-Cl, and 100mM β-mercaptoethanol for 30 mins, followed by PBST washes (5 times), blocking and incubation with primary antibody as described previously (275). The blot signals were quantified using ImageJ software for Microsoft Windows (National Institute of Health, Bethesda, MD).

2.7 Cell proliferation assay

1. Colorimetric cell proliferation assay was performed using WST-1 reagent (Cat#. 05015944001, Roche) at the indicated time according to the manufacturer's instructions.
2. Cells were seeded at a concentration of 2,500–5,000 cells/well in 96-well plates and transfected with siRNAs- si-MUF-1, si-MUF-2, and si-NS or ASO-NS, ASO-1/2 against LINC01711 at concentrations as indicated in the respective figure legends.
3. Cell proliferation was quantified at an OD of 450 nm.

2.8 Colony formation assay

1. Cells were seeded into 96 well dishes for clonogenic assays and treated with si-NS or siRNAs against lncRNA-MUF, ASO-NS, or ASO-1/2 against LINC01711.
2. 24h post-transfection, cells were trypsinized and seeded at a density of 200 cells per well in 6 well dishes and incubated at 37^0C.
3. After seeding, the media was changed every three days, and cells were observed periodically.
4. After 14 days of plating, the cells were washed with 1X PBS.
5. Cells were fixed by incubating with 4% PFA for 20min.
6. Then cells were washed with 1X PBS, and colonies were permeabilized by incubating with cold 100% methanol for 5min.
7. Methanol was removed, and cells were again washed with 1X PBS.
8. Colonies formed were stained with 0.5% crystal violet solution (Sigma, V5265) for 20min.
9. After 20min, the stain was removed, cells were washed with 1X PBS, and plates were air dried.
10. Images of the stained colonies were captured, and the number of colonies in each condition was calculated using ImageJ software.

2.9 Caspase 3/7 assay

A luminometric assay kit for caspase-3/7 (Promega – G8090) was used to determine the enzymatic activity of caspase-3/7. Glioma cells transfected with si-MUF-1 and 2 or ASO-NS, ASO-1/2 against LINC01711. 48h post-transfection proluciferin DEVD

substrate and caspase-Glo 3/7 buffer were added to the cells, and the assay was performed as per the manufacturer's instructions.

2.10 Invasion assay

1. Glioma cells were seeded in 24 well plates, and transfection conditions were carried out, as mentioned in the respective figure legends.
2. The following day, the transwell inserts (Corning; #3422) were coated with 100µl of 1µg/ml Matrigel (Corning – 356234), diluted in serum-free media in cold condition, and the inserts were incubated at 37^0C for 2h.
3. Meanwhile, spent media was removed from the transfected cells, and cells were washed with 1X PBS. Following PBS wash, cells were trypsinized and pelleted in 1.5 ml tubes.
4. The cell pellet was washed with 1X PBS to remove any residual FBS from the cells.
5. After PBS wash, the cell pellets were resuspended in 1ml serum-free media, and cells were seeded onto the upper chamber of transwell inserts precoated with Matrigel. The bottom chamber of the transwell inserts was supplemented with 20% FBS-containing media, which acts as a chemoattractant.
6. The inserts were incubated at 37^0C CO_2 incubator for 48h.
7. After 48h, Cells remaining on the upper surface of the insert were gently removed with a cotton swab.
8. Invaded cells on the lower surface of the inserts were washed twice with 1X PBS.
9. Following PBS wash, 4% PFA (diluted with PBS) was added to the inserts and incubated at room temperature for 5min.
10. PFA was removed from the cells, and the inserts were washed twice with 1X PBS.
11. After formaldehyde fixation, the cells on the lower surface of the inserts were permeabilized by adding 100% methanol and incubated for 20min.
12. Cells were then washed with 1X PBS, and 0.5% crystal violet solution was added and incubated for 15min at room temperature.
13. The stain was removed, cells were washed with 1X PBS, and the inserts were air-dried.
14. Stained cells were visualized under Magnus INVI microscopy (100x), invaded cells were counted at four different fields for each condition, and the percentage invasion was calculated using ImageJ software.

2.11 Migration Assays

1. For migration assay, cells were initially seeded in 6 well dishes for transfection.
2. Cells were transfected with negative control siRNA or si-MUF-1/si-MUF-2 or negative control ASO (ASO-NS), ASO-1/2 against LINC01711.

3. Transfected glioma cells were seeded in 12 well dishes and cultured overnight.
4. 24h post-seeding, a scratch was made using a 20µl pipette tip followed by a PBS wash.
5. Cells were maintained in 0.5% serum-containing media. Scratch images were taken periodically at 0, 24, and 48h, and the migrating length was calculated using ImageJ.

2.12 Transfection

2.12.1 siRNA and LNA transfection

Transfections of siRNAs and ASOs were performed using Lipofectamine® RNAiMAX Transfection Reagent (Life Technologies- Invitrogen, cat.No: 13778-075) and Opti-MEM (Invitrogen, 31985062) as per manufacturer's instructions. For siRNA transfection, glioma cells were transfected with 40 nM of siRNAs (Silencer Pre-designed siRNA, Ambion, Thermo Fisher Scientific) targeting lncRNA-MUF. The siRNA duplexes used in this study are as follows:

si-MUF-1 - sense 5' GCCUUCAACAUUCAGCACATT 3',

antisense 5' UGUGCUGAAUGUUCAAGGCTG 3';

si-MUF-2 - sense 5' CCUCCAUAUUCAUGAACUATT 3',

antisense 5' UAGUUCAUGAAUAUGGAGGCT 3'. Non-specific siRNA that does not target any known mammalian gene was purchased from Dharmacon – ON-TARGETplus non-targeting control pool (Cat.No: D-001810-10-20). For ASOs transfection, Glioma cells were transfected with 25 nM of ASOs (Qiagen), targeting LINC01711. The ASOs used in this study are ASO-1: 5' AGAAGAGAGAGGTCGA 3'; ASO-2: 5' TTAAACTGTGACAAAC 3'. A non-specific ASO that does not target any known mammalian gene was purchased from Qiagen (Cat.No: LG00000002-DDA).

2.12.2 miRNA mimics and inhibitors transfection

Transfection of miRNA mimics and inhibitors was performed using Lipofectamine® RNAiMAX Transfection Reagent (Life Technologies- Invitrogen, cat.No: 13778-075) and Opti-MEM (Invitrogen, 31985062) as per manufacturer's instructions (Table 2). To overexpress miR-34a, we transfected glioma cells with mimics of human miR-34a (80nM) (miRCURY LNA miRNA mimic – Qiagen – Cat.No: 339173 YM00473212-ADA) or with negative control mimics NC-mimics (Cat.No: 339173 YM00479902-ADA). To knockdown miR-34a, glioma cells were transfected with inhibitors of miR-34a (80nM) (miRCURY LNA miRNA inhibitor – Qiagen – Cat.No: 339121 YI04100982-ADA) or with negative control inhibitor (Cat.No: 339126 YI00199006-ADA).

Table 2

Cell number & conditions for transfection of LNA/siRNA/miRNA mimics/ inhibitors

Cell line	Cell number & RNAimax per well - 6 well dish	Cell number & RNAimax per well - 24 well dish	Cell number & RNAimax per well - 96 well dish
T98G	Cell number: 250,000 RNAimax: 6.5 µl Transfection volume: 1 ml	Cell number: 60,000 RNAimax: 1.5 µl Transfection volume: 250 µl	Cell number: 4000 RNAimax: 0.2 µl Transfection volume: 40 µl
U87-MG	Cell number: 250,000 RNAimax: 6.5 µl Transfection volume: 1 ml	Cell number: 60,000 RNAimax: 1.5 µl Transfection volume: 250 µl	Cell number: 4000 RNAimax: 0.2 µl Transfection volume: 40 µl
LN229	Cell number: 200,000 RNAimax: 6.5 µl Transfection volume: 1 ml	Cell number: 50,000 RNAimax: 1.5 µl Transfection volume: 250 µl	Cell number: 3500 RNAimax: 0.2 µl Transfection volume: 40 µl

2.12.3 Transfection of ZEB1/SNAIL overexpression vector and siRNAs/LNAs against lncRNA-MUF/LINC01711 for invasion rescue experiments

1. For invasion rescue experiments, cells were reverse transfected with respective amounts of LNAs/siRNAs using RNAimax transfection reagent in 24 well dishes and incubated overnight.
2. After overnight incubation, transfection media was switched with 10% growth media, and cells were incubated for 12-18h.
3. After 12-18h of incubation, cells were again transfected with ZEB1/SNAIL1 overexpression DNA constructs using jetOPTIMUS® reagent (Cat.No: 101000051) following the manufacturer's instructions.
4. 6h post-DNA transfection cells were switched with incomplete media for overnight serum starvation.
5. Invasion assays were performed as mentioned previously in section 2.10.

2.13 Cloning for luciferase reporter assay

The miR-34a binding sites containing regions of lncRNA-MUF and LINC01711 were cloned into the pmirGLO vector (Promega). The cDNA from U87-MG cells were amplified using the following primer sets containing the restriction sites for NheI and SalI enzymes (Table 3) using Phusion High-Fidelity DNA polymerase (Cat.No: M0530S). The amplified products were gel purified, ligated into the pmirGLO vector, and transformed into DH5α. Transformed colonies were picked and cultured in broth for midi-prep. Plasmids were isolated using the MN Nucleobond Xtra Midi kit for transfection-grade plasmid DNA (cat.No: 740410.50). The isolated plasmids were verified for the presence of the cloned fragment by PCR amplification.

Table 3

List of primers used for cloning

LncRNA-MUF-FP	GTAGCTAGCAGAAGTGTCTAAAGGA
LncRNA-MUF-RP	GATGTCGACTAAGCATATCAAAATTTGTGGT
LINC01711-FP	TATGCTAGCGTCTGGAGCCGTTTCTCTC
LINC01711-RP	TCTGTCGACCGTGTAACAGGCCACC

2.14 Dual-luciferase reporter assay

Dual-luciferase reporter assays confirmed the interaction between lncRNA-MUF/LINC01711 and miR-34a-5p. LncRNA-MUF/LINC01711 region containing the miR-34a-5p sites was cloned into the pmirGLO vector (Promega) using NheI and SalI restriction sites. HEK293T cells were co-transfected with pmirGLO - lncRNA-MUF reporter plasmid or pmirGLO-LINC01711 reporter plasmid and miR-34a-5p/NC mimics using Polyplus jetPRIME transfection reagent. 30h post-transfection, the cells were lysed and subjected to luciferase assays using the Dual-Luciferase® Reporter Assay System (Promega- Cat.No: E1910) according to the manufacturer's instructions on the Spectramax iD3 Luminometer (Molecular Devices Corporation). Data were normalized to Renilla luciferase activity.

2.15 Statistical analysis

Results are presented as mean ± SEM unless otherwise stated. We used paired Student's t-test for comparisons between two experimental groups. Additional statistical test information is described in the figure legends. $p < 0.05$ was considered statistically significant.

Chapter 3

Results and discussion

3.1. Identification and validation of TGF-β regulated lncRNAs using genome-wide microarray screen in T98G GBM cells

3.1.1 Validation of TGF-β treatment in T98G and U87-MG GBM cells

To validate the activation of TGF-β signaling upon treatment of GBM cells with recombinant TGF-β1, we performed western blotting of pSMAD2/3 and total SMAD2/3 upon TGF-β treatment (10 ng/ml for 30 min). Western blotting results depicted activation of the signaling pathway, which was evident from the phosphorylation of SMAD2/3 upon TGF-β treatment at the indicated time point in T98G and U87-MG cells (Fig 10 A & B). The TGF-β mediated phosphorylation of SMAD2/3 was abrogated upon pre-treatment with the inhibitor SB505124 (6 µM).

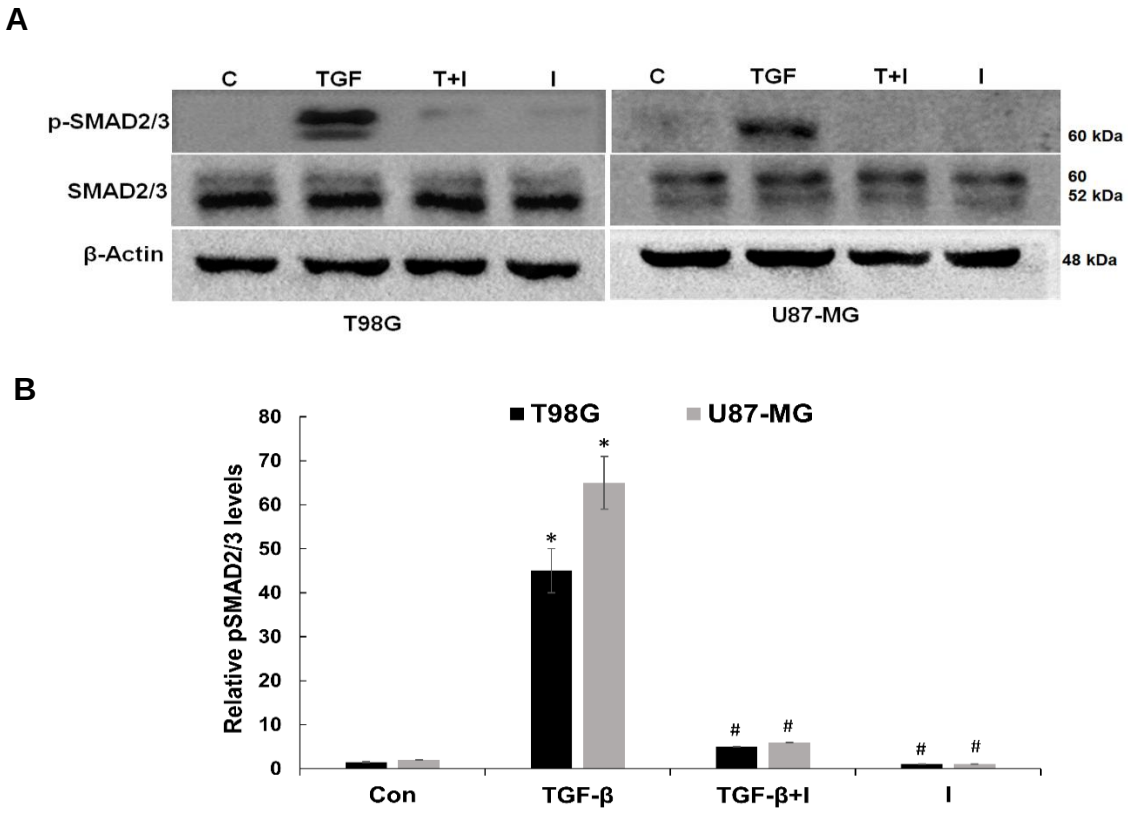


Fig 10: Validation of TGF-β treatment in T98G and U87-MG GBM cells. A. *T98G and U87-MG cells were treated with TGF-β with and without the inhibitor (SB505124). Cell lysates were collected, and total protein was isolated. Phospho-SMAD2/3 (pSMAD2/3) levels were evaluated using western blotting. The blots were reprobed for β-actin to establish equivalent loading.* ***B.*** *Quantification of pSMAD2/3 blots using ImageJ.*

3.1.2 Genome-wide microarray screen to identify TGF-β regulated lncRNAs in GBM

After validating the pSMAD2/3 levels with recombinant TGF-β1 treatment, we performed a genome-wide microarray screen to identify TGF-β-regulated lncRNAs. Microarray was performed for control, and TGF-β1 treated T98G glioma cells using the Agilent SurePrint G3 Gene Expression Microarrays for Human (v3) for lncRNAs. Cells were treated with 10 ng/ml of recombinant TGF-β1 in serum-free media, and lysates for RNA isolation and microarray analysis were collected 24 h post-treatment. 91 differentially expressed lncRNAs and 397 differentially expressed mRNAs were identified using a fold change of 1.5 and a p-value < 0.05 as a threshold (Fig 11 A & B, appendix 2 & 3). 18.3% of the differentially expressed transcripts constituted to lncRNAs (Fig 10 B).

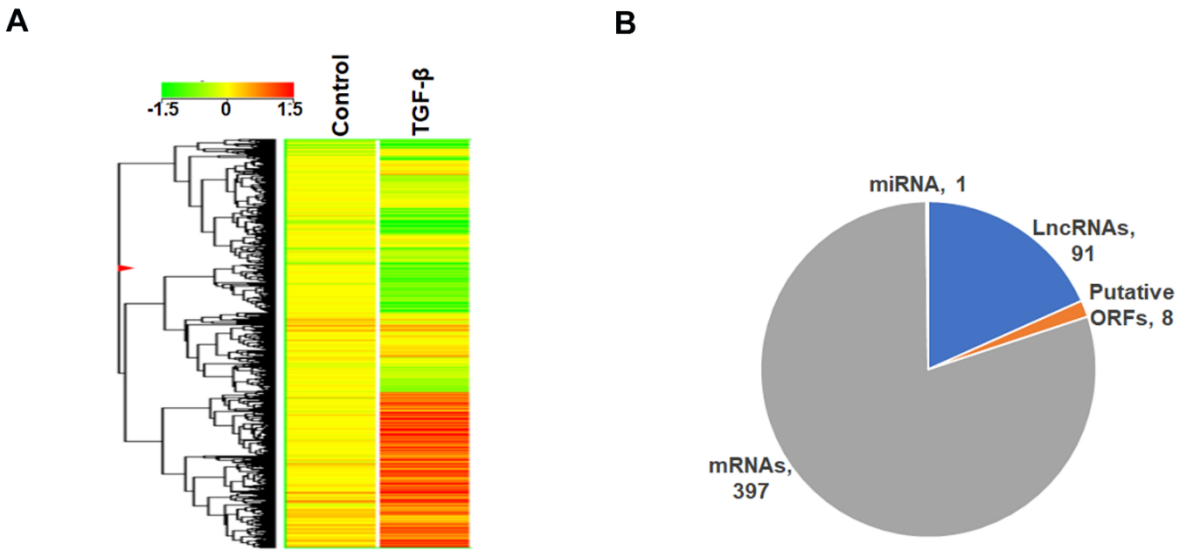


Fig 11: Genome-wide microarray screen to identify TGF-β regulated lncRNAs in GBM. A. *Heatmap representing the relative abundance of differentially expressed genes (DEGs, ≥1.5-fold, p < 0.05) in T98G cells upon TGF-β1 (10 ng/ml) treatment for 24 h.* ***B.*** *Pie chart representing the class of DEGs (p < 0.05) identified from genome-wide microarray screening in T98G GBM cells upon TGF-β1 treatment for 24 h.*

3.1.3. Verification of expression of DEGs identified from microarray screen using qRT-PCR assays

From the microarray screen, the expression of top differentially expressed lncRNAs and mRNAs in T98G cells were verified using qRT-PCR (Fig 12 A and B). The expression of several TGF-β regulated mRNA identified from the microarray screen was confirmed using qRT-PCR (Fig 12 A). Among lncRNAs, lncRNA ENST00000409910 and LOC79160 get ~ 4-fold up-regulated upon TGF-β treatment (Fig 12 B). LncRNA LINC00312, LOC101928710, lncRNA-MUF, and lnc-EGR2-1 get ~ 1.5-3-fold up-regulated upon TGF-β treatment (10 ng/ml for 24 h) (Fig 12 B). LncRNA CTB-178M22.2 and KCNMA1-AS1 are significantly down-regulated upon TGF-β treatment (Fig 12 B).

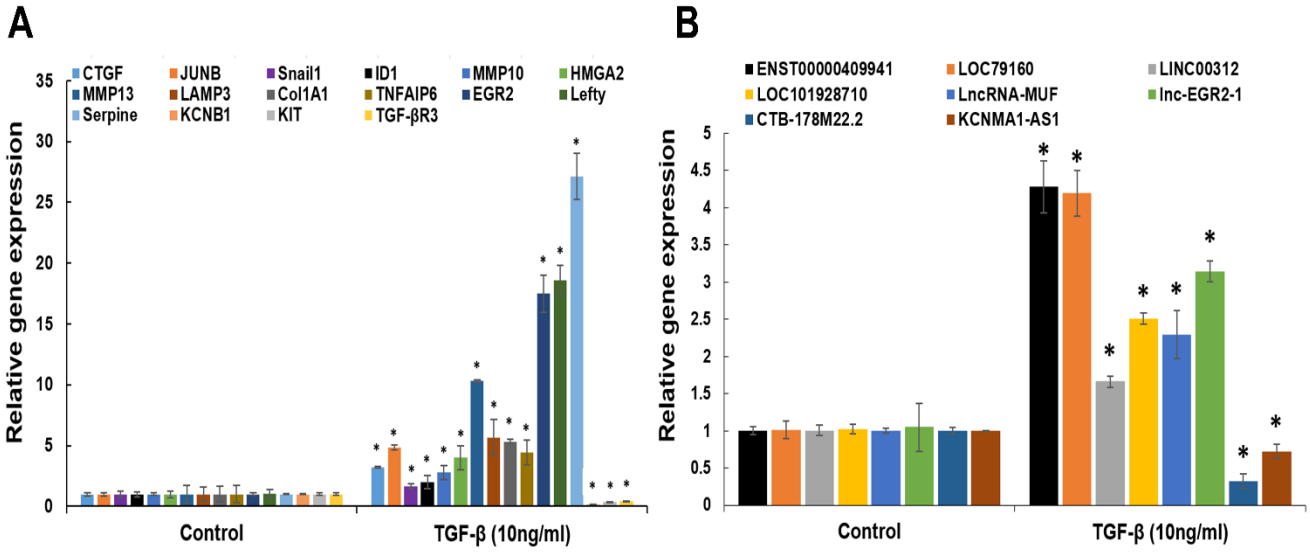


Fig 12: Verification of expression of DEGs identified from microarray screen using qRT-PCR assays. A. *Validation of top TGF-β-regulated mRNAs identified from microarray screening using qRT-PCR in T98G cells.* ***B.*** *Validation of top TGF-β-regulated lncRNAs identified from microarray screening using qRT-PCR in T98G cells. RNA samples were analyzed by qRT-PCR, and error bars represent the mean ± SEM from three independent experiments. *Significant change in TGF-β-treated cells compared to control cells ($p < 0.05$).*

3.1.4 Analysis of the role of TGF-β-induced lncRNAs in the survival of GBM patients

To identify clinically relevant TGF-β-induced lncRNAs in GBM patients' survival, we performed the survival analysis of GBM patients with high and low expression of the candidate lncRNAs using the TCGA dataset from the GEPIA online database (273). From the GEPIA database, we got the data for only lncRNA-MUF (ENSEMBL ID: ENSG00000235884) and LINC01711 (ENSEMBL ID: ENSG00000268941), while the other candidate lncRNAs were not annotated. We found that the increased expression of lncRNA-MUF and LINC01711 confer poor overall survival in GBM patients (Fig 13 A & B). Hence among the top up-regulated lncRNAs (Fig 13 B), we further set out to characterize the role of lncRNA-MUF and LINC01711 (LOC79160) in GBM pathogenesis.

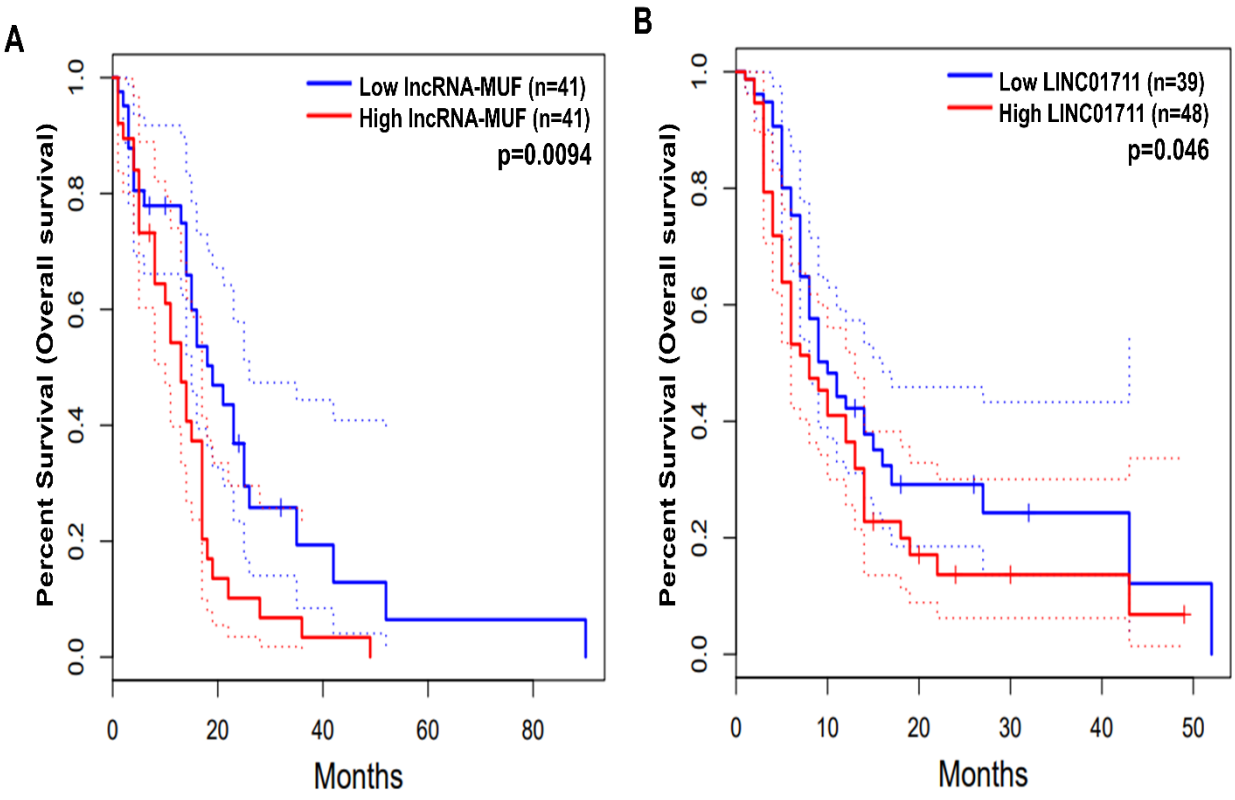


Fig 13: Kaplan–Meier survival analysis of TGF-β-induced lncRNAs in GBM patients using GEPIA database. A. *Overall survival curve of lncRNA-MUF.* ***B.*** *Overall survival curve of LINC01711.*

3.1.5 Discussion

We identified several novel differentially expressed lncRNAs upon TGF-β treatment in T98G cells (Fig 11 A & B). During the course of this work, two other groups performed genome-wide screening to identify TGF-β-regulated lncRNAs in GBM (233,236). Nie et al. reported eight significantly induced lncRNAs upon TGF-β treatment using a microarray screen in D54 and P-GBM2 glioma cells (236). Among these lncRNAs, H19 and HOXD-AS2 confer TMZ resistance (236). Tang et al. screened for TGF-β-regulated lncRNAs using RNAseq in GSCs (233). They show that LINC00115 promotes GSC's self-renewal by acting as a ceRNA for transcription factors ZEB1 and ZNF596 by sponging miR-200 (233). We observed no significant overlap of DE lncRNA targets identified from our screen in these studies (Appendix 2). This is likely due to i) the use of different cell lines for the screening, (ii) the use of different technology platforms for transcriptome analysis (Microarray/ RNA sequencing), iii) the different dosages and duration of TGF-β treatment. Among the top 6 upregulated lncRNAs from our screen, we further choose to characterize the role of two lncRNA-MUF and LINC01711in GBM pathogenesis.

3.2. Characterization of the role of TGF-β-induced LINC00941/lncRNA-MUF in GBM pathogenesis

LINC00941, also known as lncRNA - mesenchymal stem cell up-regulated factor (lncRNA-MUF), is an oncogene in several cancers. In this section characterize the role of lncRNA-MUF in various other aspects of GBM pathogenesis. We demonstrate that lncRNA-MUF acts as a ceRNA sponge for miR-34a and promotes GBM invasion by stabilizing SNAIL and ZEB1.

LncRNA-MUF

LncRNA-MUF is an oncogenic lncRNA. It was first discovered by Yan et al. as a mesenchymal stem cell upregulated factor (lncRNA-MUF) in hepatocellular carcinoma (276). It promotes HCC by binding to ANXA2 protein to activate WNT/β-catenin signaling mediated EMT (276). LncRNA-MUF is also upregulated in several other cancers, such as gastric cancer, oral squamous cell carcinoma (OSCC), papillary thyroid carcinoma, colorectal cancer (CRC), lung cancer, colon cancer, and pancreatic cancer (277–284). However, the function and mechanism of action of TGF-β induced lncRNA-MUF in GBM remain unknown.

miR-34a and its role in cancers, including GBM

miR-34a is a tumor suppressor miRNA that targets multiple oncogenes (285). Using expression profiling of the NCI-60 panel of human tumor-derived cell lines, Gaur et al. identified that miR-34a is downregulated in breast, lung, and epithelial/nasopharyngeal cancers (286). Several other studies established the tumor suppressor role of miR-34a in GBM (287,288). Expression levels of miR-34a are significantly lower in brain tumor tissues compared to normal brain tissues (289). miR-34a locus is commonly deleted in GBMs (287). Li et al., using luciferase assays, identified that miR-34a binds to and degrades the protein levels of c-Met, Notch-1, Notch-2, and CDK6 in GBM cells and glioma stem cells (GSCs) (289). Yin et al. reported that miR-34a also targets and reduces the expression of EGFR in GBM (287). The study also reported that deleting miR-34a and EGFR amplification leads to poor GBM prognosis (287). The profound antiproliferative activity of miR-34a is evident with the downregulation of Cyclin-A1, -D1, -D3, CDK2, E2F-1, -4, -3, and increased expression of the p21 and p27 CDKIs upon forced expression of miR-34a (287). Vaitkiene et al. reported that lower miR-34a levels in GBM patients are significantly associated with higher tumor volume and poor patient survival (290). miR-34a targets multiple genes that promote cancer cell proliferation, invasion, migration, drug resistance, stemness, immune response,

apoptosis, and EMT (285). Some critical target genes of miR-34a include SNAIL, Notch1, ZEB1, ZNF281, IL-6R, SIRT1, MDM4, EGFR, PD-L1, CDK6, c-Met, MYCN, BCL2, CyclinE2, CyclinD1, PDGFRA, Msi1, Akt, and Wnt (285). Guessous et al. reported that miR-34a overexpression inhibits glioma cell proliferation, cell survival, migration, and invasion (291). It also inhibits glioma stem cell malignancy, induces stem cell differentiation, and inhibits *in vivo* GBM xenograft growth (291).

3.2.1 TGF-β-induced lncRNA-MUF is upregulated in GBM tumor samples

We used the GEPIA database to identify the expression levels of lncRNA-MUF in GBM and control samples. We found that the levels of lncRNA-MUF were significantly higher (~ 1.5 fold) in GBM tumor samples (n=163) as compared to control samples (n=207) (Fig 14 A). Followed by this, survival analysis performed using the CGGA database revealed that increased expression of lncRNA-MUF correlated with poor overall survival in GBM patients (p=0.0063) (Fig 14 B). Using the mRNAseq_693 dataset of the CGGA database, we found that levels of lncRNA-MUF were significantly higher in the higher grade of glioma than in lower-grade gliomas (p=0.000000000000000016) (Fig 14 C). GBM patients with IDH mutation show a better survival rate than the IDH wildtype group (23). Hence, we evaluated lncRNA-MUF expression in IDH mutant and wild-type glioma samples. We observed that lncRNA-MUF expression is significantly higher in gliomas with the IDH wildtype group than in the IDH mutant group (p=0.00000000000000000000000000097) (Fig 14 D).

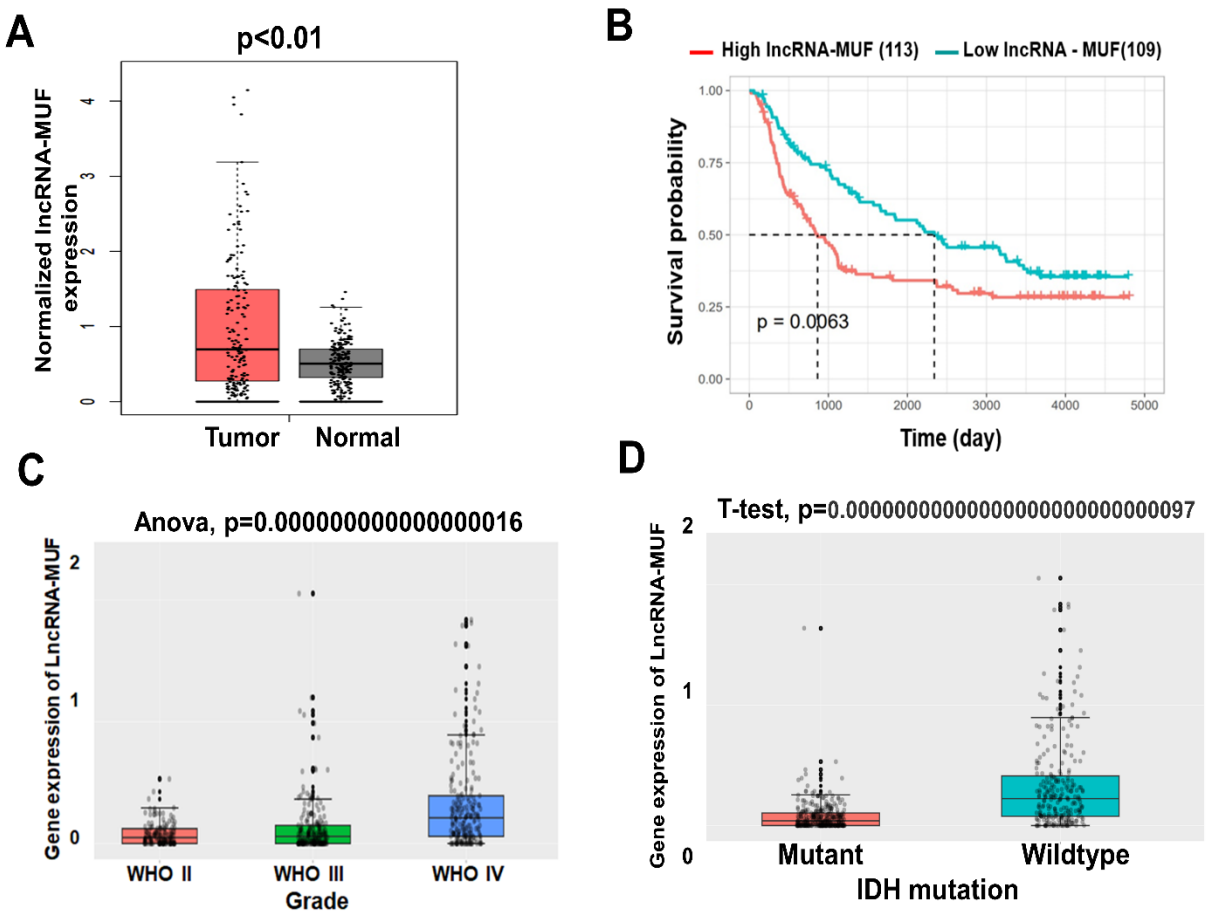


Fig 14: Analysis of expression of lncRNA-MUF in GBM samples. A. *LncRNA-MUF levels were significantly higher in GBM samples than in normal brain tissues (p<0.01), as analyzed from TCGA data using the GEPIA portal.* ***B.*** *Kaplan-Meier survival analysis of lncRNA-MUF from CGGA databases shows that high lncRNA-MUF expression is associated with poor overall survival in GBM patients (p=0.0063). The Red line represents the high lncRNA-MUF expression group, and the green line represents the low lncRNA-MUF expression group.* ***C.*** *LncRNA-MUF expression analysis in glioma tissues from the CGGA database. Elevated expression of MUF in GBM IV compared to lower-grade gliomas (p=0.000000000000000016).* ***D.*** *LncRNA-MUF levels are elevated in IDH wild-type GBM patients compared to IDH mutant cases as analyzed from the CGGA database (T-test, p = 0.0000000000000000000000000097).*

3.2.2 TGF-β-induced lncRNA-MUF is activated through the canonical SMAD2/3 signaling pathway in glioma cell lines

Given the role of lncRNA-MUF in GBM patients' survival, we further evaluated the mechanism of induction of lncRNA-MUF upon TGF-β1 treatment in GBM cells. Firstly, we identified that lncRNA-MUF induction upon TGF-β1 treatment was concentration-independent for TGF-β concentrations from 5 ng/ml- 80 ng/ml for 24 h in T98G cells (Fig 15 A). The time-course analysis (at 10 ng/ml TGF-β1 treatment) identified that lncRNA-MUF gets induced upon TGF-β1 treatment as early as 1 hr. However, a statistically significant increase of ~2-fold occurs only at 12h and 18h of TGF-β treatment and is then sustained at ~1.8-fold at 24h, 36h, and 48h (Fig 15 B).

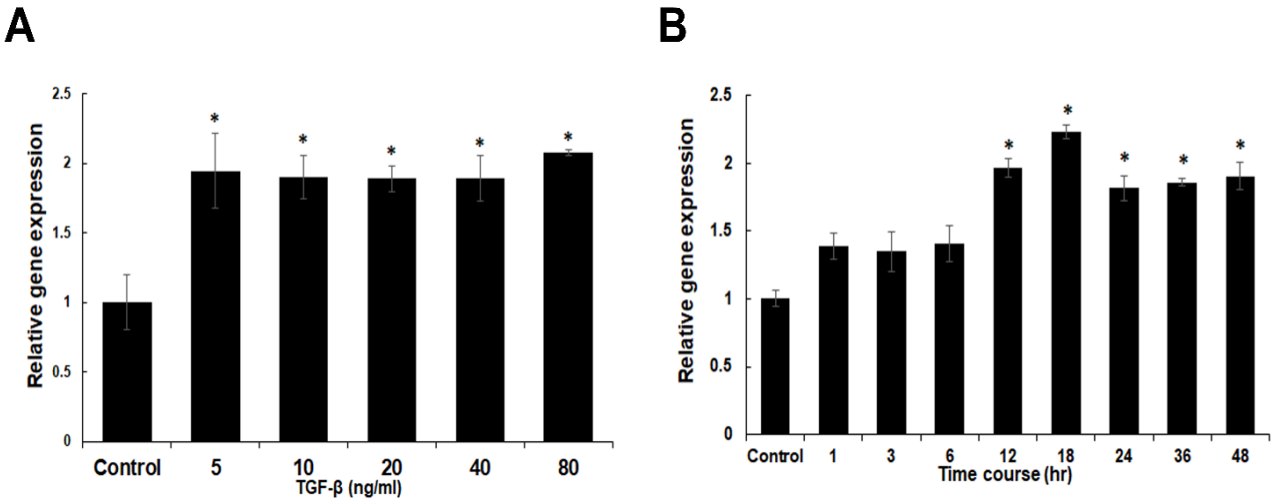


Fig 15: Evaluation of expression of lncRNA-MUF at various concentrations and time points of TGF-β treatment. A. *qRT-PCR of lncRNA-MUF with different concentrations of TGF-β1 treatment in T98G GBM cells. LncRNA-MUF induction upon TGF-β1 treatment is not concentration-dependent. Cells were treated with the indicated concentration of TGF-β1 for 24 h and analyzed for lncRNA-MUF expression by qRT-PCR.* ***B.*** *LncRNA-MUF is a delayed transcript with induction of ~2-fold at 12 h, 18 h of TGF-β1 treatment (10 ng/ml), and then reaching a plateau of ~1.8-fold at 24h, 36h, and 48h. RNA levels were measured at the indicated time points using qRT-PCR, normalized with TBP. Error bars represent the mean ± SEM from 3 independent experiments. *Significant change compared to respective control samples ($p<0.05$). Statistical comparisons were made using Student's t-test.*

To assess the impact of TGF-β1 on the lncRNA-MUF expression on additional glioblastoma cell lines, we evaluated lncRNA-MUF expression in LN18, LN229, and U87-MG glioma cells. Upon TGF-β1 stimulation for 24 hr, the expression of lncRNA-MUF was up-regulated (≥ 2-fold) in glioma cell lines (T98G: 1.89-fold; U87-MG: 1.8-fold; LN229: 2.8-fold; LN18: 2.1-fold). These results indicate that lncRNA-MUF induction upon TGF-β1 treatment is not cell line-specific (Fig 16 A). We then investigated the subcellular localization of lncRNA-MUF by measuring the lncRNA levels in nuclear and cytoplasmic fractions in T98G and U87-MG GBM cells. LncRNA-MUF displayed 75% expression in the cytoplasm and 25% in the nucleus in T98G and U87-MG cell lines, respectively (Fig 16 B). TGF-β signal transduction occurs through canonical SMAD2/3 signaling or non-canonical pathways (86). To identify the transcription factors working downstream of the TGF-β pathway to regulate lncRNA-MUF expression, we looked at the lncRNA-MUF promoter using JASPAR (http://jaspar.genereg.net/) and found functional SMAD binding elements (SBE) (5' CAGAC 3' / 5' GTCTG 3') at -498, -1321, -1850, -2413, and -2942 positions. Hence, we evaluated lncRNA-MUF expression upon TGF-β treatment in the presence and absence of TGF-β inhibitor SB505124. To this end, GBM cells were treated with 6μm SB505124 (TGFβR1/smad2/3 inhibitor) for 2 h before treatment with TGF-β1 (24 h). Blocking smad2/3 with SB505124 significantly abrogated TGF-β-induced lncRNA-MUF expression in glioma cells (~ 50% reduction in T98G, LN229, and U87-MG) (Fig 16 C). Next, we performed ChIP-qPCR to determine whether TGF-β promotes increased binding of smad2/3 to SBE on the lncRNA-MUF promoter. ChIP-qPCR revealed high binding of smad2/3 on SBE on lncRNA-MUF promoter upon TGF-β stimulation (Fig 16 D). These results suggest that TGF-β up-regulates lncRNA-MUF expression through the canonical SMAD signaling pathway.

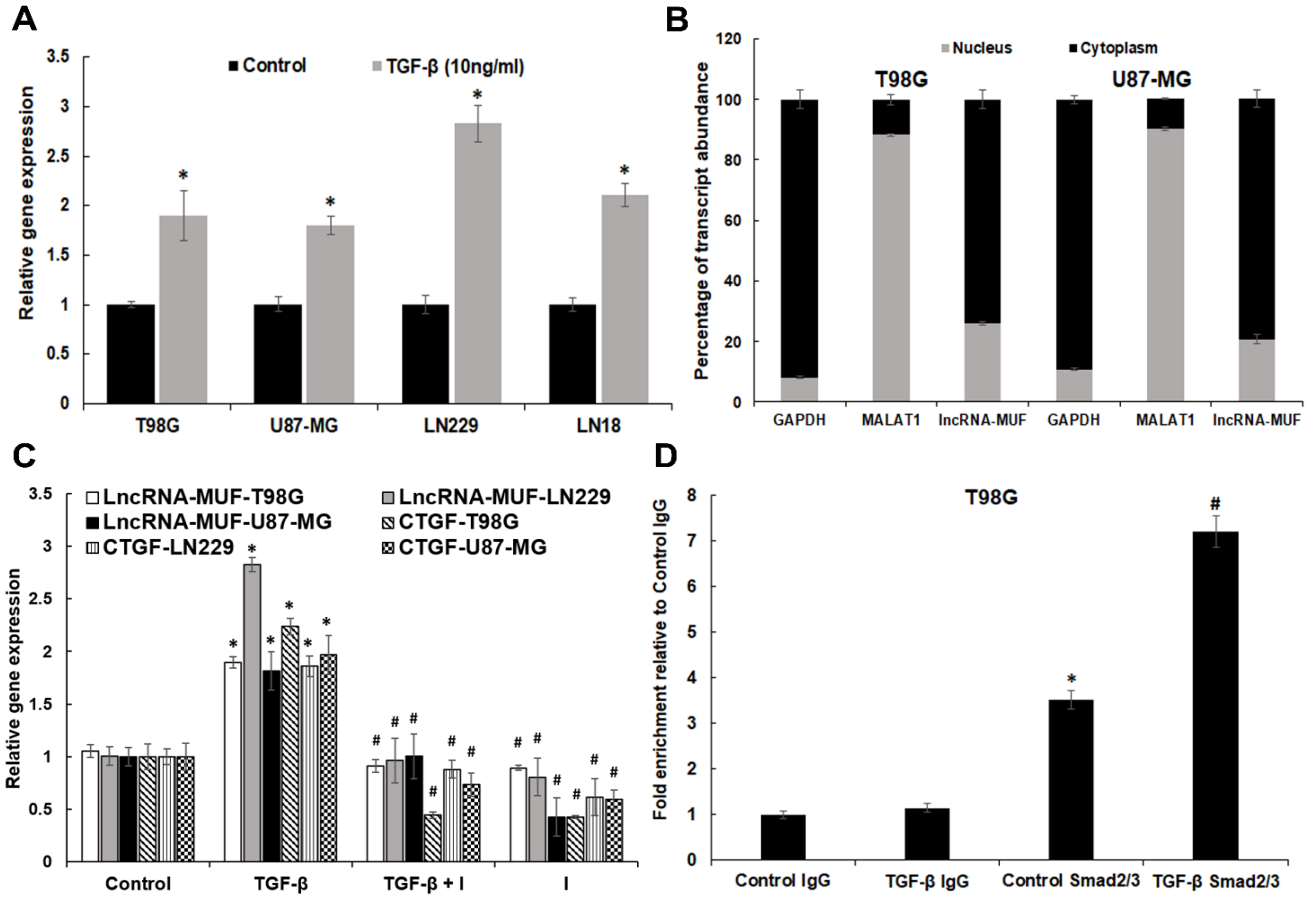


Fig 16: Identification of the mechanism of induction of lncRNA-MUF upon TGF-β treatment. ***A.*** *LncRNA-MUF induction upon TGF-β treatment is not cell type-specific. The indicated GBM cell lines were treated with 10 ng/ml TGF-β for 24 h, and lncRNA-MUF levels were measured by qRT–PCR.* ***B.*** *Fractionation of GBM cells followed by qPCR to determine the localization of lncRNA–MUF. LncRNA-MUF is localized primarily in the cytoplasm. GAPDH served as the control for cytoplasmic fraction; MALAT1 was used as a control for nuclear fraction.* ***C.*** *LncRNA-MUF induction upon TGF-β treatment is smad2/3 dependent. Human glioma cells (T98G, LN229, and U87-MG) were pre-treated with 6 μM of SB505124 (TGFβR1/Smad2/3 inhibitor) for 2 h followed by co-treatment with TGF-β1 (10 ng/ml) for 24 h, and lncRNA-MUF transcript levels were determined by qRT–PCR. CTGF is a positive control which is a known Smad2/3 target gene.* ***D.*** *ChIP-qPCR analysis of smad2/3 interaction with SBE in lncRNA-MUF promoter in control and TGF-β treated T98G cells. DNA was isolated from control and TGF-β-treated cells after immunoprecipitation with an anti-smad2/3 antibody and was amplified using specific primer sets. LncRNA-MUF promoter levels in immunoprecipitated samples were measured by qRT–PCR analysis, normalized to input, and represented as "fold enrichment relative to control IgG IP." Values represent mean ± SD from three independent experiments. *Significant change compared to IgG ($p < 0.05$) #Significant change compared to control Smad2/3 ($p < 0.05$).*

3.2.3 Knockdown of lncRNA-MUF reduces cell proliferation, induces apoptosis, and reduces GBM cells invasion and migration

To investigate the physiological function of lncRNA-MUF in glioma pathogenesis, we established lncRNA-MUF knockdown by siRNA using two different siRNAs (si-MUF-1 and si-MUF-2) in T98G and U87-MG cell lines. The knockdown of lncRNA-MUF with siMUF-1 results in ~ 85% reduction, and si-MUF-2 results in ~ 67% reduction of lncRNA-MUF levels in T98G and U87-MG cells identified using qRT-PCR (Fig 17).

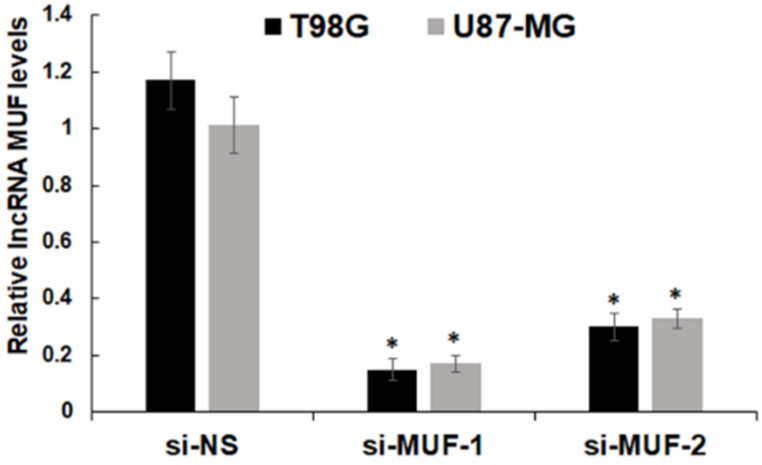


Fig 17: Validation of lncRNA-MUF expression upon knockdown using siRNAs. *siRNA mediated knock-down of lncRNA MUF in T98G and U87-MG cells. LncRNA MUF expression was efficiently knocked down by siRNAs (~ 85% by si-MUF1 and ~ 67 % by si-MUF2) in U87-MG and T98G cells as detected by qRT-PCR assays. Values represent mean ± SEM from three independent experiments. *Significant change compared to cells transfected with control siRNA (si-NS) ($p < 0.05$).*

LncRNA-MUF depletion using siRNAs results in a time-dependent decrease in cell proliferation in glioma cells. Cell proliferation was reduced by ~ 40% and ~ 55% at 48h and 72 h post-lncRNA-MUF knockdown, respectively, in T98G cells (Fig 18 A). A similar ~ 40-50% reduction in cell proliferation was observed in LN229 and U87-MG glioma cells transfected with siRNA against lncRNA MUF compared to cells transfected with non-specific siRNA (si-NS) (Fig 18 B & C). Consistent with the reduction in cell proliferation upon lncRNA-MUF depletion, lncRNA-MUF knockdown resulted in a significant decrease in colony formation of ~ 62% and 70%, respectively, in T98G and U87MG cells compared to respective control cells transfected with si-NS (Fig 18 D & E). Moreover, depletion of lncRNA-MUF by siRNA also results in apoptosis as demonstrated by ~1.75-fold, 3.6-fold, a 3.4-fold increase of caspase 3/7 activity in T98G, U87-MG, and LN229, respectively, as compared to control cells (Fig 18 F). Consistently, the levels of caspase 9 mRNA were ~2-fold increased following lncRNA-MUF knock-down in T98G and U87-MG cells (Fig 18 G).

We then investigated the effect of lncRNA-MUF knockdown on glioma cell migration and invasion. The wound healing assay revealed that lncRNA-MUF-depleted T98G and U87-MG cells show ~ 63% and ~ 58% reduction in cell migration compared to control cells. (Fig 19 A & C). Matrigel invasion assay during lncRNA-MUF depletion results in ~ 55 % and ~ 70% inhibition of cell invasion in T98G and U87-MG cells, respectively, compared to control cells (Fig 19 B & D). Thus, these results collectively suggest that lncRNA-MUF is an oncogene promoting proliferation, migration, and invasion in GBM cells. Targeting lncRNA-MUF is an attractive therapeutic strategy for GBM.

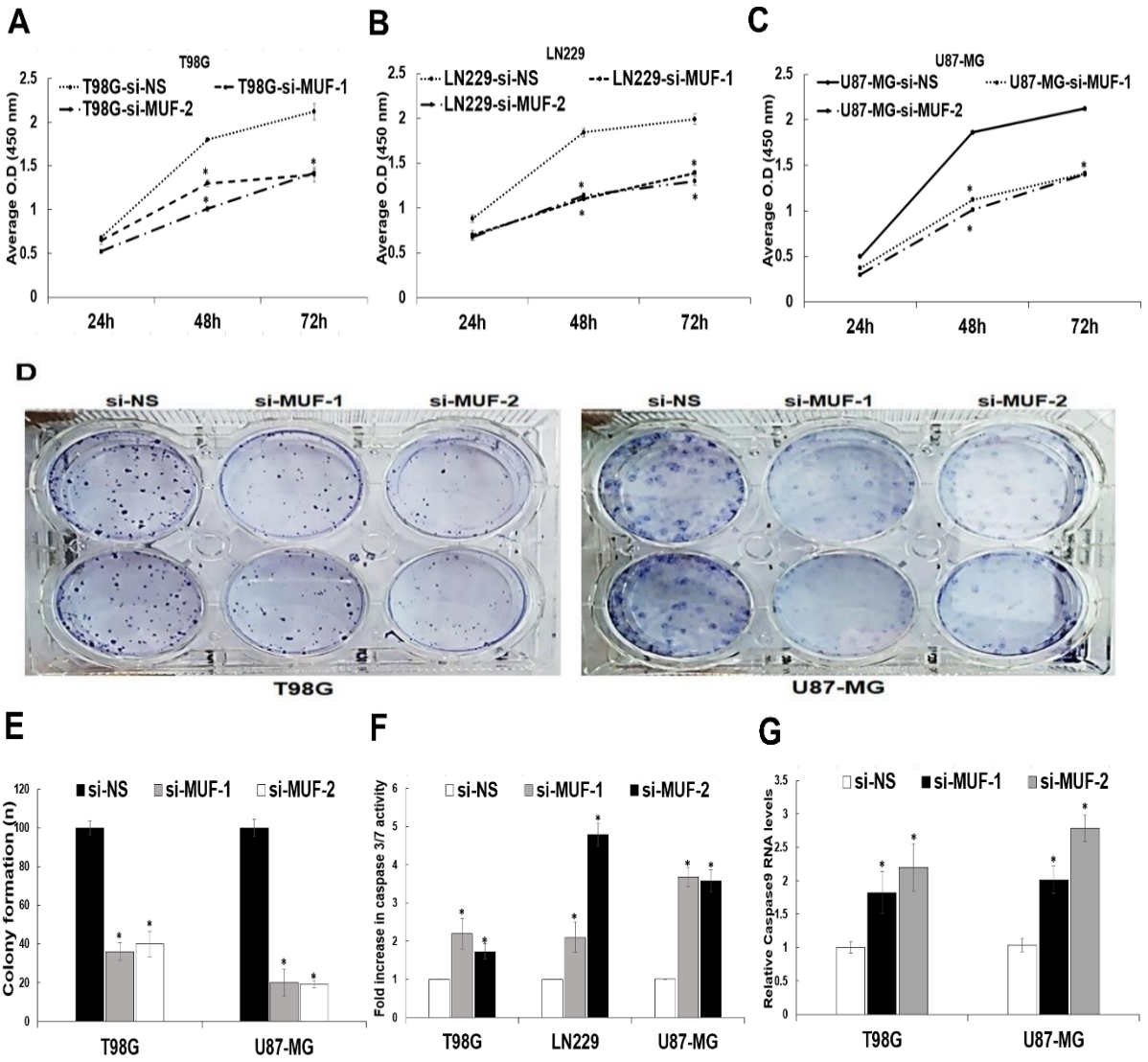


Fig 18: Physiological assays upon lncRNA-MUF knockdown. A. *T98G cells were transfected with si-NS or si-MUF-1/ si-MUF-2 (40nM), and percentage cell viability was calculated at indicated times.* ***B.*** *LN229 cells were transfected with si-NS or si-MUF-1/ si-MUF-2 (40nM), and percentage cell viability was calculated at indicated times.* ***C.*** *U87-MG cells were transfected with si-NS or si-MUF-1/ si-MUF-2 (40nM), and percentage cell viability was calculated at indicated times. Percentage cell viability was calculated at indicated times using WST1.* ***D.*** *Reduced colony formation ability of GBM cells with lncRNA-MUF knock-down.* ***E.*** *Quantification of colony formation assay using ImageJ.* ***F.*** *Caspase 3/7 activity assay shows lncRNA-MUF knock-down induces apoptosis in GBM cells.* ***G.*** *Knock-down of lncRNA-MUF shows increased caspase 9 mRNA levels in T98G and U87-MG glioma cells measured by qRT-PCR assay. Values represent mean ± SD from four independent experiments. *Significant change compared to si-NS cells at the corresponding time ($p < 0.05$). Statistical comparisons were made using Student's t-test.*

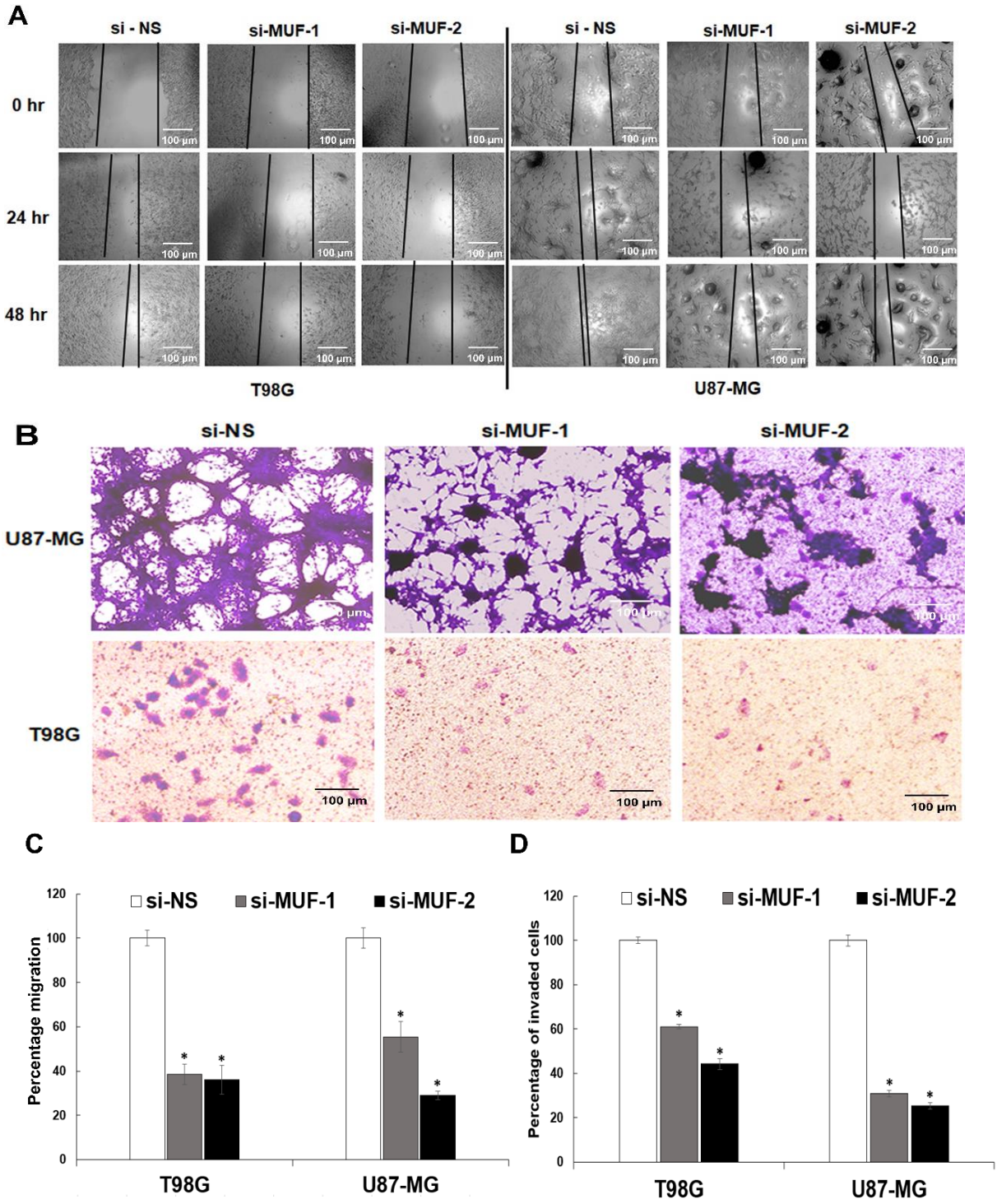


Fig 19: Migration and invasion assays upon lncRNA-MUF knockdown. A. *Wound healing assay demonstrates reduced GBM cell migration upon lncRNA-MUF knockdown.* ***B.*** *Matrigel invasion assay shows lncRNA MUF inhibition reduces glioma cell invasion.* ***C.*** *Quantification of wound healing assay using ImageJ.* ***D.*** *Quantification of invaded cells using ImageJ.*

3.2.4. LncRNA-MUF regulates gene expression of a subset of TGF-β target genes in *cis* and *trans*

LncRNA transcripts often regulate gene expression in *cis* and *trans* (119). We first evaluated the effect of lncRNA-MUF knockdown on its *cis* genes (Fig 20 A & B). We observed ~50% downregulation of the Caprin2 gene in T98G and U87-MG upon lncRNA-MUF knockdown with both the siRNAs (Fig 20 B). This is consistent with Ai et al., who reported the *cis*-regulation of the Caprin2 gene by lncRNA-MUF through chromosome looping in OSCC (277). We also observed that the Caprin2 gene is up-regulated by TGF-β in T98G GBM cells (1.7-fold) using qPCR assays (Fig 20 C). These results suggest that lncRNA-MUF regulates TGF-β induced expression of the Caprin2 gene in *cis* in glioma cells. However, the levels of other *cis* genes (IPO8, LOC645485, LOC107984476) remained unchanged upon lncRNA-MUF knockdown.

To further identify the genes regulated in *trans* by lncRNA-MUF in glioma cells, we evaluated the expression of the TGF-β gene ontology group upon its siRNA-mediated knockdown. Depleting lncRNA-MUF resulted in ~50% down-regulation of SNAIL1, ~40% down-regulation of vimentin, ~60% downregulation of CTGF, and ~30% downregulation of c-Myc in T98G cells and U87-MG cells (Fig 21 A). Several other TGF-β regulated genes did not show any change in expression with lncRNA-MUF knockdown (Fig 21 B).

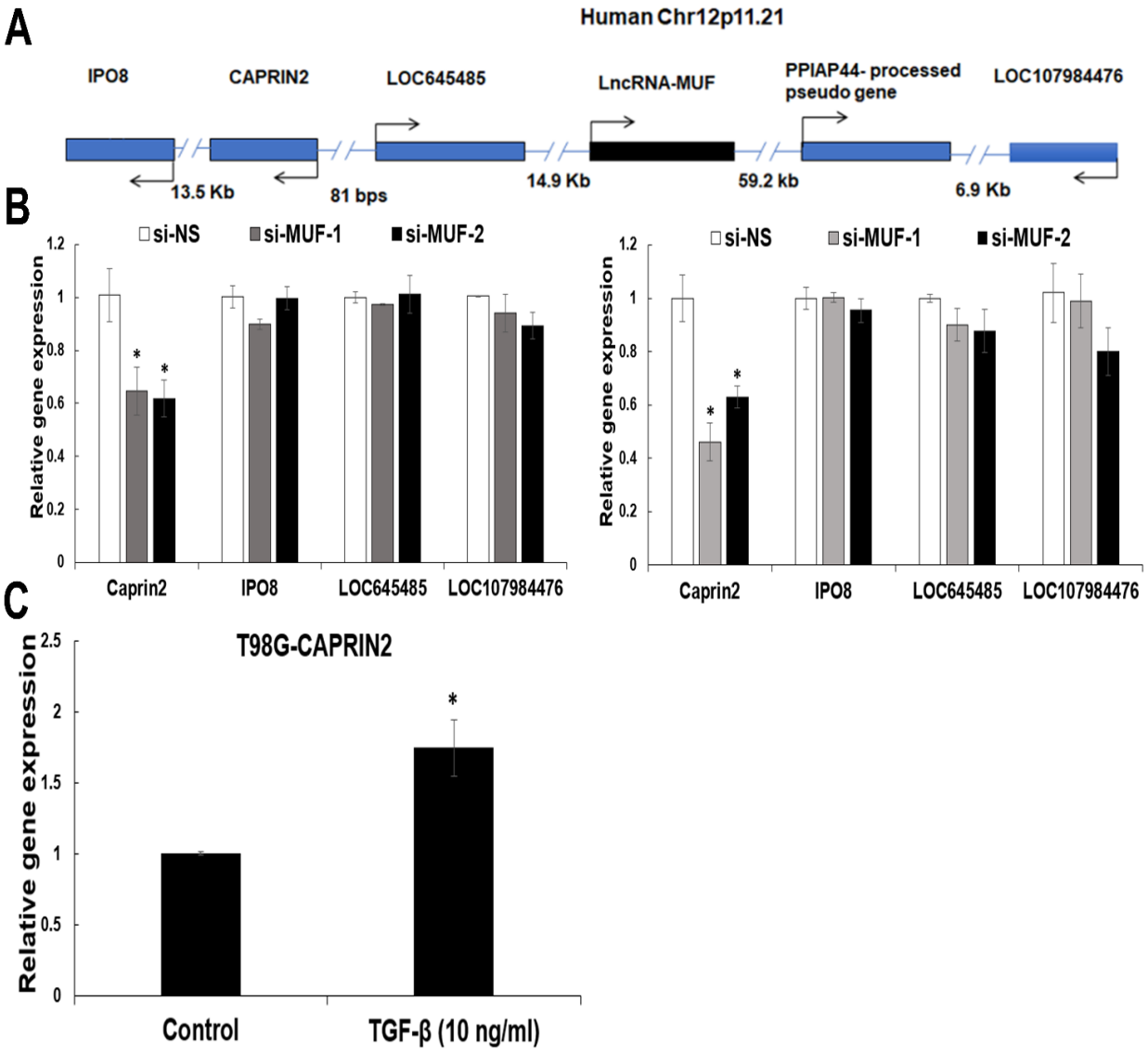


Fig 20: Validation of cis genes expression upon lncRNA-MUF knockdown. A. *Cis genes of lncRNA-MUF were identified using NCBI and ENSEMBL databases.* ***B.*** *Validation of cis gene expression of lncRNA-MUF with lncRNA knockdown. LncRNA knockdown represses cis gene expression (Caprin2).* ***C.*** *Relative mRNA levels of Caprin2 gene with TGF-β1 treatment in T98G cells identified by qRT-PCR assay.*

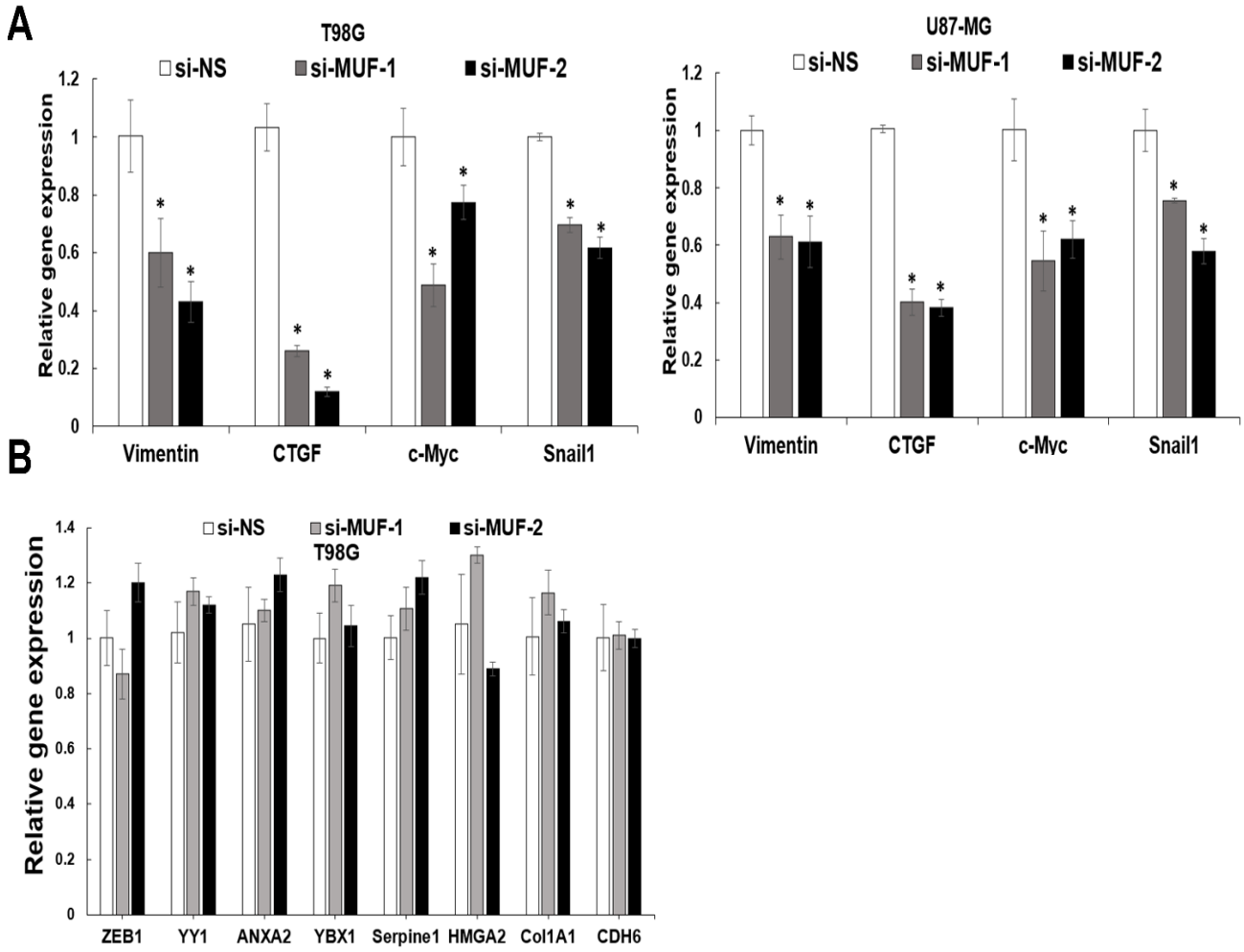


Fig 21: Validation of trans genes expression upon lncRNA-MUF knockdown. A. *LncRNA-MUF modulates TGF-β target gene expression in trans in GBM. T98G and U87-MG glioma cells transfected with si-NS, si-MUF-1, or si-MUF-2 (25 nM), and transcript levels of indicated genes were measured 48 h post-transfection. RNA samples were analyzed by qRT-PCR, and error bars represent the mean ± SEM from three independent experiments.**B.*** *Gene expression of other TGF-β target genes upon lncRNA-MUF knockdown measured by qRT-PCR assays.*

Since lncRNA-MUF regulates genes involved in the WNT/β-catenin pathway(276,277), we evaluated the impact of lncRNA-MUF knockdown on WNT/β-catenin pathway genes in glioma cells. Surprisingly we did not observe any significant change in their expression upon lncRNA-MUF depletion in glioma cells (Fig 22 A & B).

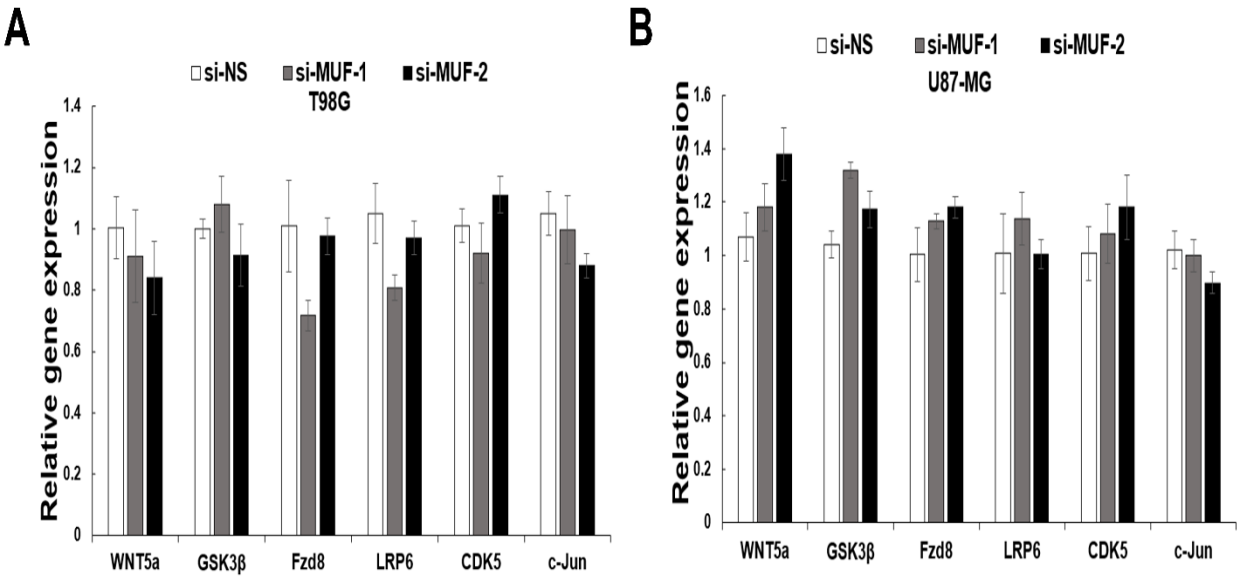


Fig 22: Validation of WNT- β catenin pathway genes expression upon lncRNA-MUF knockdown. A. *Gene expression of Wnt/ β-catenin target genes upon lncRNA-MUF knockdown measured by qRT-PCR assays in T98G cells.* ***B.*** *Gene expression of Wnt/ β-catenin target genes upon lncRNA-MUF knockdown measured by qRT-PCR assays in U87-MG cells.*

3.2.5 LncRNA-MUF regulates expression of mesenchymal markers in GBM cells

We evaluated the EMT marker expression upon lncRNA-MUF inhibition by western blotting. Depletion of lncRNA-MUF resulted in a ~ 40% decrease in ZEB1, N-cadherin, ~ 80% decrease in vimentin, and a ~ 70% decrease in Snail1 protein levels in T98G and U87-MG cells (Fig 23 A & B). These results indicate that lncRNA-MUF promotes the expression of critical mesenchymal markers in GBM cells.

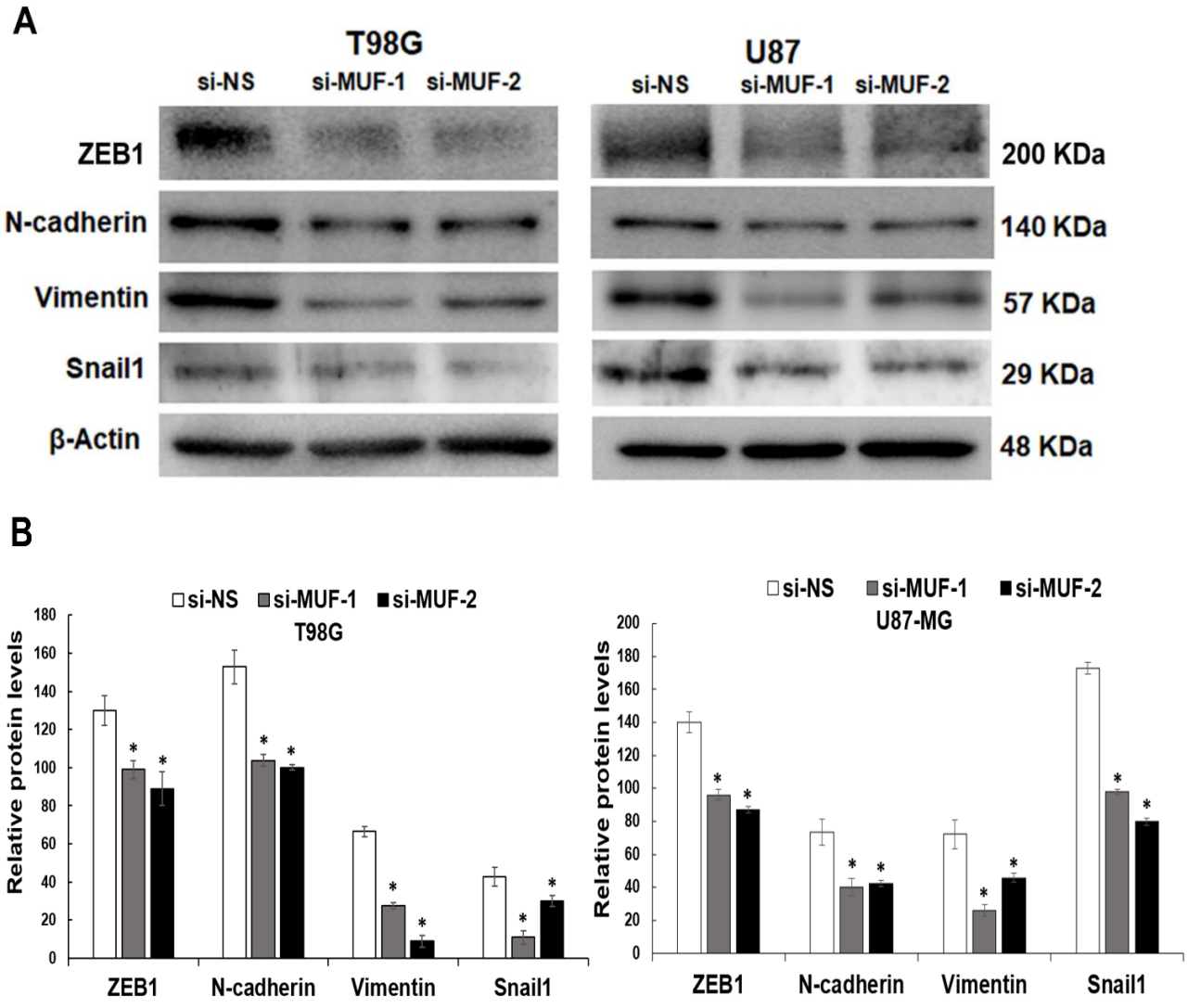


Fig 23: Validation of expression of EMT markers upon lncRNA-MUF knockdown. A. *LncRNA-MUF promotes GBM EMT. Western blot analysis of EMT markers- ZEB1, N-cadherin, Vimentin, and Snail1 followed by lncRNA-MUF knockdown in T98G and U87-MG cells.* ***B.*** *Quantification of western blots using ImageJ. *Significant change compared to cells transfected with si-NS ($p < 0.05$). Values represent mean ± SD from three independent experiments.*

3.2.6 Knockdown of lncRNA-MUF attenuates TGF-β signaling

TGF-β-induced lncRNAs are known to regulate the TGF-β signaling pathway via an autocrine signaling loop (292). Hence, we asked if lncRNA-MUF is also involved in regulating TGF-β signaling. To test this, we evaluated the impact of lncRNA-MUF knockdown on TGF-β-induced phosphorylation of SMAD2/3 (Fig 24 A & B).

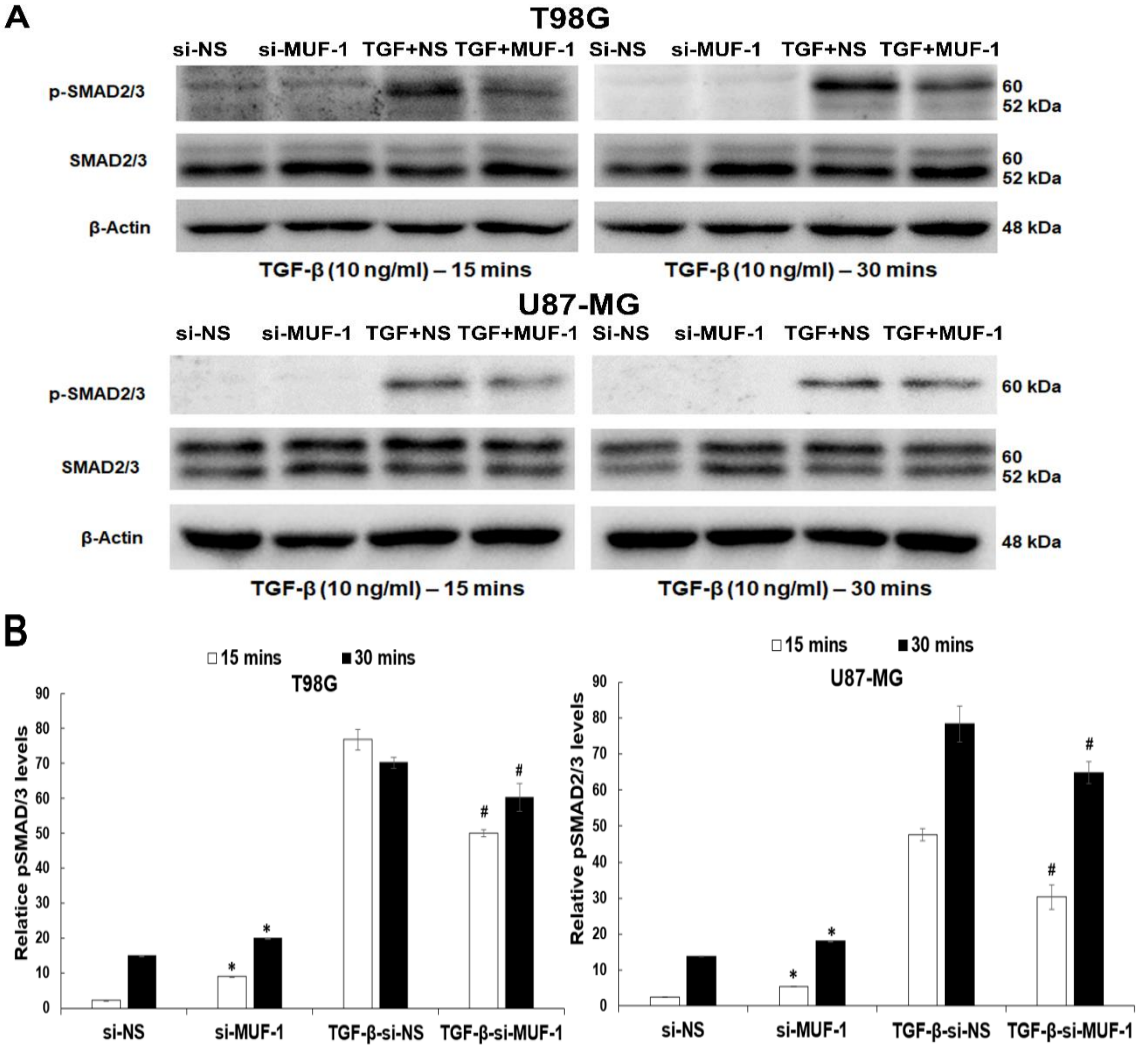


Fig 24: Evaluation of TGF-β signaling upon lncRNA-MUF knockdown. A. *LncRNA-MUF knockdown impairs phosphorylation of the SMAD2/3 complex. Western blot analysis of pSMAD 2/3 and total SMAD2/3 levels in T98G and U87-MG cells treated with 10 ng/ml TGF-β1 (15 mins and 30 mins), 48 h after lncRNA-MUF knockdown with si-MUF-1. A representative blot is shown from three independent experiments with similar results. Blots were reprobed for β-actin to establish equivalent loading.* ***B.*** *Quantification of western blots using ImageJ. A representative blot is displayed from three independent experiments with similar results. Blots were reprobed for β-actin to establish equivalent loading.*

3.2.7. LncRNA-MUF modulates TGF-β-induced invasion in glioma via miR-34a-5p/SNAIL1/ZEB1 axis

LncRNAs function as endogenous miRNA sponges and participate in the ceRNA regulatory network (260,265). Yan et al. have reported the direct binding of lncRNA-MUF and miR-34a using RNA immunoprecipitation (RIP) and RNA pulldown assays (276). In addition, they show that lncRNA-MUF regulates SNAIL1 expression by sponging miR-34a to modulate EMT in HCC cells (276). Using RNAhybrid and IntaRNA databases, we identified putative miR-34a binding sites in lncRNA-MUF, ZEB1, and SNAIL1 (Fig 25 A). Also, the direct binding of miR-34a with SNAIL1 and ZEB1 UTRs has been previously reported (285,293–296). To confirm the interaction between lncRNA-MUF and miR-34a-5p, we cloned the region of lncRNA-MUF with the miR-34a binding sites into the pmirGLO vector downstream of the firefly luciferase gene. Co-transfection with the pmirGLO-lncRNA-MUF reporter plasmid and miR-34a mimics significantly reduced reporter activity (~ 70%) compared to the control cells (Fig 25 B).

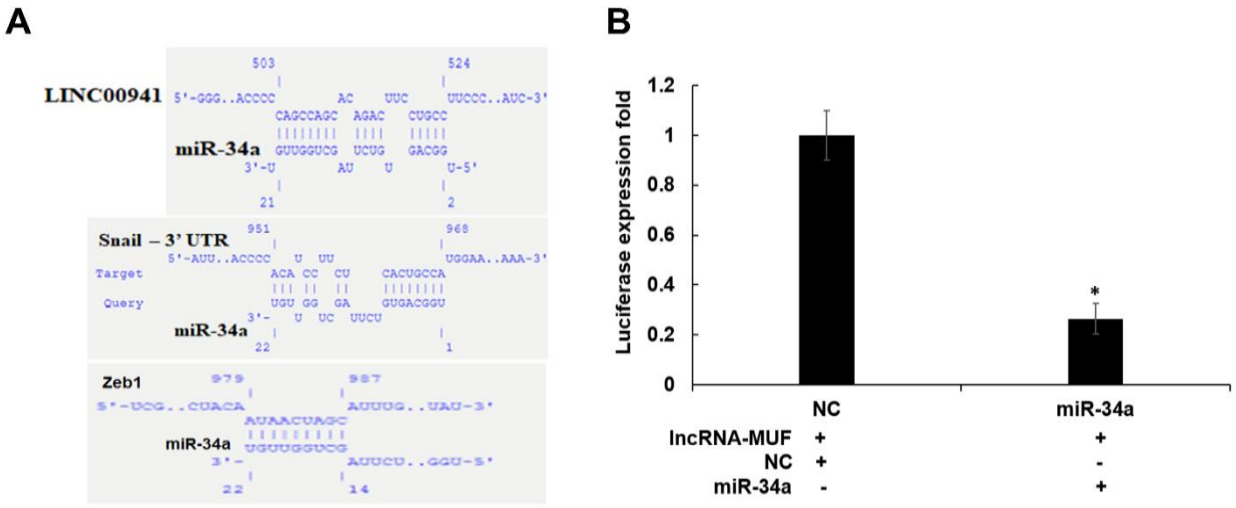


Fig 25: Interaction of miR-34a with lncRNA-MUF, SNAIL1, and ZEB1. A. *The predicted miR-34a binding sites on lncRNA-MUF, SNAIL1, and ZEB1 3' UTR.* ***B.*** *Luciferase activity assay demonstrated that lncRNA-MUF could bind with miR-34a; relative luciferase activity was measured in HEK293T cells co-transfected with miR-34a-5p mimics and pmiRGLO-lncRNA-MUF constructs. Luminescence signals were measured 30 hrs post-transfection using dual luciferase assay. Data are shown as mean ± SD of three independent experiments; *p<0.05*

Since miR-34a has a well-established tumor suppressor role in several cancers, including GBM (297), we first evaluated its expression in glioblastoma tissue using the CGGA dataset. Expression of miR-34a is lowest in grade IV GBM (p=0.0038) (Fig 26 A). In addition, Kaplan–Meier survival curve demonstrates that high expression of miR-34a positively correlates with better survival of glioma patients (p=0.016) (Fig 26 B).

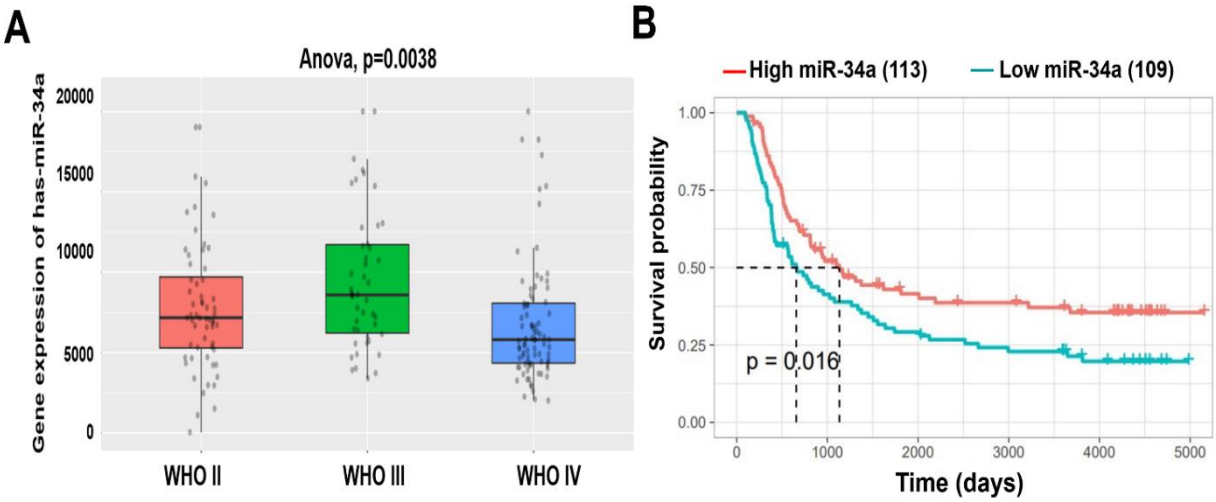


Fig 26: Expression of miR-34a-5p in GBM samples. A. *Low expression of miR-34a in grade IV GBM compared to lower grade gliomas (p=0.0038). The red bar represents miR-34a expression in the WHO grade II group; the green bar represents miR-34a expression in WHO grade III, and the blue bar represents miR-34a expression in the WHO grade IV glioma group.* ***B.*** *Kaplan-Meier survival analysis showed high miR-34a expression correlated with better survival in primary and recurrent GBM patients identified from the CGGA database (p=0.016). The Red line represents the high miR-34a expression group, and the green line represents the low miR-34a expression group.*

To understand the impact of miR-34a on lncRNA-MUF regulation, we first determined its levels upon miR-34a overexpression using miRNA mimics. We observed a significant ~ 40-50 % reduction in lncRNA-MUF expression in T98G and U87-MG cells upon treatment with miR-34a mimic (Fig 27 A). ZEB1 and SNAIL1 are well-known targets of miR-34a (293–296,298); consistent with this, we observed that transfection of miR-34a mimics in T98G and U87-MG glioma cells significantly reduced ZEB1 and SNAIL transcript and protein levels (Fig 27 B-E).

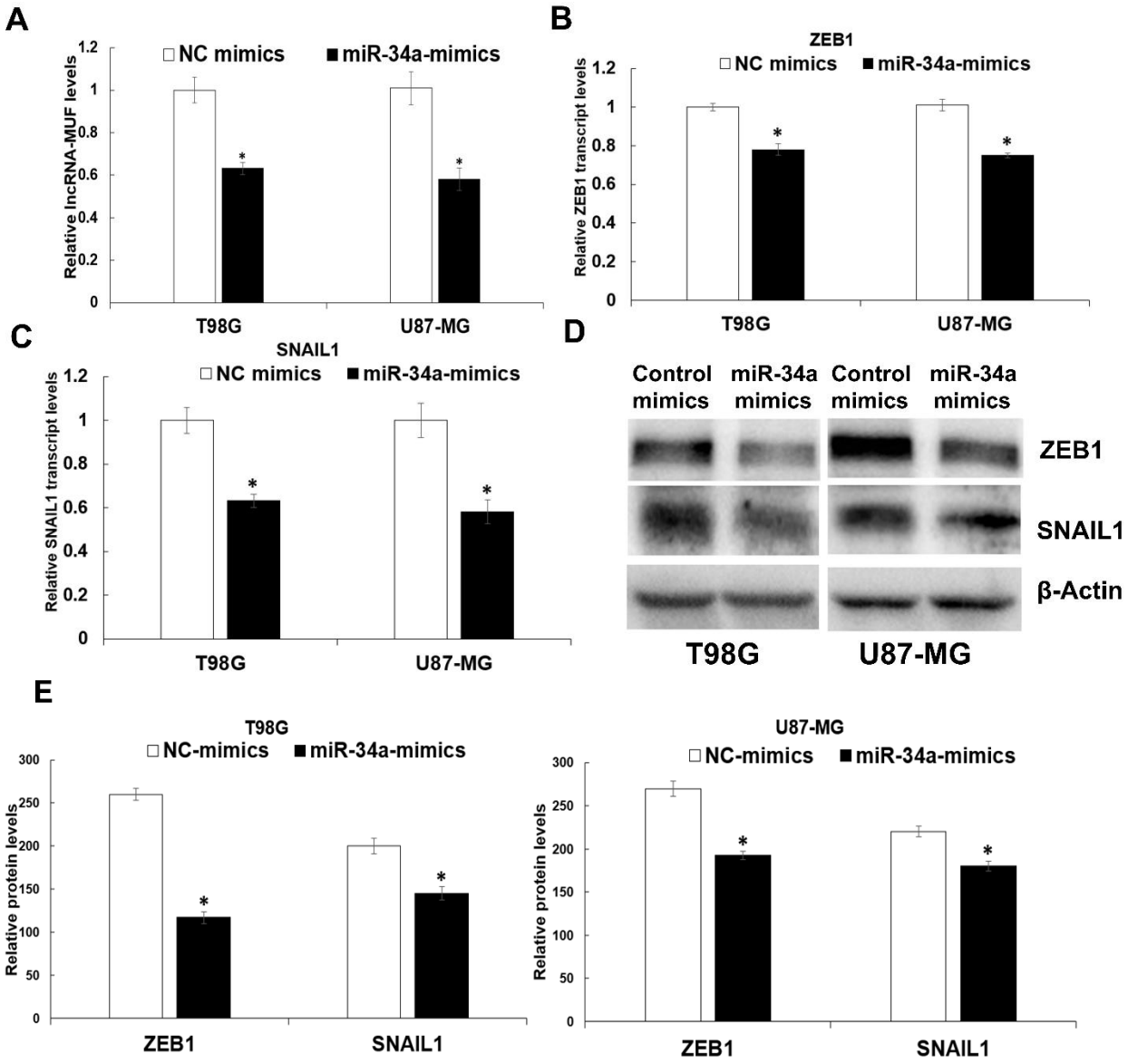


Fig 27: Expression of lncRNA-MUF, ZEB1, and SNAIL1 upon miR-34a mimic A. *Down-regulation of lncRNA-MUF transcript levels upon miR-34a overexpression measured by qRT-PCR in T98G and U87-MG cells.* ***B.*** *Down-regulation of ZEB1 transcript levels upon miR-34a overexpression measured by qRT-PCR.* ***C.*** *Down-regulation of SNAIL1 transcript levels upon miR-34a overexpression measured by qRT-PCR.* ***D.*** *Downregulation of ZEB1 and SNAIL1 protein levels upon miR-34a overexpression.* ***E.*** *Quantification of ZEB1 and SNAIL1 blots using ImageJ. T98G & U87-MG cells transfected with 80nM of negative control mimics and miR-34a mimics. 48h post-transfection, RNA was isolated, and transcript levels were measured by qRT-PCR. For western blotting, 48 h post-transfection protein lysates were collected, and ZEB1 and SNAIL1 protein levels were analyzed by western blotting. A representative blot is shown from three independent experiments with similar results. Blots were reprobed for β-actin to establish equivalent loading.*

Given that miR-34a targets lncRNA-MUF, SNAIL1, and ZEB1 expression and because we observed downregulation of SNAIL1 and ZEB1 upon lncRNA-MUF depletion, we explored if lncRNA-MUF could act as a ceRNA to sponge miR-34a for stabilizing SNAIL1 and ZEB1 to regulate invasion in glioma cells. Invasion analysis revealed that reduction in invasion upon lncRNA-MUF knockdown is significantly reversed upon co-transfection with miR-34a inhibitor (Fig 28 A & B).

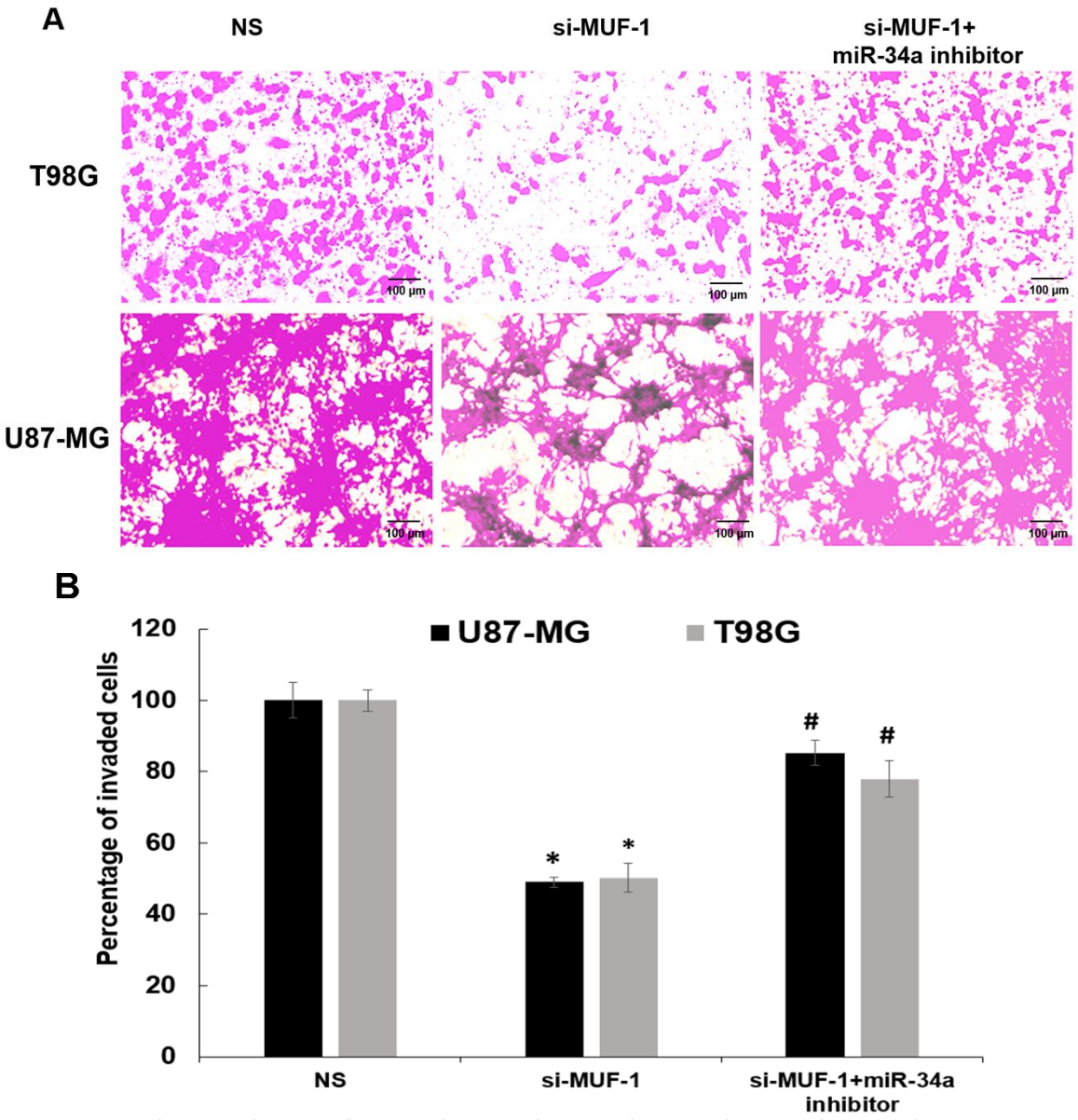


Fig 28: Rescue of invasion upon combined knockdown of lncRNA-MUF and miR-34a. A. *Rescue of invasion caused by lncRNA-MUF knock-down in T98G and U87-MG glioma cells upon miR-34a inhibition.* ***D.*** *Quantification of invasion using ImageJ.*

Further, we observed that ZEB1 or SNAIL1 overexpression could partly reverse the lncRNA-MUF knockdown-mediated reduction in T98G and U87-MG GBM cell invasion (Fig 29 & 30).

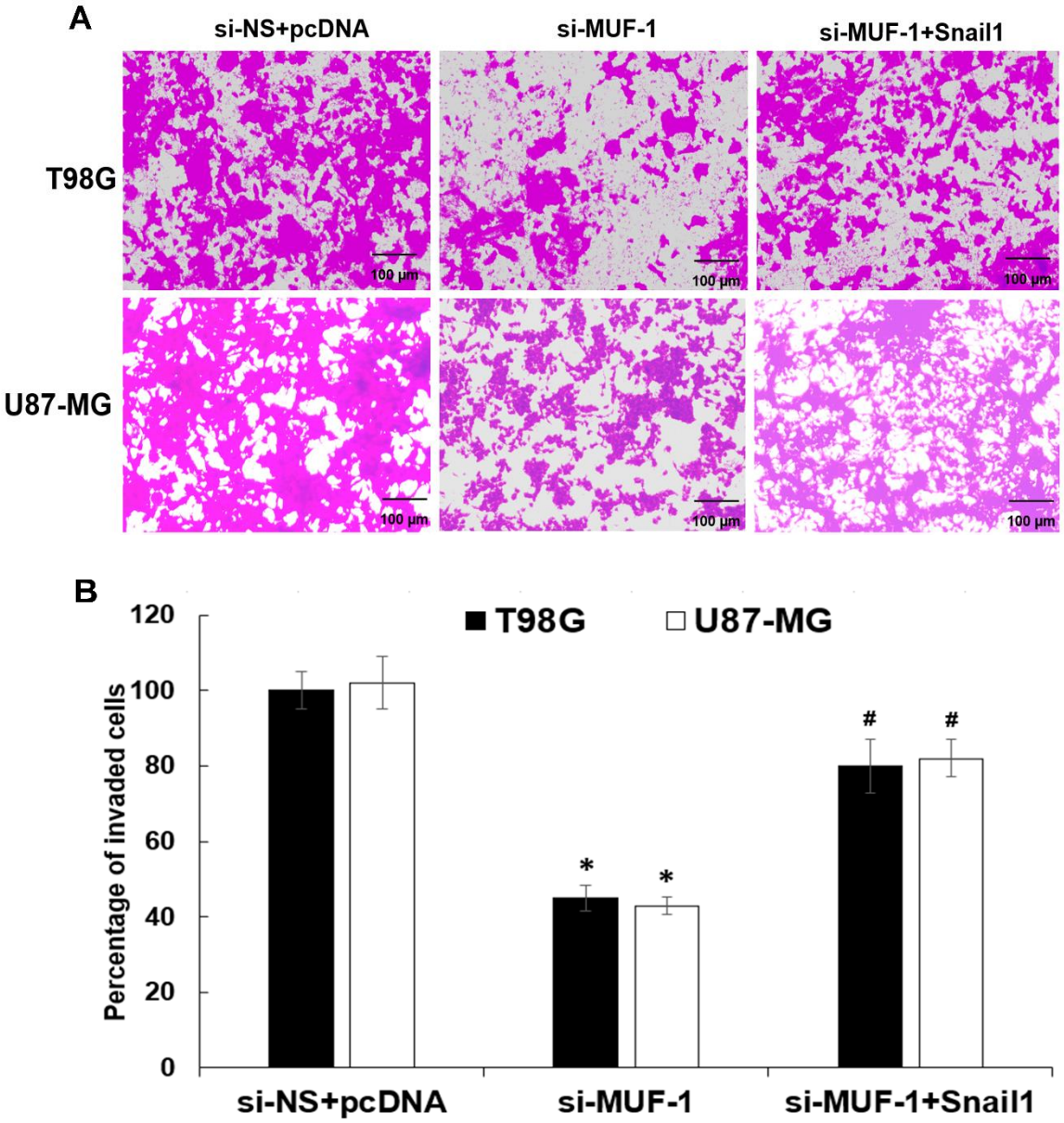


Fig 29: Rescue of invasion upon knockdown of lncRNA-MUF and SNAIL1 overexpression. A. *Rescue of invasion caused by lncRNA-MUF knockdown in T98G and U87-MG glioma cells upon SNIAIL1 overexpression.* ***B.*** *Quantification of invasion using ImageJ.*

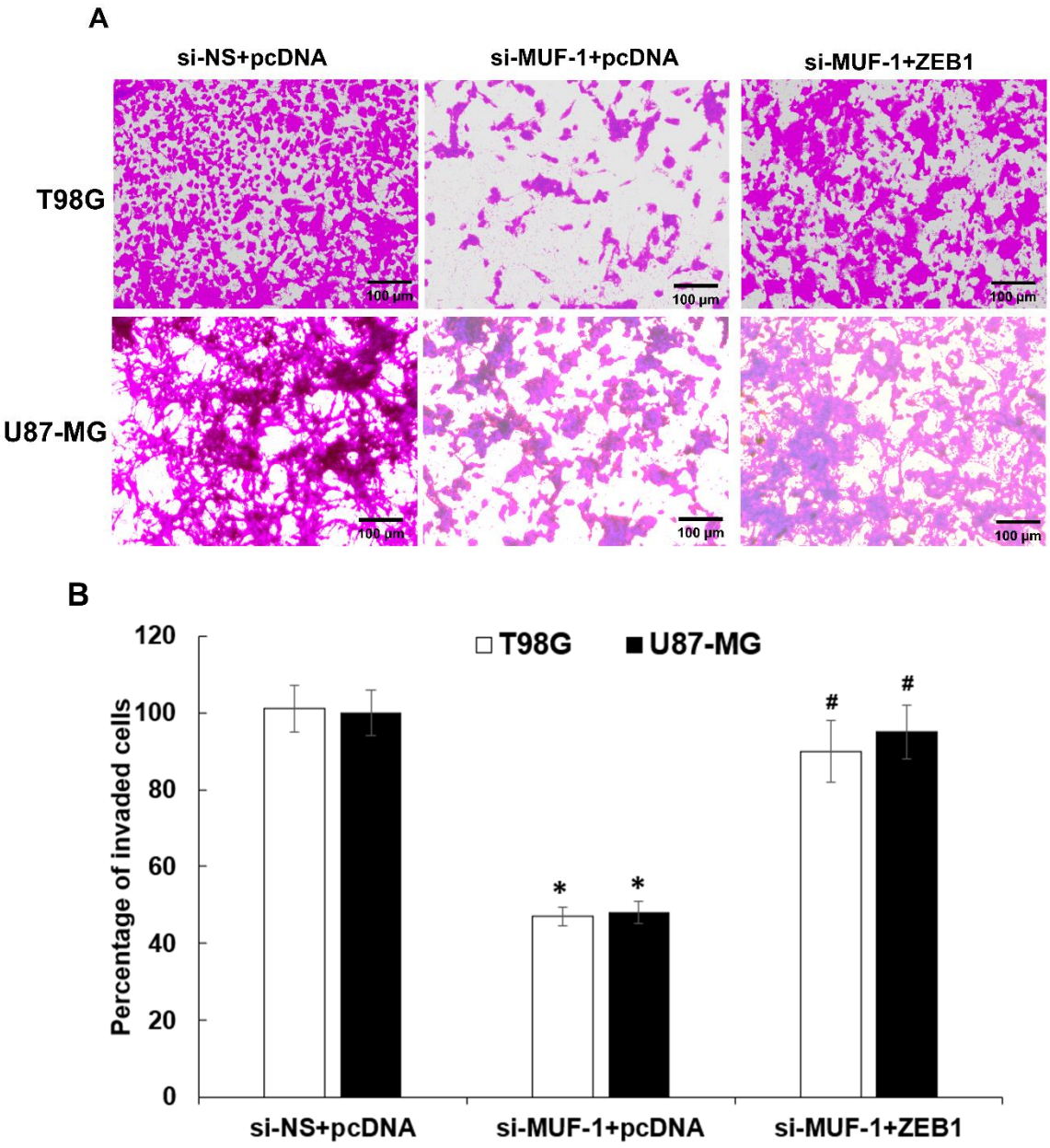


Fig 30: Rescue of invasion upon knockdown of lncRNA-MUF and ZEB1 overexpression. A. *Rescue of invasion caused by lncRNA-MUF knockdown in T98G and U87-MG glioma cells upon ZEB1 overexpression.* ***B.*** *Quantification of invasion using ImageJ.*

We observed that the miR-34a inhibitor significantly restores SNAIL1 and ZEB1 protein downregulation caused by lncRNA-MUF depletion (Fig 31 A & B). Also, These experiments indicate that TGF-β induced lncRNA-MUF sponges miR-34a to promote ZEB1 and SNAIL1-induced invasion.

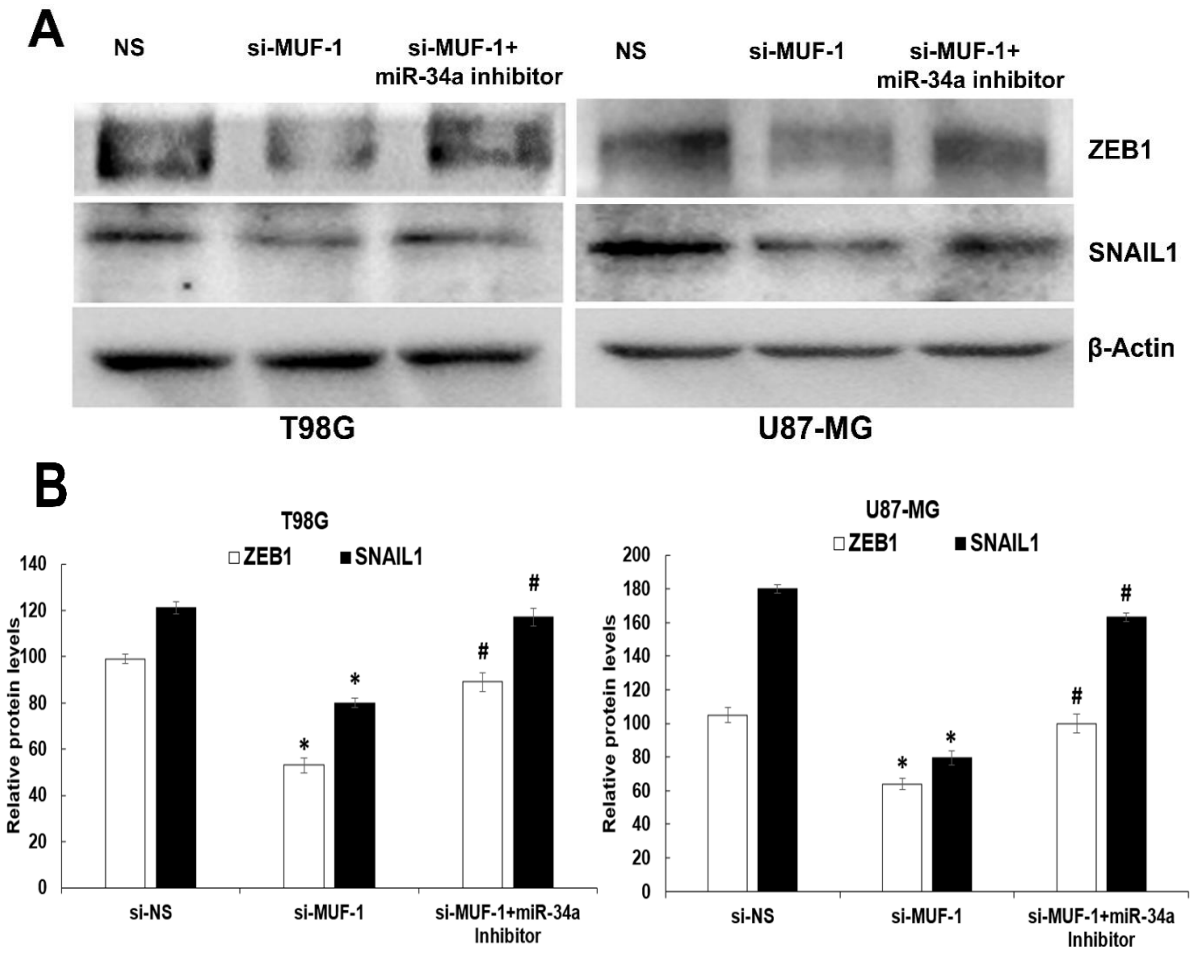


Fig 31: Rescue of ZEB1 and SNAIL1 protein expression upon combined knockdown of lncRNA-MUF and miR-34a. A. *Reduction in ZEB1 and SNAIL1 protein levels caused by lncRNA-MUF knockdown in T98G and U87-MG glioma cells is restored upon miR-34a inhibition. A representative blot is shown from three independent experiments with similar results. Blots were reprobed for β-actin to establish equivalent loading.* ***B.*** *Quantification of blots by ImageJ.*

3.2.8 LncRNA-MUF promotes drug resistance in GBM

TMZ is an oral alkylating drug used to treat GBM; however, 50% of GBM cases develop resistance to TMZ. Several GBM cell lines, such as T98G and LN229, show resistance to TMZ, and TGF-β-induced lncRNAs are known to promote TMZ resistance (111,236). Therefore, we evaluated the effect of lncRNA-MUF knockdown on TMZ sensitivity in T98G and LN229 cells.

LncRNA-MUF depletion with low siRNA levels (20nM) significantly reduced cell proliferation in TMZ-treated T98G and LN229 cells compared to si-NS transfected cells treated with TMZ (Fig 32.1 A). In addition, TMZ treatment in lncRNA-MUF knockdown resulted in a significant increase in caspase 3/7 activity (~ 5-fold) compared to si-NS transfected T98G and LN229 cells treated with TMZ (Fig 32.1 B). These results suggest that lncRNA-MUF knockdown sensitizes glioma cells to TMZ-induced apoptosis.

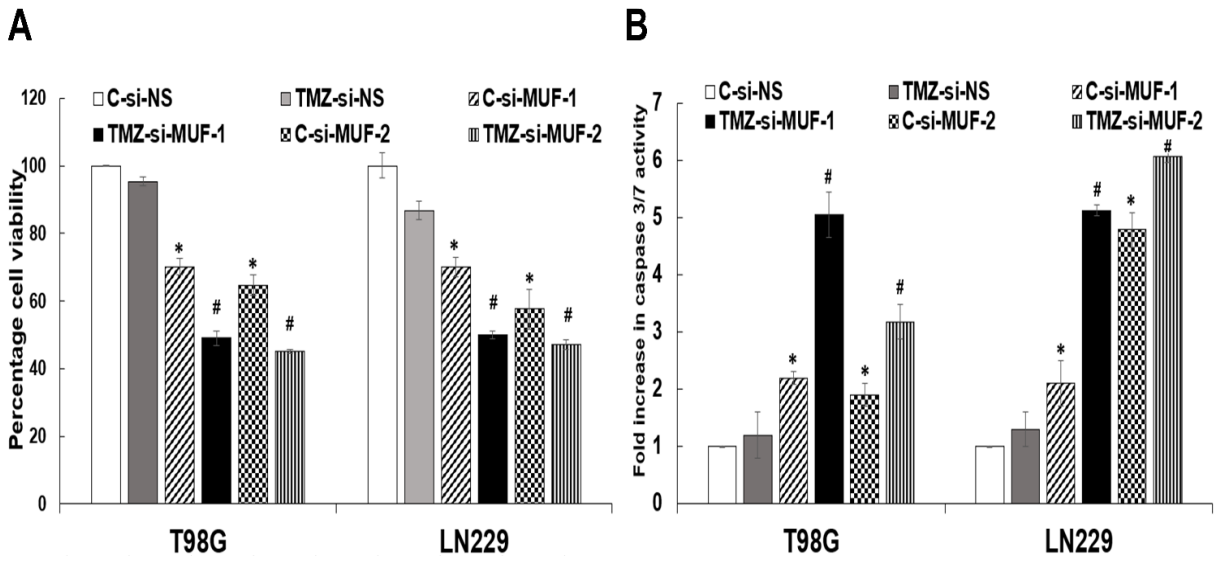


Fig 32.1: LncRNA-MUF induces TMZ resistance in GBM cells. A. *LncRNA-MUF knock-down with a low concentration of si-MUF1/ si-MUF-2 (20 nM) in combination with TMZ treatment (600 µM) shows enhanced reduction of glioma cell (T98G and LN229) proliferation and increased sensitivity to TMZ, as analyzed using WST1 assay.* ***B.*** *LncRNA-MUF knock-down (20nM of si-MUF-1/ si-MUF-2) combined with TMZ treatment (600 µM) show enhanced caspase 3/7 activity to TMZ alone.*

Further, we also tested the ability of lncRNA-MUF knockdown to induce cisplatin sensitivity in T98G, U87-MG, and LN229 cells. LncRNA-MUF depletion with low siRNA levels (20nM) significantly reduced cell proliferation in cisplatin-treated T98G, U87-MG, and LN229 cells compared to si-NS transfected cells treated with cisplatin (Fig 32.2 A). In addition, cisplatin treatment in lncRNA-MUF knockdown resulted in a significant increase in caspase 3/7 activity (~ 5-fold) compared to si-NS transfected T98G, U87-MG, and LN229 cells treated with cisplatin (Fig 32.2 B). These results suggest that lncRNA-MUF knockdown sensitizes glioma cells to TMZ and cisplatin-induced apoptosis.

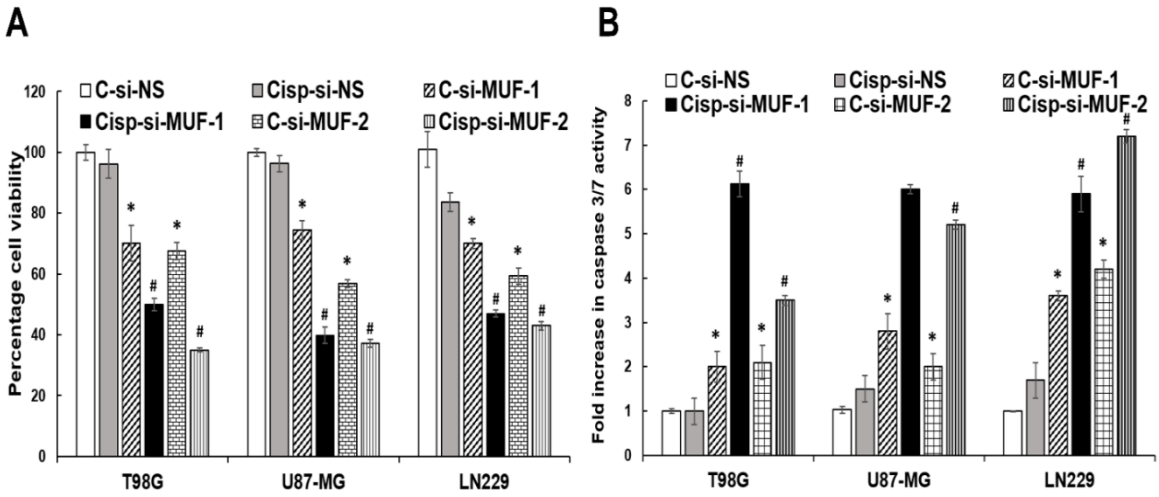


Fig 32.2: LncRNA-MUF induces cisplatin resistance in GBM cells. A. *LncRNA-MUF knockdown with a low concentration of si-MUF1/ si-MUF-2 (20 nM) in combination with cisplatin treatment shows enhanced reduction of glioma cell (T98G, U87-MG, and LN229) proliferation and increased sensitivity to cisplatin, as analyzed using WST1 assay.* ***B.*** *LncRNA-MUF knockdown (20nM of si-MUF-1/ si-MUF-2) combined with cisplatin treatment show enhanced caspase 3/7 activity to cisplatin alone.*

3.2.9 Discussion

Glioma is the most lethal and invasive malignant brain neoplasm with a poor prognosis and frequent recurrence after surgery. Prominent features of GBM that contribute to recurrence include the presence of glioma stem cells, resistance to TMZ, and invasion (233,299). TGF-β secreted by glioma cells confers them with an aggressive pro-invasive phenotype and TMZ resistance (236). TGF-β induces the expression of several lncRNAs (LINC00645, LINC00115, UCA1, lncRNA-ATB) through the canonical or non-canonical signaling pathway to promote glioma progression (212,213,231,232).
We demonstrate that lncRNA-MUF levels are elevated in GBM tumor samples (Fig 14 A). Its high expression is associated with poor survival and prognosis of GBM patients (Fig 14 B). This is consistent with that lncRNA-MUF levels are upregulated in multiple cancers (272,276–283,300).
ChIP-seq data analysis has revealed that TGF-β stimulation results in the accumulation of activating H3K27ac marks at the lncRNA-MUF promoter (278). Also, Wu et al. reported that lncRNA-MUF induction by TGF-β in colorectal cancer (CRC) cells are abrogated upon treatment with disitertide, an inhibitor of TGFBR1 (279). In line with these findings, we identified that lncRNA-MUF induction by TGF-β is completely abrogated upon treatment with TGFBR1/ SMAD2/3 inhibitor SB505124 in glioma cells (Fig 16 C). Our ChIP-qPCR data also indicated substantial enrichment of lncRNA-MUF promoter in the pull-downs of SMAD2/3 antibodies upon TGF-β stimulation (Fig 16 D). These results suggest that lncRNA-MUF is induced through the SMAD2/3 transcription factors downstream of TGF-β in GBM.

Loss-of-function studies demonstrated that lncRNA-MUF promotes proliferation, migration, invasion, and TMZ resistance in GBM (Fig 18, 19, 32.1). Further, we studied the molecular mechanisms of gene regulation by lncRNA-MUF in glioma cells. TGF-β induced lncRNAs are known to regulate the signaling pathway by forming a positive feedback loop. For example, lncRNA-MIR100HG regulates SMAD2/3 phosphorylation in prostate carcinoma (292). We demonstrate for the first time that lncRNA-MUF downregulation attenuates TGF-β-induced phosphorylation of SMAD2/3 in glioma cells (Fig 24 A & B).

Next, we explored the role of lncRNA-MUF *in cis* and *trans* gene regulation. Ai et al. previously reported that lncRNA-MUF promotes OSCC progression by mediating chromosome looping to the promoter of its *cis* gene, Caprin2, to activate the WNT/β-

catenin signaling mediated progression of OSCC (277). Although we observed a significant downregulation of the Caprin2 gene with lncRNA-MUF knockdown (Fig 20 B), we did not observe any change in the WNT/β-catenin signaling genes at the transcript level. Apart from regulating Caprin2 expression, lncRNA-MUF modulates the expression of ZEB1, N-cadherin, vimentin, CTGF, c-Myc, and SNAIL1 in the TGF-β pathway in GBM (Fig 21 A).

The mechanism of action of a lncRNA, by and large, depends on the subcellular localization of the lncRNA. Cellular fractionation experiments indicated that lncRNA-MUF is primarily located in the cytoplasm (Fig 25 A & B). Cytoplasmic lncRNAs act as endogenous miRNA sponges for binding to miRNAs or participate in the ceRNA regulatory network (260). Hence, we tested the ceRNA hypothesis-mediated gene regulation of lncRNA-MUF in GBM.

Bioinformatics analysis demonstrated that miR-34a is the primary miRNA target of lncRNA-MUF. Overexpression of miR-34a significantly downregulated SNAIL1 and ZEB1 transcript and protein levels (Fig 27 D & E). miR-34a suppresses the proliferation and invasion in glioma (291). It is downregulated in human glioma tumors compared to normal brain tissue (291). miR-34a has a potential tumor-suppressor role in glioma by targeting several oncogenes and also induces differentiation of glioma stem cells (291). SNAIL1 and ZEB1 are master transcription factors that promote invasion in GBM (301–306). SNAIL1 and ZEB1 are often upregulated in glioma cells, and high expression of SNAIL1/ZEB1 is associated with poor survival of glioma patients (304,307). ZEB1 and SNAIL1 promote expression of each other, and both are essential factors for the mesenchymal phenotype in the cancer cell (305,308–310). A significant positive correlation is seen between SNAIL1 and ZEB1 in lung cancer cells (311).
Also, literature evidence proves the direct interaction of miR-34a with SNAIL1 and ZEB1 (293–296). We also show that lncRNA-MUF depletion in glioma cells reduces migration and invasion. Mechanistically, lncRNA-MUF promotes GBM invasion by acting as an endogenous sponge for miR-34a and causing stabilization of its targets SNAIL1 and ZEB1 (Fig 49). Our findings suggest that the TGF-β-regulated lncRNA-MUF/miR-34a/SNAIL1/ZEB1 signaling axis is a critical regulator of invasion in GBM (Fig 49).
The mechanism of regulation of other *trans* genes, vimentin, N-cadherin, CTGF, and c-Myc by lncRNA-MUF, needs further investigation. miR-34a targets several other

oncogenes such as c-Myc, VEGF, Sox2, Notch1, ZNF281, IL-6R, SIRT1, MDM4, EGFR, PD-L1, CDK6, c-Met, MYCN, BCL2, CyclinE2, CyclinD1, PDGFRA, Msi1, Akt, and Wnt. The abundance of individual transcripts, the number of miRNA binding sites in the target genes, and the stoichiometry between the lncRNA and miRNA greatly influence the outcomes of the ceRNA hypothesis (312). Hence, the impact of other miR-34a targets relevant to GBM could be further studied upon lncRNA-MUF knockdown. In this study, we have reported that lncRNA-MUF promotes TMZ resistance in GBM cells. Since lncRNA-MUF primarily regulates SNAIL1 and ZEB1, and they have a predominant role in promoting TMZ resistance in GBM cells (302,313), it is tempting to hypothesize that lncRNA-MUF promotes TMZ resistance through SNAIL/ZEB1 in GBM.

3.3. Characterization of the role of TGF-β-induced LINC01711 in GBM pathogenesis

In this section, we characterized the role of the second clinically relevant non-coding RNA LINC01711 from our microarray screen (235). LINC01711 is upregulated in esophageal squamous cell carcinoma (ESCC), thyroid carcinoma, low-grade glioma, and breast cancer (314–318). However, the exact function and mechanism of action of LINC01711 in GBM pathogenesis were unknown. Here, we characterize the role of LINC01711 in promoting GBM pathogenesis by acting as a sponge for miR-34a to stabilize ZEB1 and SNAIL1.

3.3.1 LINC01711 is upregulated in GBM tumor samples and is associated with poor patient prognosis

Using the TCGA database, a previous study reported that LINC01711 is upregulated in low-grade gliomas (315). However, its role in GBM (grade IV glioma) pathophysiology remains unknown. Hence to understand the role of LINC01711 in GBM pathophysiology, we evaluated its expression in GBM tumor samples using the GEPIA database. Expression of LINC01711 was ~1.5-fold higher in GBM samples compared to normal tissue (Fig 33 A). Increased expression of *LINC01711* correlated with poor overall survival in both primary and recurrent GBM patients ($p<0.0001$) (Fig 33 B). These results suggest that *LINC01711* expression is associated with aggressive phenotype and poor survival in glioma patients. In addition, using the mRNAseq_693 dataset of the CGGA database, we found that *LINC01711* levels are substantially higher in grade IV GBM (~ 2-fold) than in lower-grade gliomas ($p=0.000000000024$) (Fig 33 C). GBM patients with IDH mutation show a better survival rate than the IDH wild-type group (23). Hence, we evaluated the expression of LINC01711 in IDH mutant and wild-type glioma samples. We observed that the levels of *LINC01711* are significantly higher in gliomas with the IDH wild-type group than in the IDH mutant group ($p=0.0000046$) (Fig 33 D).

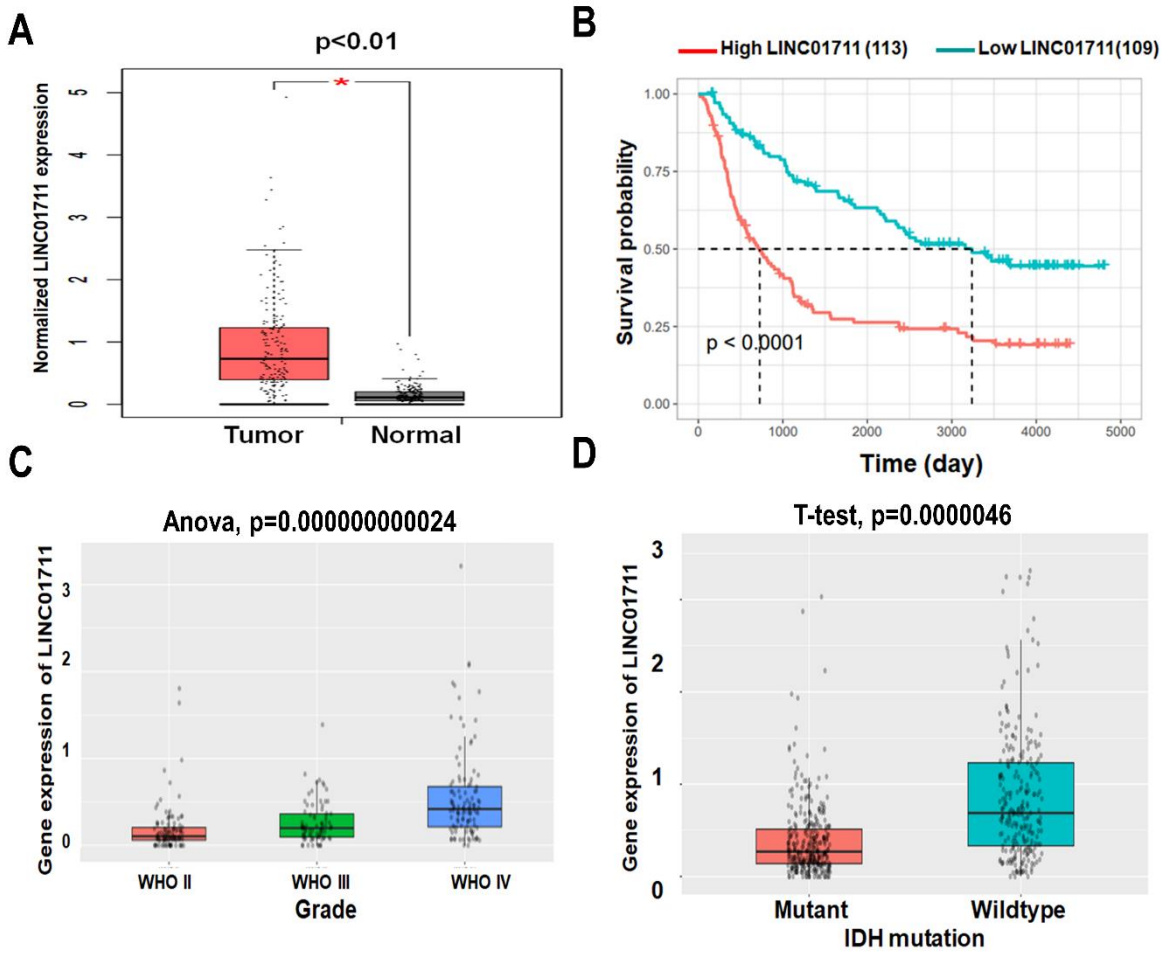


Fig 33: Expression of LINC01711 in GBM samples. A. *LINC01711 levels were significantly higher in GBM samples than in normal brain tissues (p<0.01), as analyzed from TCGA data using the GEPIA portal.* ***B.*** *LINC01711 expression analysis in glioma tissues from CGGA database. Elevated expression of LINC01711 in GBM IV compared to lower-grade gliomas (p=0.000000000024).* ***C.*** *LINC01711 levels are elevated in IDH wild-type GBM patients compared to IDH mutant cases as analyzed from the CGGA database (T-test, p=0.0000046).* ***D.*** *Kaplan-Meier survival analysis of LINC01711 expression in glioma samples from CGGA databases shows that high LINC01711 expression is associated with poor overall survival in GBM patients (p<0.0001). The Red line represents the high LINC01711 expression group, and the green line represents the low LINC01711 expression group.*

3.3.2 LINC01711, a long non-coding RNA transcriptionally regulated by TGF-β1 in GBM

LINC01711 is generated from the genomic locus Chr20q13.32 (LOC79160). LINC01711 is upregulated ~ 3.98 fold upon TGF-β treatment in the microarray screen. LINC01711 induction upon TGF-β1 treatment was concentration-independent for TGF-β concentrations from 5 ng/ml- 80 ng/ml for 24h in T98G cells with a fold change of ~ 3.5-fold (Fig 34 A). Further, the time-course analysis (at 10 ng/ml TGF-β1 treatment) identified that LINC01711 gets induced upon TGF-β1 treatment as early as 1 hr. However, a statistically significant increase of ~2-fold occurs only at 12 h, followed by ~5-fold and ~ 4.5-fold at 18h and 24h of TGF-β treatment, respectively. After that, levels are sustained at ~ 3-fold at 36h and 48h post-TGF-β treatment (Fig 34 B).

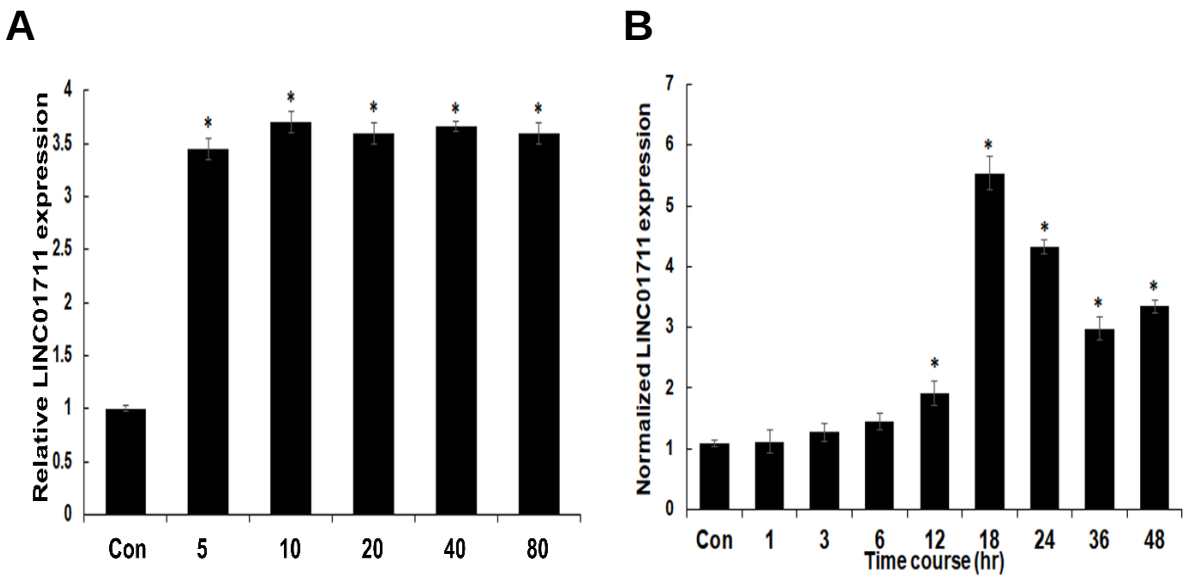


Fig 34: Evaluation of expression of LINC01711 at various concentrations and time points of TGF-β treatment. A. *qRT-PCR of LINC01711 with different concentrations of TGF-β1 treatment in T98G GBM cells. LINC01711 induction upon TGF-β1 treatment is not concentration-dependent. Cells were treated with the indicated concentration of TGF-β1 for 24 h and analyzed for LINC01711 expression by qRT-PCR.* ***B.*** *LINC01711 is a delayed transcript with induction of ~2-fold at 12 h, followed by ~5-fold and ~ 4.5-fold at 18h and 24h of TGF-β treatment, respectively, and then sustained at ~ 3-fold at 36 h and 48 h. *Significant change compared to control cells ($p < 0.05$).*

3.3.3 TGF-β-induced LINC01711 is activated through the canonical SMAD2/3 signaling pathway in glioma cell lines

To assess the impact of TGF-β1 on the LINC01711 expression on additional glioblastoma cell lines, we evaluated its levels in LN18, U87-MG, and LN229 glioma cells. Upon TGF-β1 stimulation for 24 hr, the expression of LINC01711 was upregulated (≥ 2.5-fold) in glioma cell lines (T98G: ~ 4-fold; U87-MG: ~ 2.5-fold; LN18: ~ 4-fold; LN229: ~ 7-fold) (Fig 35 A). These results indicate that LINC01711 induction upon TGF-β1 treatment in GBM is not cell line-specific. We then investigated the subcellular localization of LINC01711 using cellular fractionation experiments. Xu et al., using FISH analysis, demonstrated that LINC01711 is mainly present in the cytoplasm (314). Our cellular fractionation experiments in T98G GBM cells also confirmed that LINC01711 is primarily located in the cytoplasm (Fig 35 B).

TGF-β regulates gene expression through the canonical SMAD2/3 signaling pathway. To identify the transcription factors working downstream of the TGF-β pathway, we analyzed the LINC01711 promoter using JASPAR (http://jaspar.genereg.net/) and found functional SBE (5' CAGAC 3' / 5' GTCTG 3') at -1642, -2029, -2121, -2549, and -3073 positions. We further evaluated LINC01711 expression upon TGF-β treatment in the presence and absence of TGF-β inhibitor SB505124. GBM cells were treated with 6μM SB505124 (TGFβR1/smad2/3 inhibitor) for 2 hrs before treatment with TGF-β1 (24 hr). Blocking SMAD2/3 with SB505124 significantly abrogated TGF-β-induced LINC01711 expression in glioma cells (~ 70% reduction in T98G, U87-MG, and LN-18 cells) (Fig 35 C). To confirm the role of the SMAD2/3 transcription factor in regulating LINC01711 expression, we performed ChIP-qPCR to determine whether TGF-β promotes increased binding of SMAD2/3 to SBE on the LINC01711 promoter. ChIP-qPCR revealed ~5-fold increased binding of SMAD2/3 on SBE of LINC01711 promoter upon TGF-β stimulation (Fig 35 D). These results suggest that TGF-β upregulates LINC01711 expression through the canonical SMAD signaling pathway.

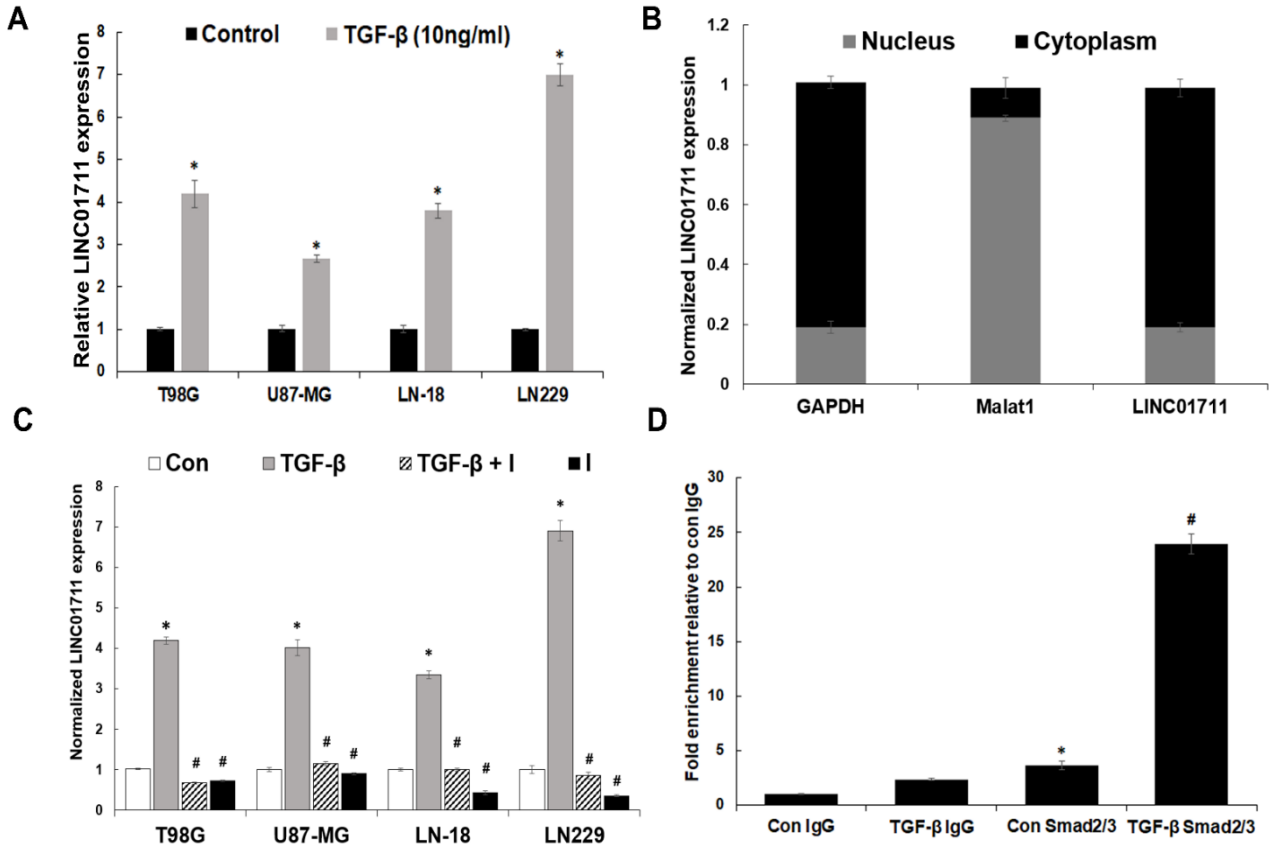


Fig 35: Identification of the mechanism of induction of LINC01711 upon TGF-β treatment. A. *LINC01711 induction upon TGF-β treatment is not cell type-specific. The indicated GBM cell lines were treated with 10 ng/ml TGF-β for 24 h and evaluated for LINC01711 expression.* ***B.*** *Fractionation of T98G GBM cells followed by qRT-PCR indicates LINC01711 is localized primarily in the cytoplasm. GAPDH served as a control for cytoplasmic fraction; MALAT1 was used as a control for nuclear fraction.* ***C****. LINC01711 induction upon TGF-β treatment is SMAD2/3 dependent. Human glioma cells were pre-treated with 6 μM of SB505124 (TGFβR1/SMAD2/3 inhibitor) for 2 h, followed by co-treatment with TGF-β1 (10 ng/ml) for 24 h.* ***D.*** *ChIP-qPCR analysis of SMAD2/3 interaction with SBE in the LINC01711 promoter in control and TGF-β-treated T98G cells. DNA was isolated from control and TGF-β-treated cells after immunoprecipitation with the anti-SMAD2/3 antibody and was amplified using specific primer sets. LINC01711 promoter levels in immunoprecipitated samples were measured by qRT-PCR analysis, normalized to input, and represented as fold enrichment relative to control IgG I.P. Data information: RNA samples were analyzed by qRT–PCR, normalized with TBP. Error bars represent the mean ± SEM from 3 independent experiments. *Significant change compared to control ($p<0.05$). #Significant decrease from TGF-β-treated cells ($p<0.05$). Statistical comparisons were made using Student's t-test.*

3.3.4. LINC01711 promotes GBM cell proliferation, migration, and invasion

To further evaluate the role of LINC01711 in glioma pathogenesis, we established lncRNA knockdown using two different ASOs against LINC01711 (ASO-1 and ASO-2) in glioma cell lines. Knockdown of LINC01711 with ASO-1 results in ~ 70% reduction, and ASO-2 results in ~ 75% reduction of the LINC01711 levels in T98G and U87-MG cells (Fig 36).

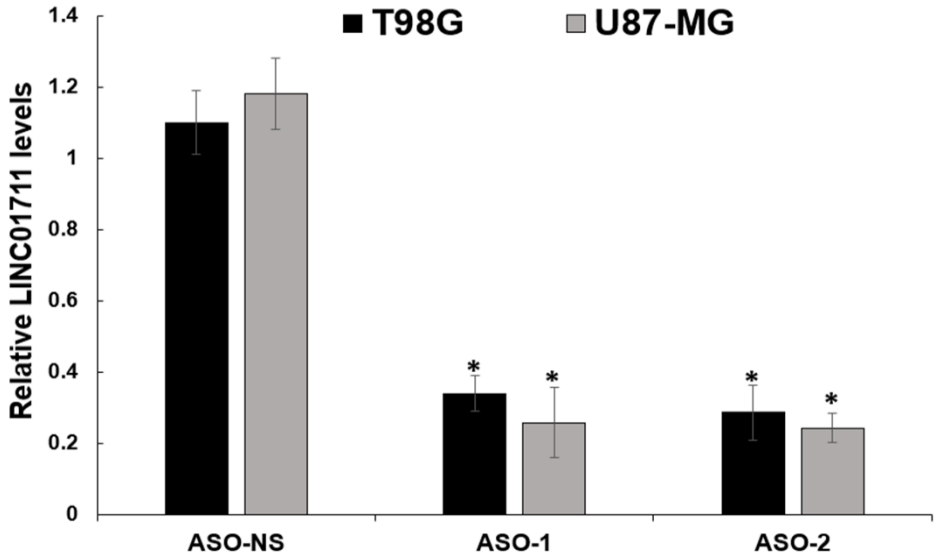


Fig 36: Validation of LINC01711 expression upon knockdown using ASOs. *ASO-mediated knockdown of LINC01711 in T98G and U87-MG cells. LINC01711 expression was efficiently knocked down by ASOs (~ 70% by ASO-1 and ~ 75 % by ASO-2) in T98G and U87-MG cells as detected by qRT-PCR assays. Values represent mean ± SEM from three independent experiments. *Significant change compared to cells transfected with control ASO (ASO-NS) ($p < 0.05$).*

WST cell proliferation assay indicated that LINC01711 depletion using ASOs results in a time-dependent reduction in cell proliferation in glioma cells. Cell proliferation was reduced by ~ 50% and ~ 75% at 48h and 72h post-LINC01711 knockdown in T98G cells (Fig 37 A). A similar ~ 65% reduction in cell proliferation was observed in LN229 and U87-MG glioma cells transfected with ASOs against LINC01711 compared to cells transfected with non-specific ASO (ASO-NS) (Fig 37 B & C). Depletion of LINC01711 significantly reduced colony formation by ~ 78% in T98G and U87-MG cells. (Fig 37 D & E). Knockdown of LINC01711 also results in apoptosis of GBM cells as demonstrated by an increase of ~ 3.5-fold, 2.5-fold, and 3-fold caspase 3/7 activity in T98G, LN229, and U87-MG, respectively, as compared to control cells (Fig 37 F).

Moreover, the depletion of LINC01711 also results in a decrease in glioma cell invasion and migration. Matrigel invasion assay revealed LINC01711 knockdown results in ~ 55% and ~ 70% inhibition of cell invasion in T98G and U87-MG cells, respectively (Fig 38 A & C). Wound healing assay revealed ~ 63% and ~ 58% reduction in migration in T98G and U87-MG cells upon LINC01711 knockdown (Fig 38 B & D).

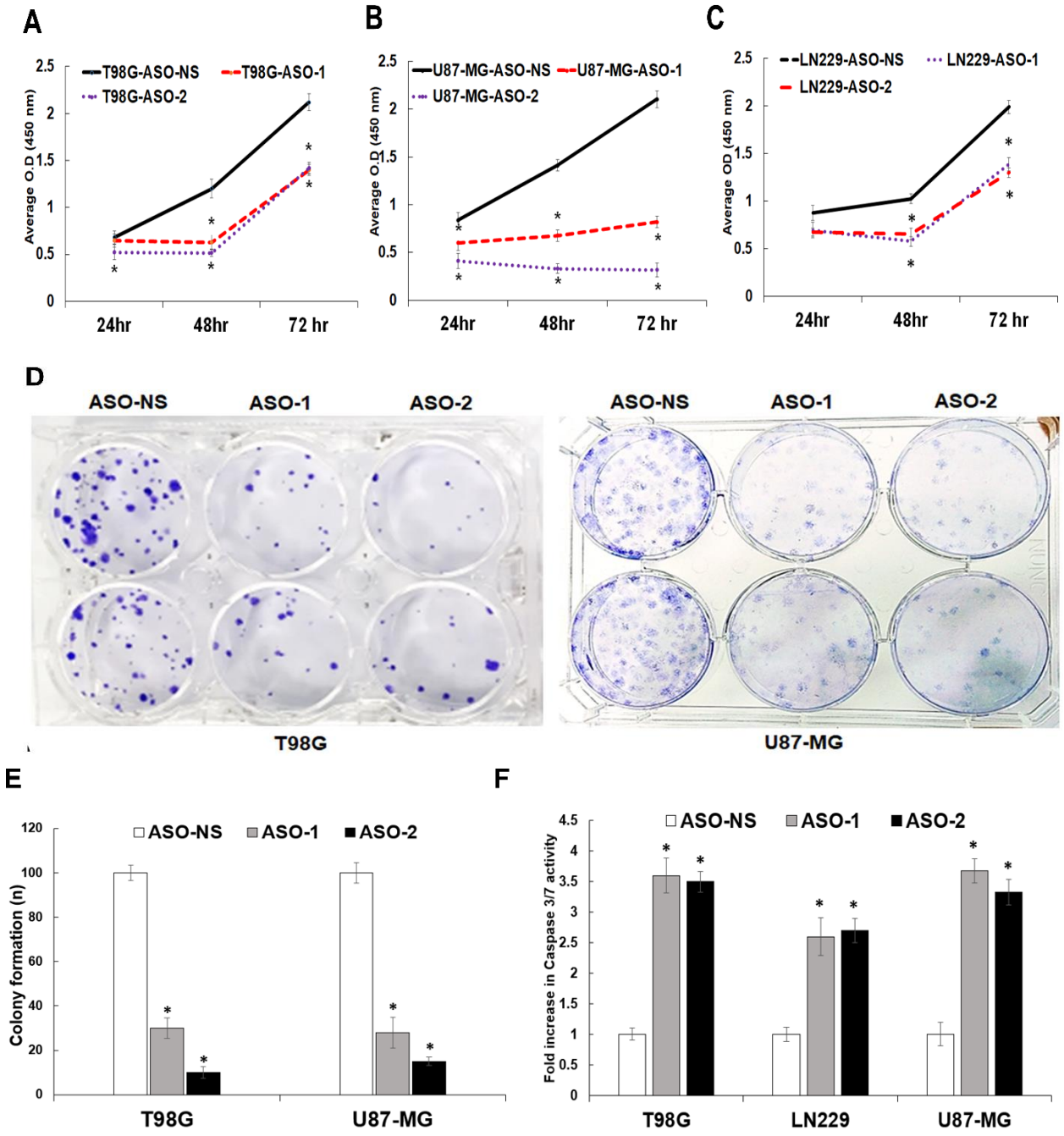


Fig 37: Physiological assays upon LINC01711 knockdown A. *T98G were transfected with ASO-NS or ASO-1/ ASO-2 (40nM), and percentage cell viability was calculated at indicated times.* ***B.*** *U87MG were transfected with ASO-NS or ASO-1/ ASO-2 (40nM), and percentage cell viability was calculated at indicated times.* ***C.*** *LN229 were transfected with ASO-NS or ASO-1/ ASO-2 (40nM), and percentage cell viability was calculated at indicated times. *Significant change compared to ASO-NS cells at the corresponding time ($p < 0.05$).* ***D.*** *Reduced colony formation ability of GBM cells with LINC01711 knockdown.* ***E.*** *Quantification of colony formation assay using ImageJ.* ***F.*** *LINC01711 knockdown induces apoptosis in GBM cells identified by Caspase 3/7 activity assay.*

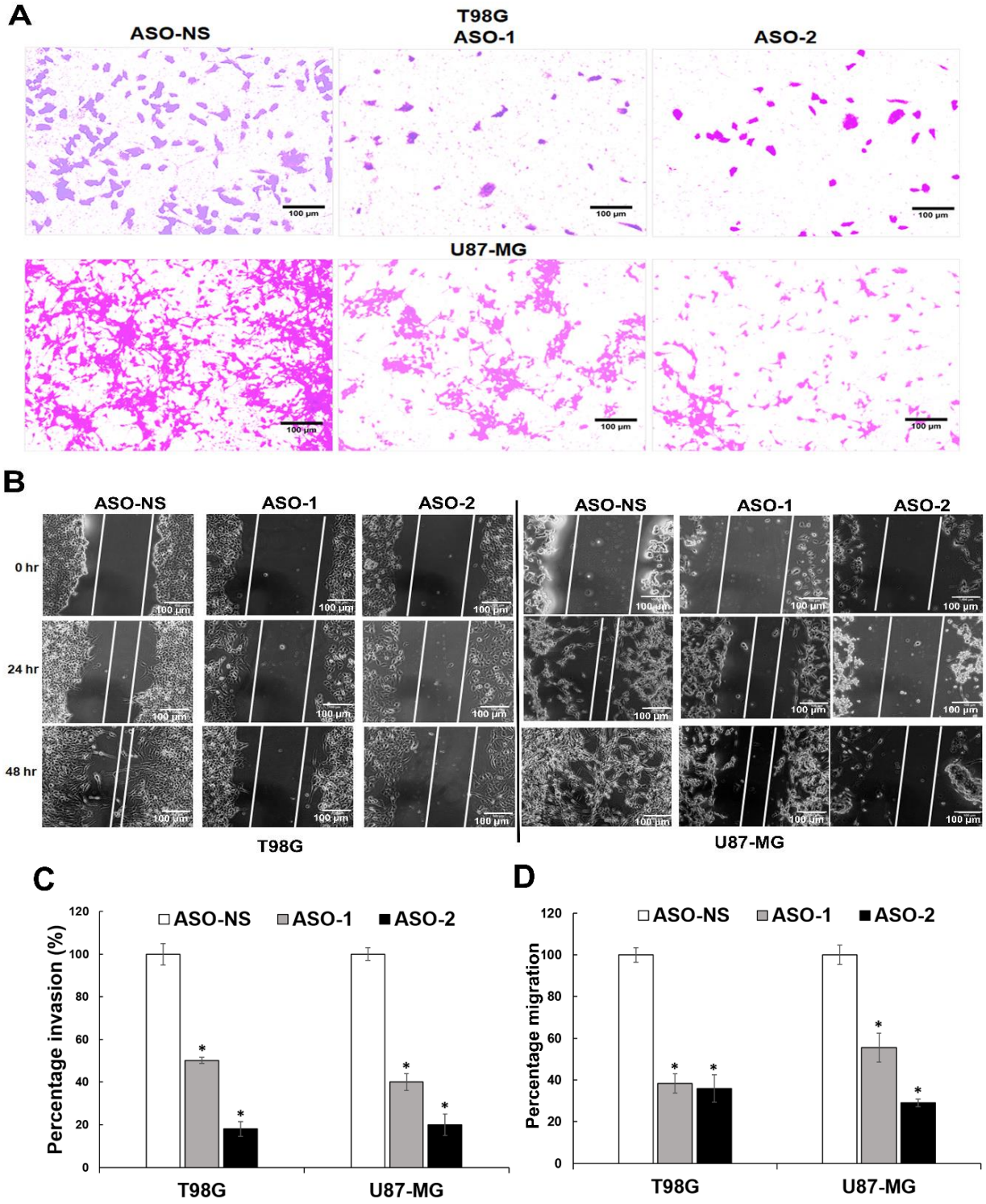


Fig 38: invasion and migration assays upon LINC01711 knockdown. A. *Matrigel invasion assay shows LINC01711 inhibition reduces glioma cell invasion.* ***B.*** *The wound healing assay demonstrates reduced GBM cell migration upon LINC01711 knockdown.* ***C.****Quantification of invasion assay using ImageJ.* ***D.*** *Quantification of invasion assay using ImageJ.*

3.3.5 LINC01711 regulates gene expression of a subset of TGF-β target genes in *cis* and *trans*

Since lncRNAs regulate gene expression in *cis* and *trans*, we first evaluated the effect of LINC01711 knockdown on its *cis* genes (Fig 39 A & B). We observed ~ 40% downregulation of the APCDD1L, ~ 44% downregulation of APCDD1L-DT, and ~ 80% downregulation of LOC107985410 in T98G and U87-MG upon LINC01711 knockdown with both the ASOs against LINC01711 by qRT-PCR (Fig 39 B). However, other *cis* genes (VAPB, RAB22A, STX16, and NPEPL1) remained unchanged upon LINC01711 knockdown. To further identify the genes regulated in *trans* by LINC01711, we evaluated the expression of the TGF-β gene ontology group upon LINC01711 knockdown. LINC01711 depletion resulted in ~ 40% downregulation of Slug, and VEGF, ~ 30% downregulation of ZEB1, ~ 37% downregulation of Col1A1, ~ 28% downregulation of Sox2 transcripts in T98G, and U87-MG cells (Fig 39 C). Several other TGF-β regulated genes did not show any change in expression with LINC01711 knockdown (Fig 39 D).

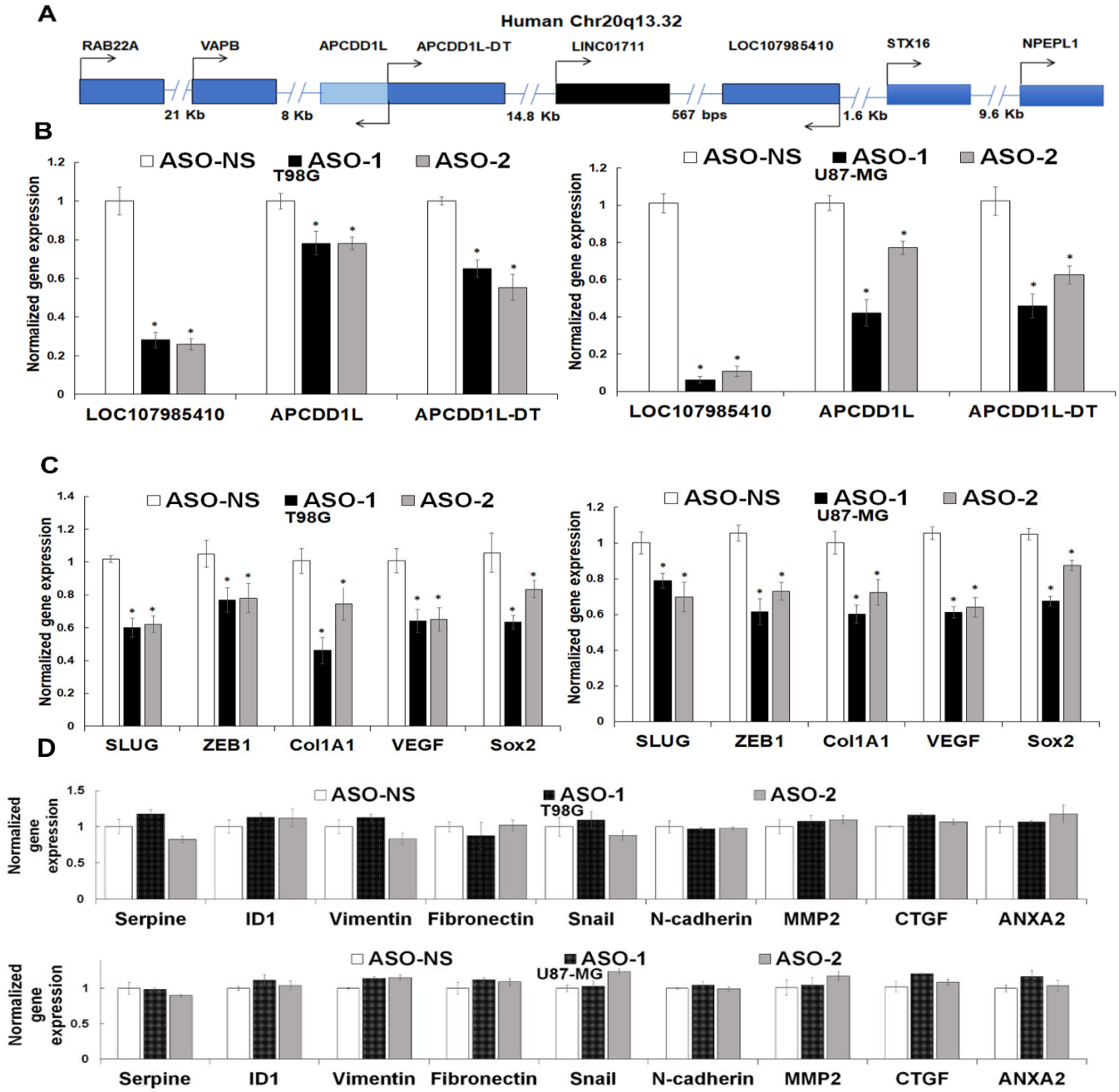


Fig 39: LINC01711 modulates gene expression in cis and trans. A. *Genomic location of LINC01711 and its cis genes.* ***B.*** *Validation of cis genes expression of LINC01711 with lncRNA knockdown. LncRNA knockdown represses cis genes – APCDD1L, APCDD1L-DT, and LOC107985410.* ***C.*** *LINC01711 modulates TGF-β target gene expression in trans in GBM. T98G and U87-MG glioma cells were transfected with ASO-NS or ASO-1/ ASO-2 (25 nM), and transcript levels of indicated genes were measured 48 h post-transfection. RNA samples were analyzed by qRT-PCR, and error bars represent the mean ± SEM from three independent experiments.* ***D.*** *Gene expression of TGF-β target genes upon LINC01711 knockdown measured by qRT-PCR assays in T98G and U87-MG cells.*

3.3.6 LINC01711 regulates the expression of mesenchymal markers in GBM cells

We next evaluated mesenchymal markers expression upon LINC01711 inhibition by western blotting. The knockdown of LINC01711 resulted in a ~ 60% decrease in ZEB1, a ~ 30% decrease in vimentin and N-cadherin, and a ~ 20% decrease in Snail1 protein levels in T98G and U87-MG cells (Fig 40 A & B). These results indicate that LINC01711 promotes the expression of mesenchymal markers in GBM.

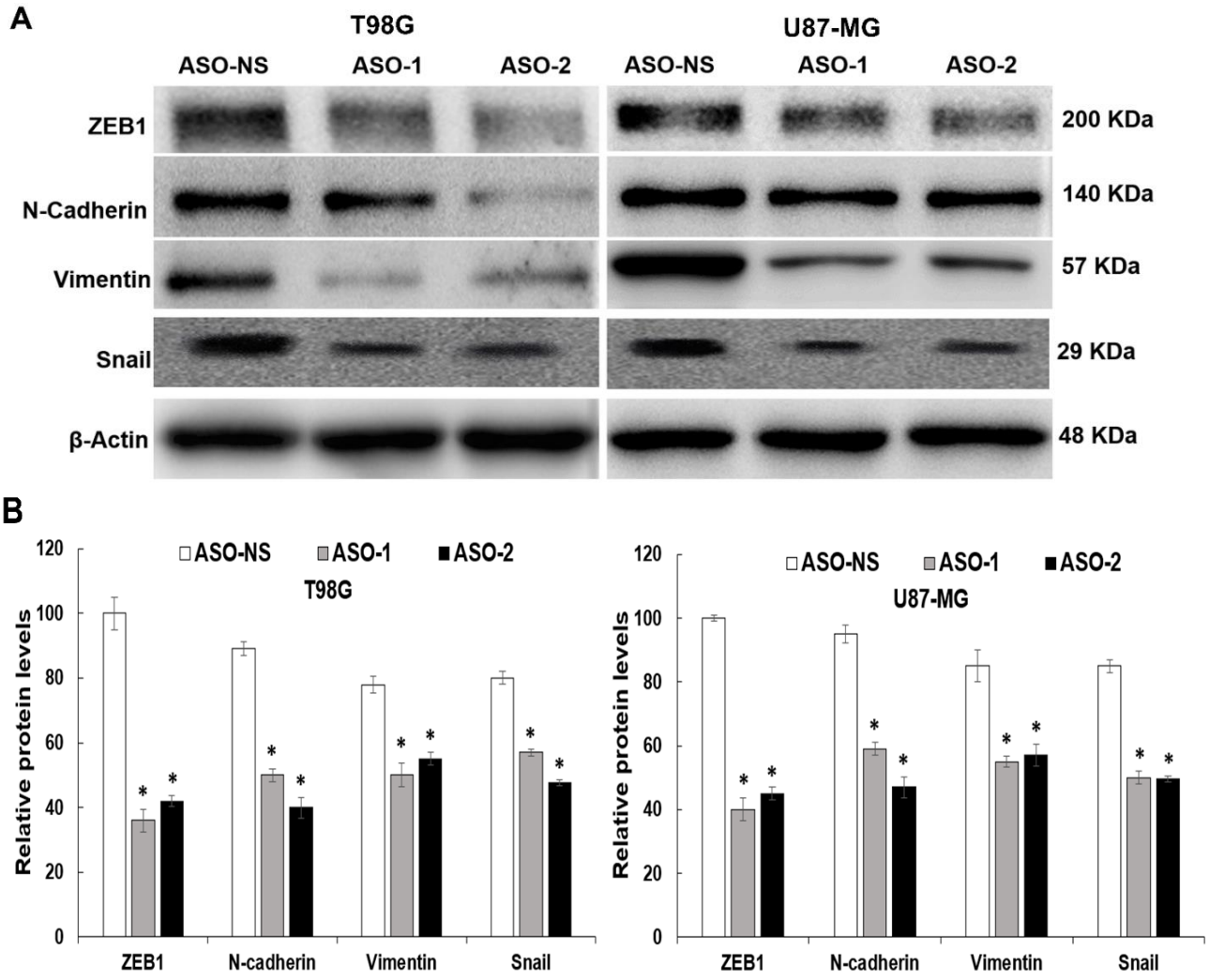


Fig 40: Evaluation of EMT markers upon LINC01711 knockdown by western blotting. A. *Western blot analysis of EMT markers- ZEB1, Vimentin, N-cadherin, and Snail1 followed by LINC01711 knockdown in T98G and U87-MG cells.* ***B.*** *Quantification of western blots using ImageJ. *Significant change compared to cells transfected with si-NS ($p < 0.05$). Values represent mean ± SD from three independent experiments.*

TGF-β-regulated lncRNAs regulate SMAD2/3 phosphorylation (292); therefore, we evaluated the impact of LINC01711 knockdown on TGF-β-induced phosphorylation of SMAD2/3. Silencing LINC01711 did not affect SMAD2/3 phosphorylation in GBM cells, indicating that LINC01711 acts downstream of SMAD2/3 in GBM (Fig 41).

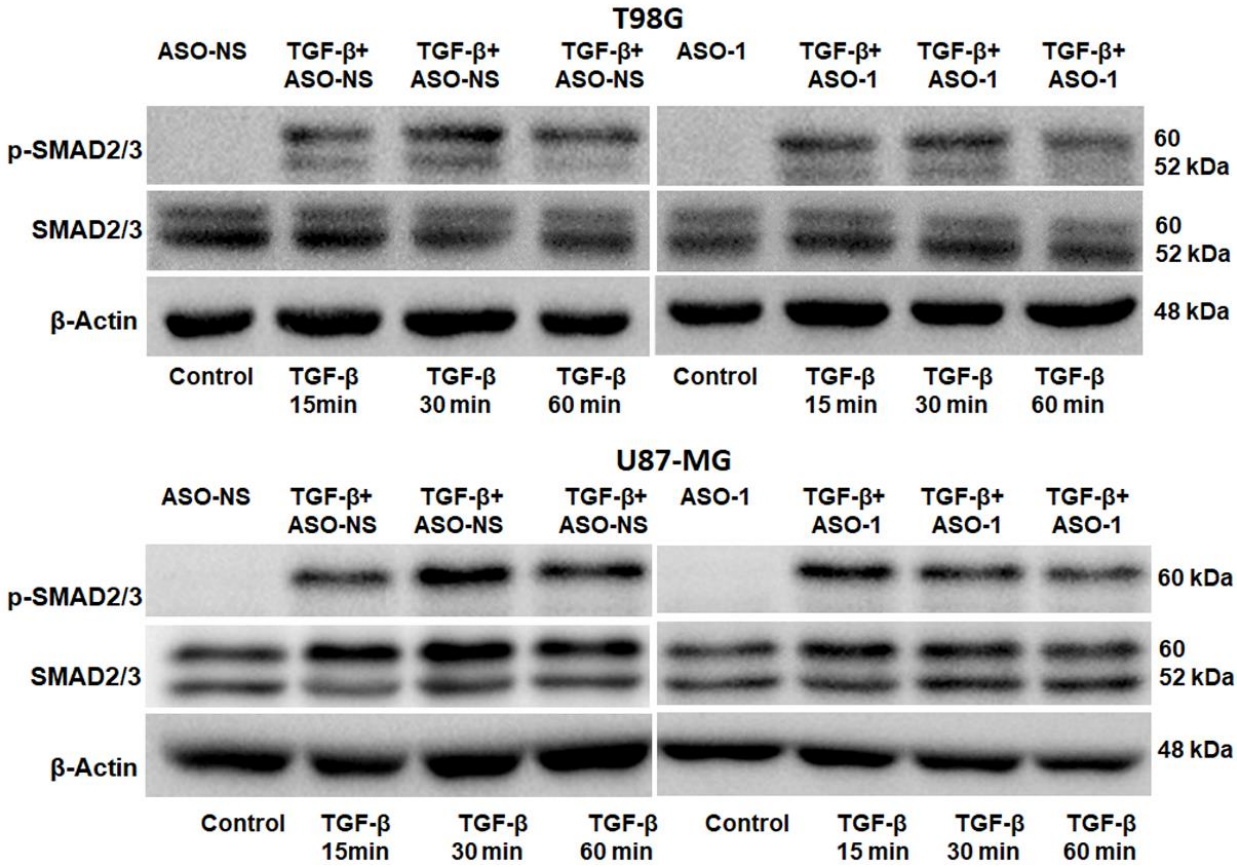


Fig 41: Effect of LINC01711 knockdown on TGF-β signaling. *LINC01711 knockdown does not affect the phosphorylation of the SMAD2/3 complex. Western blot analysis of pSMAD2/3 and total SMAD2/3 levels in T98G and U87-MG cells treated with 10 ng/ml TGF-β1 (15, 30, and 60 min), 48 h after LINC01711 knockdown with ASO-1.*

3.3.7 LINC01711 modulates TGF-β-induced invasion in glioma via miR-34a-5p/ZEB1/SNAIL1 axis

Previously reported results and our fractionation experiments indicated that LINC01711 is primarily located in the cytoplasm. LncRNAs localized in cytoplasm usually function as competing endogenous RNA (ceRNA) for miRNAs and modulate gene expression (260,265). Hence, we identified miRNA targets of LINC01711 using online databases: IntaRNA, DINABASE lncBASE V2, and TANRIC. Among these three databases, we found miR-34a-5p as a common miRNA target of LINC01711 (Fig 42 A). miR-34a has a potential tumor suppressor role in GBM and several other cancers (274,282). To confirm the interaction between LINC01711 and miR-34a-5p, we performed a luciferase reporter assay using the pmirGLO vector. However, we were unsuccessful in cloning the full-length LINC01711 despite multiple attempts due to multiple repeat regions. But we were successful in cloning the 421 base pair fragment from nucleotide 21 bps - 442 bps part of LINC01711 containing 7- miR-34a binding sites into the pmirGLO vector downstream of the firefly luciferase gene. Co-transfection of the pmirGLO-LINC01711 reporter plasmid and miR-34a mimics reduced the reporter activity significantly (~ 60%) compared to the control cells (Fig 42 B).

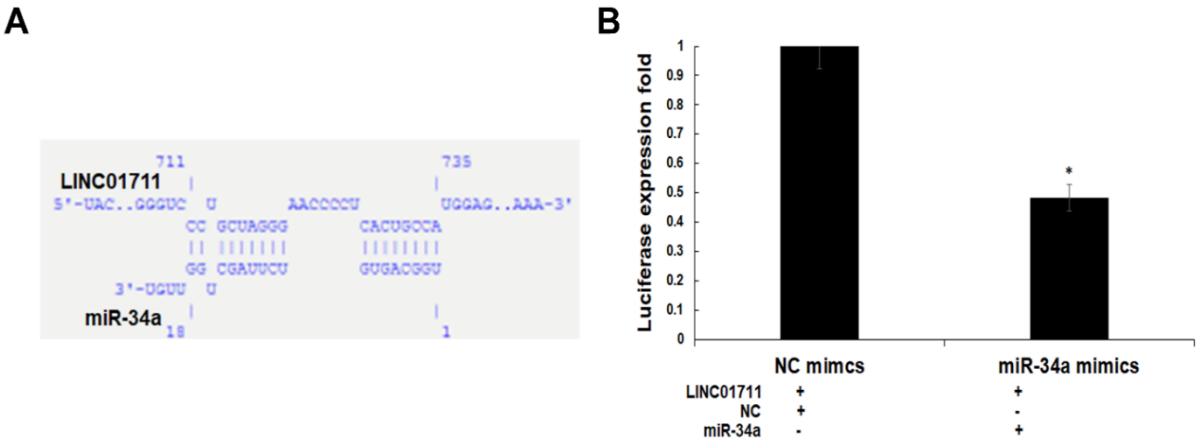


Fig 42: miR-34a interacts with LINC01711. A. *The predicted miR-34a binding sites on LINC01711.* ***B.*** *Luciferase activity assay indicates LINC01711 binds with miR-34a; relative luciferase activity was measured in HEK293T cells co-transfected with miR-34a-5p mimics and pmiRGLO-LINC01711 constructs. Luminescence signals were measured 30 h post-transfection using a dual luciferase assay. Data are shown as mean ± S.D. of three independent experiments; *p < 0.05.*

To understand the regulation of LINC01711 expression by miR-34a, we determined LINC01711 levels upon miR-34a overexpression using miRNA mimics. We observed a significant ~ 35% reduction in LINC01711 expression in T98G, LN229, and U87-MG cells upon treatment with miR-34a mimics (Fig 43).

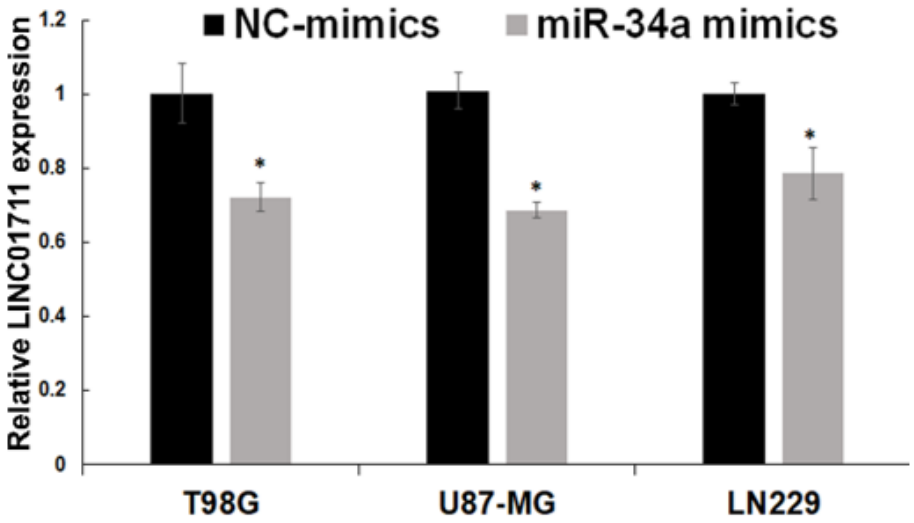


Fig 43: miR-34a targets LINC01711. A. *Downregulation of LINC01711 transcript levels upon miR-34a overexpression measured by qRT-PCR in T98G, U87-MG, and LN229 cells. T98G & U87-MG cells were transfected with 80nM of negative control mimics and miR-34a mimics. 48 h post-transfection, RNA was collected, and LINC01711 levels were analyzed by qRT-PCR.*

Given that miR-34a targets LINC01711, ZEB1, and SNAIL1, and because we observed downregulation of ZEB1 and SNAIL1 upon LINC01711 depletion, we explored if LINC01711 could act as a ceRNA to sponge miR-34a for stabilizing ZEB1 and SNAIL1 in glioma cells. Matrigel invasion assay revealed that miR-34a inhibition could partly rescue the reduction in invasion upon LINC01711 knockdown in T98G and U87-MG cells (Fig 44 A & B).

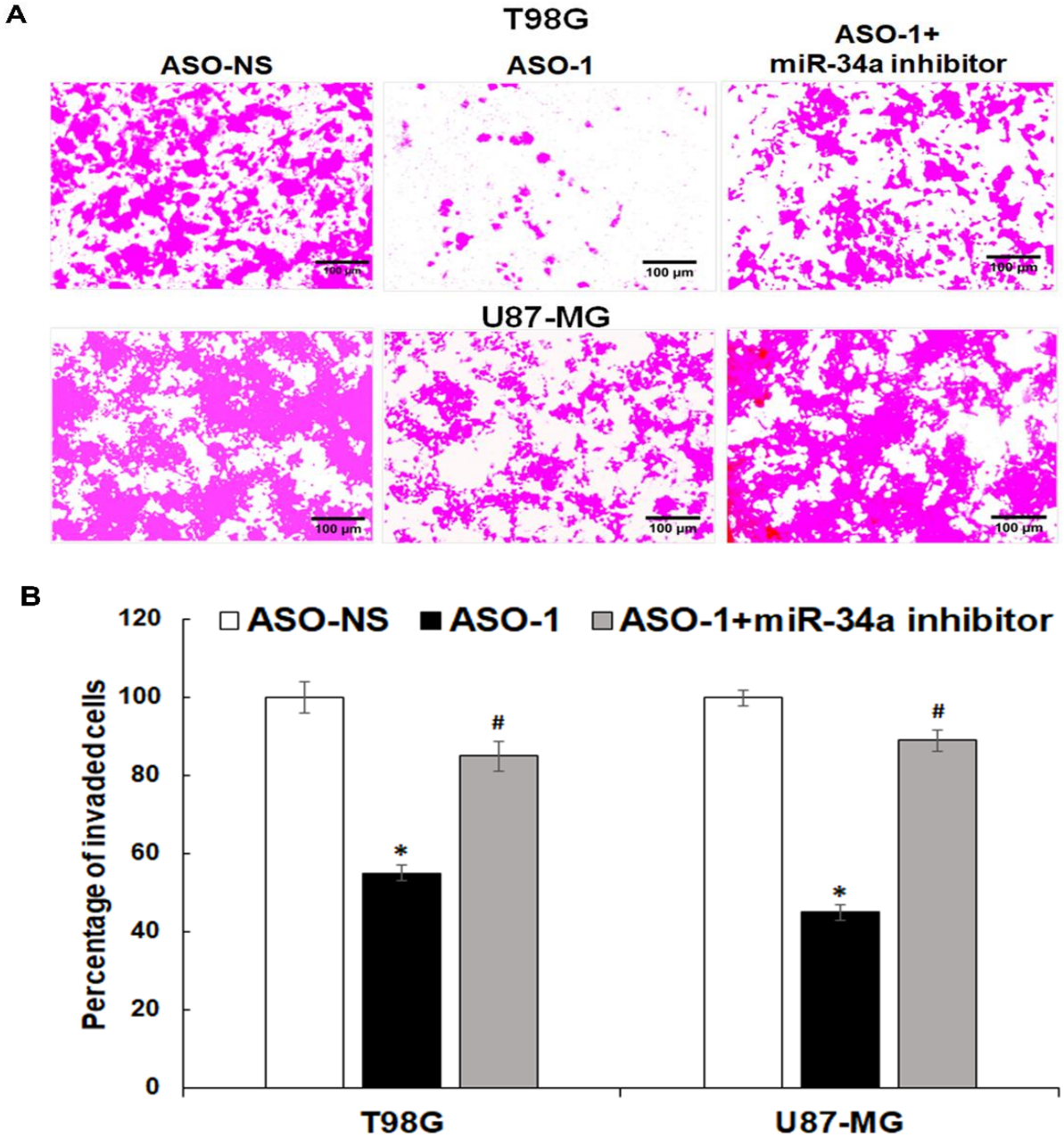


Fig 44: Rescue of invasion upon LINC01711 knockdown and miR-34a inhibition. A. *Rescue of invasion by LINC01711 knockdown upon miR-34a inhibition.* ***B.*** *Quantification of invasion using ImageJ.*

We observed that ZEB1 overexpression could partly reverse the LINC01711 knockdown-mediated reduction in the invasion of T98G and U87-MG GBM cells (Fig 45 A & B).

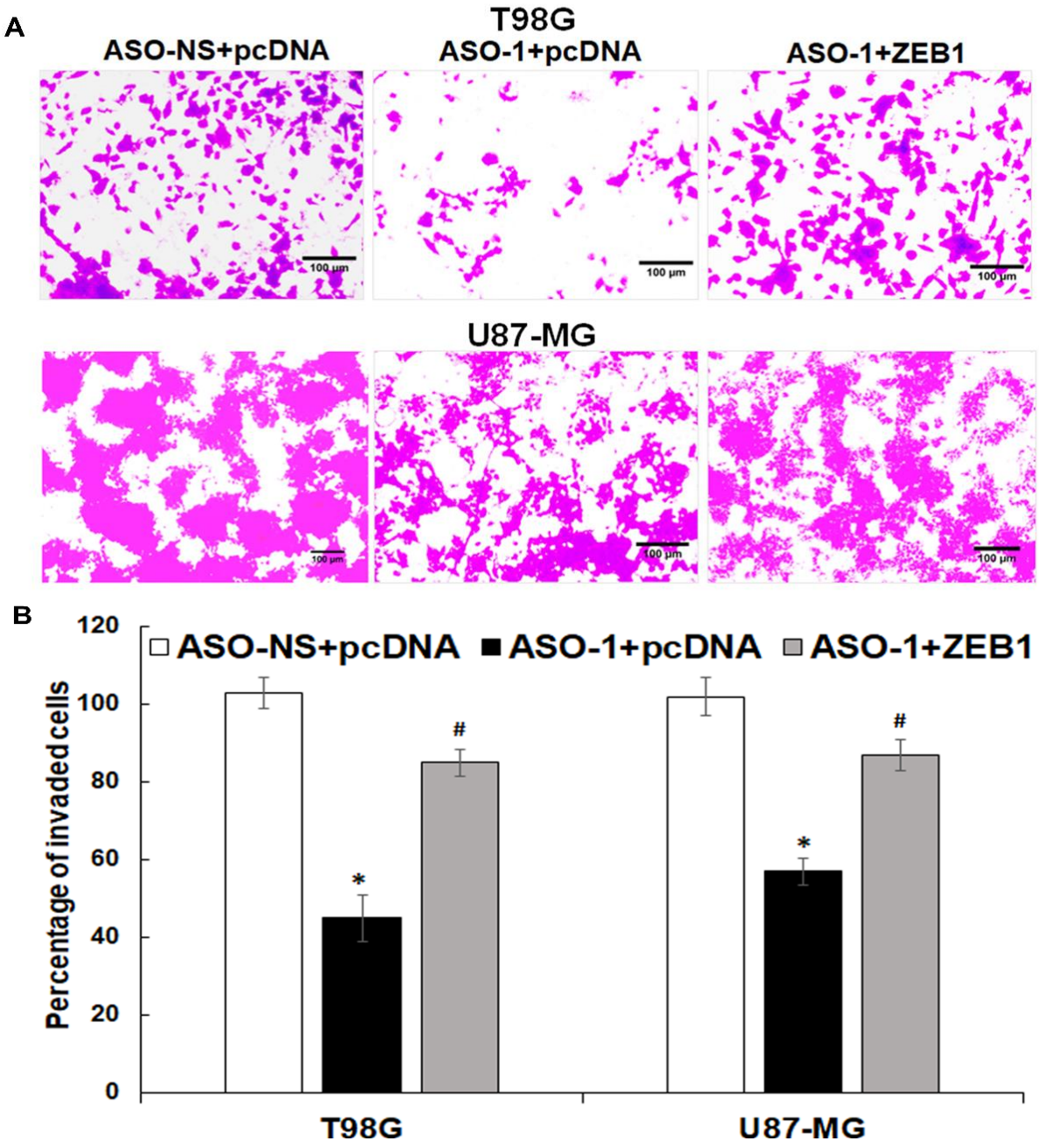


Fig 45: Rescue of invasion upon LINC01711 knockdown and ZEB1 overexpression. ***A.*** *Rescue of invasion during LINC01711 knockdown in GBM cells upon ZEB1 overexpression.* ***B.*** *Quantification of invasion using ImageJ.*

Also, SNAIL1 overexpression could partly reverse the LINC01711 knockdown-mediated reduction in the invasion of T98G and U87-MG GBM cells (Fig 46 A & B).

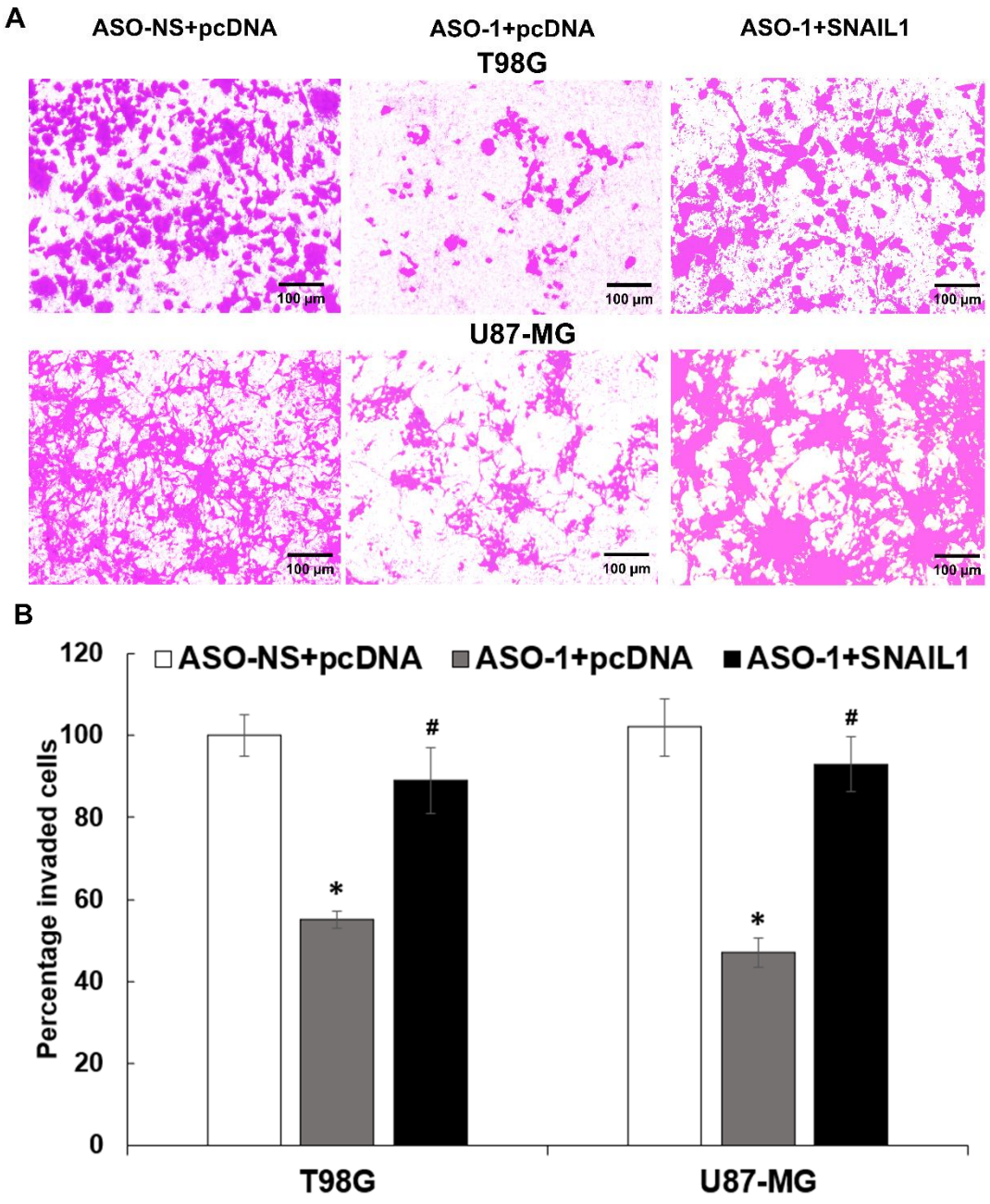


Fig 46: Rescue of invasion upon LINC01711 knockdown and SNAIL1 overexpression. A. *Rescue of invasion during LINC01711 knockdown in GBM cells upon SNAIL1 overexpression.* ***B.*** *Quantification of invasion using ImageJ.*

Further, western blot analysis revealed that the downregulation of ZEB1 and SNAIL1 protein levels caused by LINC01711 knockdown was partially rescued upon combined depletion of LINC01711 and miR-34a (Fig 47 A & B). These experiments indicate that TGF-β induced LINC01711 sponges miR-34a to promote ZEB1 and SNAIL1-induced invasion in GBM.

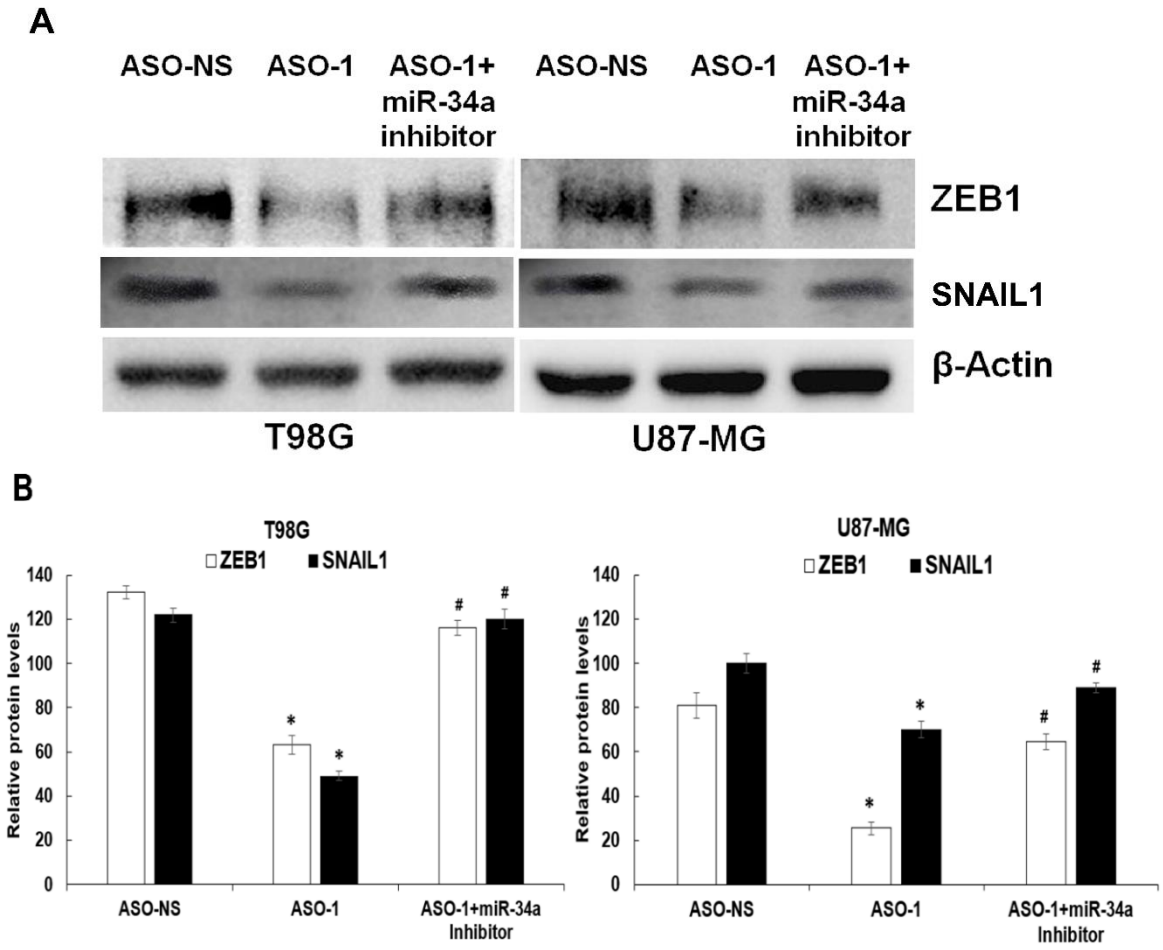


Fig 47: Rescue of ZEB1 and SNAIL1 expression upon combined knockdown of LINC01711 and miR-34a. A. *Reduction in ZEB1 and SNAIL1 protein levels caused by LINC01711 knockdown in T98G and U87-MG glioma cells upon miR-34a inhibition. A representative blot is shown from three independent experiments with similar results. Blots were reprobed for β-actin to establish equivalent loading.* ***B.*** *Quantification of blots by ImageJ.*

3.3.8 LINC01711 promotes drug resistance in GBM

We next tested if LINC0711 knockdown could sensitize TMZ-resistant glioma cell lines T98G and LN229 to TMZ-mediated apoptosis. LINC01711 depletion with low ASO (15nM) levels sensitized T98G and LN229 cells to TMZ-mediated inhibition of cell proliferation (Fig 48.1 A). In addition, TMZ treatment in LINC01711 knockdown cells significantly increased caspase 3/7 activity (~ 4-fold in T98G and ~5.5 fold in LN229 cells) compared to cells treated with TMZ alone (Fig 48.1 B). These results suggest that LINC01711 knockdown sensitizes glioma cells to TMZ-induced apoptosis.

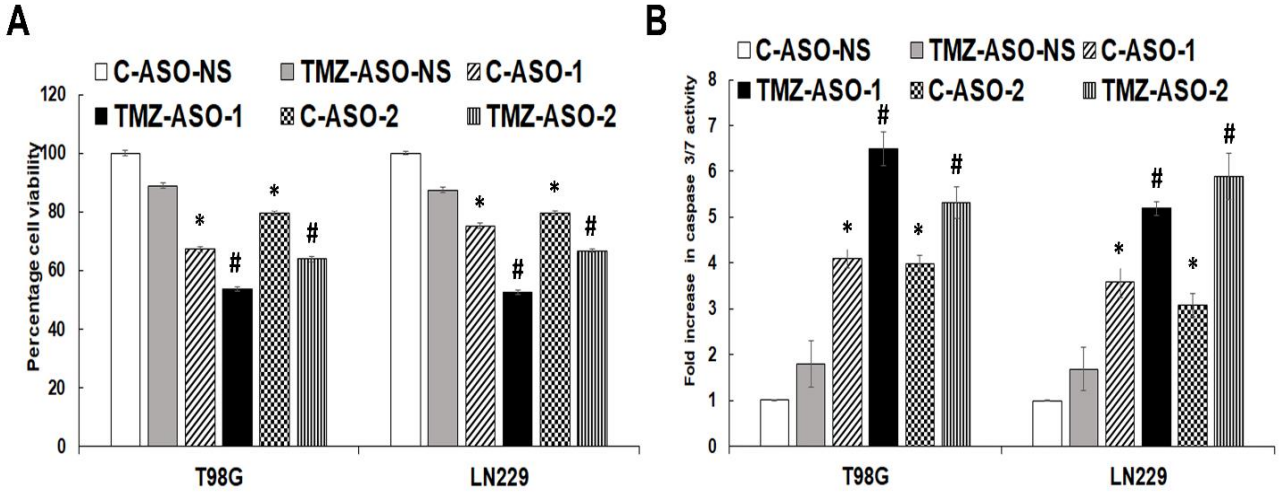


Fig 48.1: LINC01711 induces TMZ resistance in GBM cells. A. *LINC01711 knockdown with a low concentration of ASO-1/ ASO-2 (15 nM) in combination with TMZ treatment (600 µM) shows enhanced reduction of glioma cell (T98G and LN229) proliferation and increased sensitivity to TMZ, as analyzed using WST1 assay.* ***B.*** *LINC01711 knockdown (15nM of ASO-1/ ASO-2) combined with TMZ treatment (600 µM) shows enhanced caspase 3/7 activity compared to TMZ alone. *Significant increase in fold change compared to ASO-NS treated cells ($p<0.05$). $^{\#}$Significant change compared to ASO-1/2 treated cells ($p<0.05$). Values represent mean ± S.D. from three independent experiments.*

We also tested the effect of LINC01711 depletion on cisplatin-mediated apoptosis in GBM cells. Knockdown of LINC01711 also sensitized GBM cells to cisplatin-induced apoptosis (Fig 48.2 A & B).

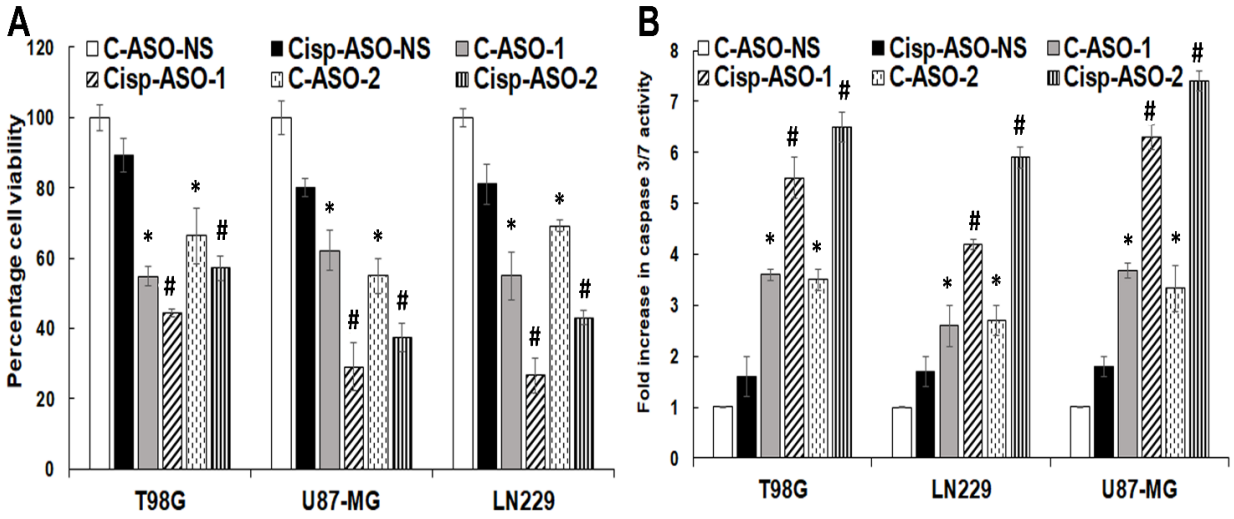


Fig 48.2: LINC01711 induces cisplatin resistance in GBM cells. A. *LINC01711 knockdown with a low concentration of ASO-1/ASO-2 (15 nM) in combination with cisplatin (20 µM) treatment shows enhanced reduction of glioma cell (T98G, LN229, and U87-MG) proliferation and increased sensitivity to cisplatin, as analyzed using WST-1 proliferation assay.* ***B.*** *LINC01711 knockdown with a low concentration of ASO-1/ASO-2 (15 nM) in combination with cisplatin (20 µM) treatment shows enhanced glioma cell (T98G, LN229, and U87-MG) apoptosis and increased sensitivity to cisplatin, as analyzed using caspase 3/7 assay. *Significant change compared to cells transfected with control ASO (ASO-NS); #Significant change compared to cells transfected with C-ASO-1 and C-ASO-2 ($p < 0.05$).*

3.3.9 Discussion

Li et al. reported that LINC01711 is an immune-related lncRNA, and it is associated with a poor prognosis of low-grade glioma (315). However, the exact function and mechanism of action of LINC01711 in GBM pathogenesis were unknown. We show that TGF-β upregulates LINC01711 in GBM (Fig 35 A). LINC01711 is overexpressed in GBM tissues (Fig 33 A), and high expression of LINC01711 confers poor survival in GBM patients (Fig 33 B).

Next, we looked at the transcription machinery causing the induction of LINC01711 upon TGF-β stimulation. In a previous study, Vishnubalaji et al. analyzing SMAD2/3 ChIP-seq datasets from the GEO database, reported the possible SMAD2/3 binding site in the promoter of LINC01711 (317). In line with these findings, we observed direct binding of SMAD2/3 at the promoter of LINC01711 using ChIP-qPCR assays (Fig 35 C & D).

Loss of function studies demonstrated that LINC01711 promotes GBM cell proliferation, migration, and invasion in GBM. Moreover, LINC01711 depletion also sensitizes GBM cells to TMZ-mediated apoptosis (Fig 37, 38, 48.1 & 48.2).

Further, we looked at the *cis* and *trans* genes regulated by LINC01711 in GBM. Our results suggest that LINC01711 induces the expression of its *cis* genes APCDD1L, APCDD1L-DT, and LOC107985410 (Fig 39 A & B). Notably, APCDD1L-DT lncRNA is upregulated in lung squamous cell carcinoma, lung adenocarcinoma, and high-grade gliomas (320–322). Also, the APCDD1L gene is reported to play an oncogenic role in papillary thyroid cancer (323). Further studies are required to confirm if the regulation of these genes by TGF-β and LINC01711 contributes to GBM pathogenesis. Among the TGF-β ontology group, LINC01711 regulates the expression of ZEB1, N-cadherin, vimentin, SNAIL1, SLUG, VEGF, and Col1A1 in *trans* (Fig 39 C; Fig 40 A & B).

Like lncRNA-MUF, LINC01711 is primarily localized in the cytoplasm and directly binds with miR-34a (Fig 42 A & B). Moreover, overexpression of miR-34a significantly reduced LINC01711 levels. Since miR-34a binds to and reduces the levels of LINC01711, SNAIL1, and ZEB1, we hypothesized that, like lncRNA-MUF, LINC01711 also acts as a ceRNA for miR-34a to promote SNAIL1 and ZEB1-mediated invasion in glioma cells. Consistent with our hypothesis, we observed that ZEB1 or SNAIL1 overexpression could partly reverse the LINC01711 knockdown-mediated

reduction in GBM invasion. However, the mechanism of regulation of SLUG, VEGF, and Col1A1 by LINC01711 needs further investigation.

Chapter 4

Summary and conclusion

GBM diagnosis and therapy have not significantly improved despite decades of efforts. LncRNAs regulate all aspects of cancer pathogenesis. Aberrant TGF-β signaling in GBM alters lncRNA expression and *vice versa* to promote malignant phenotypes of glioma. The essential role played by TGF-β in cancer progression makes it an attractive therapeutic target for cancer treatment. However, given the multiple functions of the TGF-β in normal physiology, its complete inactivation for cancer treatment is not ideal. LncRNAs regulated by the TGF-β pathway modulate numerous aspects of GBM pathogenesis and may serve as potential diagnostic tools and attractive therapeutic targets.

Our microarray screen identified 91 differentially expressed lncRNAs upon TGF-β treatment in T98G GBM cells comprising 18.3% of the DEGs. Among the DE lncRNAs, we further characterized the role of lncRNA-MUF and LINC01711 in GBM pathogenesis. TCGA dataset analysis revealed a significant increase in the expression of lncRNA-MUF and LINC01711 in GBM tumor tissues compared to control tissues. Moreover, this increase in the expression of lncRNA-MUF and LINC01711 in GBM is correlated with an increase in GBM grade. Overexpression of lncRNA-MUF and LINC01711 is also correlated with poor survival in GBM patients, as revealed by analysis from the CGGA database. These results demonstrate the potential of these lncRNAs as tools for diagnosis and prognosis for GBM.

We found several putative SBEs in the promoters of lncRNA-MUF and LINC01711. qRT-PCR analysis of lncRNA-MUF and LINC01711 with TGF-β treatment, with the TGFBR1 or SMAD2/3 inhibitor, and ChIP-qPCR assays demonstrated that lncRNA-MUF and LINC01711 are induced through SMAD2/3 transcription factors upon TGF-β stimulation.

LncRNA-MUF and LINC01711 depletion studies suggest they promote cell proliferation, migration, and invasion in GBM. In addition, lncRNA-MUF and LINC01711 depletion sensitize glioma cells to TMZ-mediated apoptosis. These results indicate the potential of these lncRNAs as therapeutic targets for GBM.

LncRNA-MUF depletion studies revealed that it regulates the expression of Caprin2, vimentin, CTGF, c-Myc, ZEB1, and SNAIL1. Loss-of-function studies of LINC01711 revealed that it regulates the expression of APCDD1L, APCDD1L-DT, and LOC107985410 *in cis*. LINC01711 also regulates the expression of ZEB1, SNAIL1,

SLUG, VEGF, and Col1A1 in *trans*. We show that miR-34a directly binds to lncRNA-MUF and LINC01711 to downregulate them. miR-34a also targets SNAIL1 and ZEB1, which are regulated by both lncRNA-MUF and LINC01711. Overall our results demonstrate that lncRNA-MUF and LINC01711, sponge miR-34a, to stabilize ZEB1 and SNAIL1 expression in GBM (Fig 49). Our results provide compelling evidence for targeting miR-34a and lncRNA-MUF/LINC01711 axis for GBM therapy.

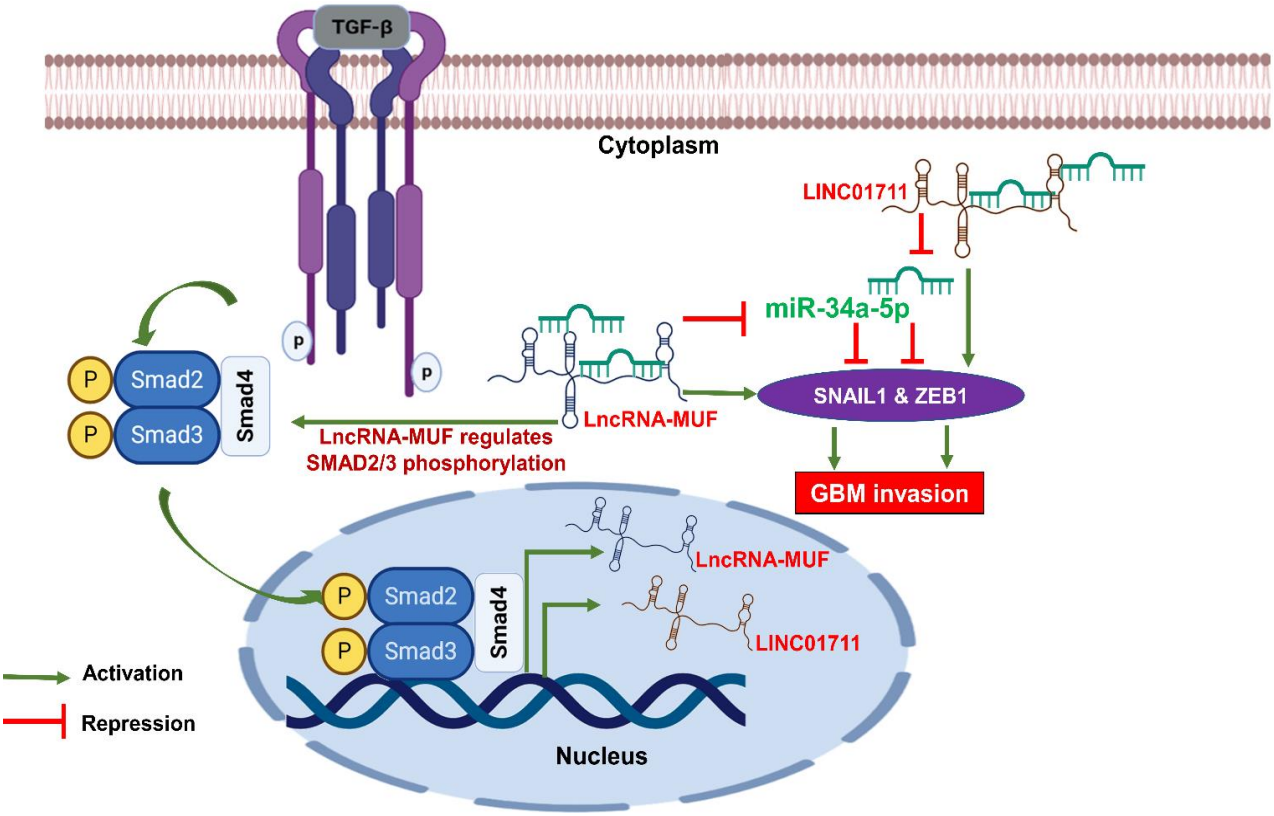


Fig 49: A working model of TGF-β-induced lncRNA-MUF and LINC01711 in promoting GBM invasion. *TGF-β-regulated lncRNA-MUF and LINC01711 promote GBM invasion by sponging miR-34a, titrating its levels, and preventing its binding to its target genes, ZEB1 and SNAIL1.*

Specific contributions

TGF-β-regulated lncRNAs modulate several aspects of tumor development, such as proliferation, invasion, metastasis, EMT, and chemoresistance in various cancers, including GBM. We discovered that TGF-β induces lncRNA-MUF and LINC01711 expression in GBM. Their expression is significantly upregulated in glioma tissues and is associated with poor overall survival of GBM patients. LncRNA-MUF and LINC01711 promote proliferation, migration, invasion, and drug resistance in GBM cells. LncRNA-MUF and LINC01711 act as ceRNA for miR-34a and promote ZEB1 and SNAIL1-mediated GBM invasion. Hence collectively, our results suggest lncRNA-MUF and LINC01711 as attractive therapeutic targets for GBM.

Future scope of work

We have characterized the role of TGF-β-regulated lncRNA-MUF and LINC01711 in promoting GBM pathogenesis by acting as a ceRNA for miR-34a and promoting ZEB1 and SNAILl expression. Our studies point to post-transcriptional gene regulation of ZEB1 and SNAIL1 by lncRNA-MUF and LINC01711. However, there is a need to do a whole transcriptome analysis upon their depletion to identify the complete set of their target genes. LncRNAs also exert their function by interacting with proteins. Further studies are required to determine the interacting protein partners of lncRNA-MUF and LINC01711 to understand their complete role in the TGF-β pathway and GBM pathogenesis.

Moreover, our findings need to be extended by studying the expression and function of lncRNA-MUF and LINC01711 in low-passage glioma patient-derived cell models and glioma stem cells. The use of ASOs as therapeutic options is gaining acceptance, and it is approved for a few diseases, such as hypercholesterolemia and Duchenne muscular dystrophy (324). However, oligonucleotide therapies for cancers are still in their infancy. Pre-clinical studies and *in vivo* experiments to explore the role of ASO targeting lncRNA-MUF and LINC01711 along with TMZ treatment in GBM are required to evaluate the true potential of lncRNA-MUF and LINC01711 as a therapeutic target for GBM.

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Appendix 1
List of primers used for qRT-PCR

LncRNA-MUF-FP1	CCTCCAACCCCCTTTTCTCC
LncRNA-MUF-RP1	GAAGGCAGGAAGTCTGTGCT
LncRNA-MUF-FP2	ACCACTACACTCAGCCAAATAC
LncRNA-MUF-RP2	GGCTATCAACTGTCTCCTTTAGAC
ENST00000409910-FP	CACTTGCTTCCTTTGCCACC
ENST00000409910-RP	TGCTTTGCCAACCAAAACCA
LINC01711-FP1	GGAGACCTGTGGTTCTCAGC
LINC01711-RP1	GAGAGAAACGGCTCCAGACC
LINC01711-FP2	GGTCTGGAGCCGTTTCTCTC
LINC01711-RP2	GGAGGAGAGGGGTTCTCCAT
LINC00312-FP	CTGTGAGAGCAACAGGAGCA
LINC00312-RP	TCCTGTGTGAGCTTCCGTTC
LOC101928710-FP	ATCCAACACAGTCAGCCAGG
LOC101928710-RP	AAGACAGCATAGCCCACACC
lnc-EGR2-1-FP	GAGTTGGCGGATGAACACGA
lnc-EGR2-1-RP	ATGGCAAGAGCAACTCAGGC
CTB-178M22.2-FP	AGAGCAACTGGAGCAAGTCC
CTB-178M22.2-RP	GCTGGAAGAGGATAGGCCAC
KCNMA1-AS1-FP	TCTTCCCAACCTGCCAAGAC
KCNMA1-AS1-RP	GTTCCTCCCAATGTCCCCAG
Vimentin-FP	CAGCTAACCAACGACAAA
Vimentin-RP	CGTGGAGTTTCTTCAAAAAG
SNAI1-FP	CAATCGGAAGCCTAACTA
SNAI1-RP	CAGATGAGCATTGGCAGCG
CTGF-FP	GTTTGGCCCAGACCCAACTA
CTGF-RP	GGCTCTGCTTCTCTAGCCTG
cMYC-FP	TCAAGAGGCGAACACACAAC
cMYC-RP	GGCCTTTTCATTGTTTTCCA
Caspase9-FP	CGAACTAACAGGCAAGCAG
Caspase9-RP	ACCTCACCAAATCCTCCAGAAC
CAPRIN2-FP	TGCCTGGGGAGTCACCTT

CAPRIN2-RP	TGACTGTGGTTCACTTGGGG
ANXA2-FP	CTTTCCTGGAGAGGGAGAAATG
ANXA2-RP	ATCGCTTTGGCTACTCGTAAA
Zeb1-FP	ACTCTGATTCTACACCGC
Zeb1-RP	TGTCACATTGATAGGGCTT
YY1-FP	ACGGCTTCGAGGATCAGATTC
YY1-RP	TGACCAGCGTTTGTTCAATGT
YBX1-FP	GGGTGCAGGAGAACAAGGTA
YBX1-RP	TCTTCATTGCCGTCCTCTCT
Serpine1-FP	GGCTGACTTCACGAGTCTTTCA
Serpine1-RP	ATGCGGGCTGAGACTATGACA
HMGA2-RP	AGCGCCTCAGAAGAGAGGAC
HMGA2-RP	GAGCTGCTTTAGAGGGACTCTTGT
Col1A1-FP	CCCCTGGTGCTACTGGTTTCCC
Col1A1-RP	GACCTTTGCCGCCTTCTTTGC
TBP-FP	GAGCTGTGATGTGAAGTTTCC
TBP-RP	TCTGGGTTTGATCATTCTGTAG
HPRT-FP	TGAGGATTTGGAAAGGGTGT
HPRT-RP	GAGCACACAGAGGGCTACAA
JUNB-FP	ACTCATACACAGCTACGGGATACG
JUNB-RP	GGCTCGGTTTCAGGAGTTTG
MMP10-FP	GTCTCTCTACGGACCTCCCC
MMP10-RP	GGGTTCCAGTGGGATCTTCG
MMP13-FP	TGGCTGCCTTCCTCTTCTTG
MMP13-RP	GCTCTGCAAACTGGAGGTCT
LAMP3-FP	GGAAATGTGGATGAGTGCTCG
LAMP3-RP	CCCCCGGGCAACAATTAGAT
TNFAIP6-FP	GCTACAACCCACACGCAAAG
TNFAIP6-RP	CCAAGCAACCTGGGTCATCT
EGR2-FP	CAGGAGAGAGTCAGTGGCAAA
EGR2-RP	CTCGCTACCTGGAGTGTCAG
Lefty-FP	AGAGCTGGCGATGACTGAAC
Lefty-RP	AAACTGAGCAAGGGCTCTCC

KCNB1-FP	CCCGCTACCACCAGAAGAAA
KCNB1-RP	TGGCAAGGATCTTGGCAGC
KIT-FP	TACTTGGAGCCTGCACCATT
KIT-RP	TATCGCTGCAGGAAGACTCC
SMAD2 FP	GGGTTTTGAAGCCGTCTATCAGC
SMAD2 RP	CCAACCACTGTAGAGGTCCATTC
SMAD3 FP	GCCTGTGCTGGAACATCATC
SMAD3 RP	TTGCCCTCATGTGTGCTCTT
WNT5a-FP	AGGGCTCCTACGAGAGTGCT
WNT5a-RP	GACACCCCATGGCACTTG
GSK3β-FP	TCGAGAGCTCCAGATCATGAGAA
GSK3β-RP	CGGAACATAGTCCAGCACCAGA
FZD8-FP	GCTCTACAACCGCGTCAAGA
FZD8-RP	GCTGAAAAAGGGGTTGTGGC
LRP6-FP	TTGTTGCTTTATGCAAACAGACG
LRP6-RP	CGTTTAATGGCTTCTTCGCTGAC
CDK5-FP	GGGAAGGCACCTACGGAACTG
CDK5-RP	GGCGGAACTCGGCACACC
c-JUN-FP	TCCAAGTGCCGAAAAAGGAAG
c-JUN-RP	CGAGTTCTGAGCTTTCAAGGT
IPO8-FP	CATGATGCCTCTCCTGCATAA
IPO8-RP	CTTCTCCTGCATCTCCACATAG
LOC645485-FP	AAGATGCTCCTGCCACCTTC
LOC645485-RP	GTCAAAACGCCAGAGGGAGA
LOC107984476-FP	CAATGTCTCGCATGAAGCGG
LOC107984476-RP	AGCTCTTTCCAGATGTGCCC
miR-34a-5p-FP	TGGCAGTGTCTTAGCTGGTTGT
U6-FP	CTCGCTTCGGCAGCACA
U6-RP	AACGCTTCACGAATTTGCGT

Appendix 2

List of differentially expressed lncRNAs upon TGF-β1 (10 ng/ml) treatment in T98G GBM cells identified by microarray screen

S.No	LncRNA ID	Fold change	p-value
1	ENST00000409910	6.732	0.025
2	lnc-CLEC18B-3	4.287	0.002
3	ENST00000435044	4.127	0.000
4	LOC79160	3.989	0.000
5	LINC01049	3.609	0.005
6	LINC00312	3.551	0.000
7	ENST00000563424	3.379	0.000
8	lnc-MSRB3-2	3.336	0.001
9	FAM222A-AS1	3.292	0.023
10	LINC01405	3.264	0.001
11	lnc-CNIH3-1	3.126	0.020
12	lnc-FSIP1-1	2.949	0.000
13	lnc-AIRE-1	2.930	0.001
14	lnc-CHAF1B-2	2.833	0.004
15	ENST00000511422	2.800	0.019
16	lnc-CLCN3-3	2.789	0.034
17	ENST00000539116	2.738	0.010
18	LOC101927253	2.660	0.032
19	lnc-COX10-1	2.624	0.000
20	ENST00000564772	2.596	0.003
21	LOC101928710	2.593	0.000
22	lnc-EGR2-1	2.565	0.001
23	lnc-RP11-597K23.2.1-2	2.448	0.024
24	XLOC_l2_010963	2.440	0.004
25	TMEM92-AS1	2.403	0.001
26	LOC102725378	2.394	0.001
27	A_33_P3247072	2.284	0.000

28	LOC102723834	2.105	0.000
29	lnc-ZNF643-1	2.075	0.024
30	lnc-NACA2-2	2.008	0.047
31	lnc-RCL1-1	2.007	0.015
32	ENST00000418271	1.955	0.042
33	LINC01137	1.942	0.000
34	LOC100130417	1.922	0.002
35	ENST00000444114	1.914	0.002
36	ENST00000576365	1.882	0.015
37	LINC01546	1.876	0.005
38	lnc-ICOSLG-2	1.853	0.001
39	lnc-CCNB2-1	1.833	0.011
40	lnc-KCNN4-1	1.810	0.002
41	lnc-SRP9-1	1.759	0.001
42	lnc-FAM43A-2	1.746	0.027
43	LINC00941	1.735	0.004
44	FAM226A	1.730	0.001
45	FOXP4-AS1	1.695	0.000
46	ENST00000504578	1.693	0.001
47	DKFZp434J0226	1.684	0.002
48	ENST00000507391	1.631	0.049
49	ENST00000541888	1.607	0.005
50	LOC100507002	1.527	0.000
51	LOC102724348	0.636	0.001
52	DKFZP586I1420	0.633	0.004
53	ENST00000511928	0.629	0.004
54	lnc-SRBD1-1	0.616	0.001
55	PXN-AS1	0.603	0.004
56	THC2651904	0.599	0.000
57	lnc-RAP1GDS1-3	0.598	0.003
58	PRKAG2-AS1	0.593	0.001
59	ENST00000623174	0.588	0.011
60	LOC729950	0.579	0.006

61	lnc-SNRNP200-1	0.574	0.005
62	LOC101929027	0.559	0.000
63	lnc-OSBPL10-1	0.521	0.003
64	GHRLOS	0.520	0.011
65	LOC101929626	0.514	0.036
66	ENST00000520544	0.508	0.047
67	lnc-AHR-2	0.498	0.006
68	ENST00000445534	0.497	0.004
69	LOC101927415	0.487	0.000
70	lnc-KLRG2-1	0.477	0.000
71	lnc-THNSL1-2	0.476	0.006
72	ENST00000527434	0.454	0.004
73	A_33_P3227457	0.450	0.002
74	AK002210	0.450	0.024
75	ZNF295-AS1	0.427	0.000
76	THC2548955	0.424	0.009
77	A_23_P90470	0.424	0.033
78	lnc-CHSY1-5	0.393	0.012
79	LINC01564	0.338	0.058
80	lnc-TIAL1-1	0.331	0.013
81	lnc-SOX6-1	0.314	0.048
82	lnc-TCL1B-2	0.302	0.025
83	lnc-RPP30-2	0.299	0.044
84	lnc-DYDC1-4	0.287	0.047
85	LOC101928152	0.253	0.000
86	lnc-SUPT3H-1	0.245	0.024
87	lnc-HMCN1-2	0.242	0.045
88	PARD3-AS1	0.238	0.000
89	lnc-TMEM64-2	0.212	0.016
90	KCNMA1-AS1	0.182	0.005
91	CTB-178M22.2	0.157	0.001

Appendix 3

List of differentially expressed mRNAs upon TGF-β1 (10 ng/ml) treatment in T98G GBM cells identified by microarray screen

S.No	Gene	Fold change	p-value
1	GPA33	19.790	0.000
2	SERPINE1	18.523	0.000
3	FOXS1	16.556	0.000
4	LEFTY1	15.429	0.000
5	EGR2	13.478	0.000
6	COL1A1	13.325	0.000
7	AMTN	13.241	0.002
8	MFAP2	13.057	0.000
9	RPS21	12.287	0.000
10	BTBD16	11.422	0.001
11	PMEPA1	10.922	0.000
12	LAMP3	10.229	0.000
13	TSPAN2	10.220	0.003
14	APCDD1L	10.210	0.000
15	IL11	9.894	0.005
16	HAVCR2	8.430	0.005
17	PLEKHG1	7.515	0.001
18	TNFAIP6	7.406	0.001
19	MYOZ1	6.816	0.003
20	MMP10	6.637	0.019
21	SFRP4	6.551	0.010
22	PDGFB	6.408	0.002
23	MMP13	6.282	0.000
24	F2RL1	6.254	0.000
25	SERPINE2	6.136	0.000
26	ENST00000390547	5.931	0.010
27	RGS16	5.742	0.000

28	JUNB	5.302	0.000
29	PROC	5.132	0.000
30	MOV10L1	5.130	0.000
31	SLC29A1	5.035	0.000
32	RGS9	5.019	0.000
33	KCNK12	4.741	0.003
34	CCL7	4.735	0.011
35	AK098835	4.603	0.041
36	RET	4.380	0.003
37	ENST00000508366	4.253	0.001
38	ARHGAP22	4.246	0.000
39	ZNF468	4.242	0.000
40	MIR181A2HG	4.211	0.000
41	DACT1	4.065	0.000
42	FRMD5	4.052	0.013
43	SYT12	3.911	0.000
44	HTRA1	3.867	0.000
45	HTR1D	3.835	0.007
46	AMIGO2	3.734	0.000
47	LDLRAD4	3.718	0.000
48	TYRP1	3.707	0.000
49	ENST00000407780	3.674	0.000
50	FHOD3	3.595	0.004
51	BPGM	3.568	0.000
52	CARD14	3.525	0.000
53	SKIL	3.518	0.000
54	SMAD7	3.460	0.000
55	ZNF365	3.447	0.023
56	NRP2	3.433	0.000
57	ATP2B2	3.416	0.001
58	CALHM3	3.323	0.000
59	SAMD11	3.295	0.000
60	ELSPBP1	3.256	0.000

61	PRICKLE1	3.232	0.002
62	CSF1R	3.209	0.000
63	IHH	3.059	0.002
64	CD82	3.042	0.000
65	NKD1	3.004	0.001
66	PRDM1	2.941	0.000
67	PBXIP1	2.912	0.000
68	CTGF	2.899	0.000
69	DNM3	2.896	0.001
70	DAAM1	2.883	0.000
71	SYTL2	2.821	0.000
72	CEMIP	2.804	0.011
73	SPOCD1	2.801	0.000
74	HMGA2	2.786	0.001
75	ITGB3	2.728	0.000
76	CTHRC1	2.709	0.000
77	TMIE	2.707	0.016
78	FGF1	2.688	0.010
79	LMCD1	2.657	0.000
80	CRLF1	2.645	0.000
81	PRRX2	2.621	0.004
82	THC2755556	2.604	0.001
83	KCNK6	2.592	0.023
84	ZNF423	2.584	0.008
85	PFKFB3	2.576	0.000
86	NDP	2.556	0.009
87	SORCS2	2.553	0.000
88	SIK1	2.553	0.000
89	CAMK4	2.533	0.002
90	TGFB1	2.528	0.002
91	PLAT	2.512	0.000
92	XKR7	2.483	0.042
93	STEAP3	2.465	0.000

94	FST	2.426	0.000
95	PPAPDC1A	2.423	0.001
96	DRAXIN	2.420	0.001
97	ENC1	2.394	0.000
98	ID1	2.387	0.000
99	ADO	2.375	0.018
100	NAV1	2.374	0.000
101	ATP1B1	2.374	0.000
102	CDKN2B	2.371	0.000
103	VCAN	2.340	0.000
104	MEF2C	2.333	0.001
105	SPSB1	2.328	0.000
106	HILS1	2.321	0.009
107	ANKH	2.317	0.000
108	LPL	2.300	0.032
109	TMCC2	2.296	0.001
110	TNFRSF12A	2.281	0.004
111	PIK3IP1	2.271	0.037
112	FGF18	2.257	0.002
113	NREP	2.230	0.000
114	KCNMB4	2.229	0.000
115	HECW2	2.222	0.001
116	XYLT1	2.219	0.030
117	GAD1	2.218	0.005
118	RAMP1	2.215	0.000
119	SPINT2	2.197	0.003
120	PTGDR	2.193	0.032
121	TPM1	2.188	0.000
122	KLF7	2.185	0.001
123	IL7R	2.184	0.001
124	LRIG3	2.169	0.000
125	EYA2	2.161	0.000
126	KIF26B	2.159	0.000

127	MFAP4	2.152	0.002
128	MAP3K4	2.149	0.000
129	ATP13A2	2.143	0.001
130	SIGLEC17P	2.141	0.000
131	PTPRR	2.133	0.029
132	ADAM19	2.117	0.001
133	TNFSF4	2.112	0.025
134	FAM26E	2.083	0.000
135	IFITM10	2.071	0.000
136	ESP33	2.058	0.048
137	EMP1	2.053	0.001
138	MDFI	2.050	0.003
139	COL7A1	2.049	0.001
140	KLF10	2.046	0.002
141	GEM	2.043	0.001
142	COL4A1	2.033	0.002
143	BHLHE40	2.029	0.002
144	S1PR3	2.027	0.000
145	CYR61	2.018	0.001
146	DHRS3	2.017	0.006
147	LPCAT2	2.015	0.002
148	ARHGEF4	2.000	0.004
149	FZD8	1.992	0.001
150	HEY2	1.980	0.000
151	PTHLH	1.965	0.017
152	USP18	1.961	0.000
153	HLA-DOB	1.951	0.027
154	THBS1	1.948	0.000
155	F2R	1.936	0.000
156	PHACTR1	1.929	0.004
157	CDH6	1.927	0.001
158	HTR7	1.926	0.001
159	DLX2	1.924	0.000

160	B3GNT5	1.922	0.002
161	DLC1	1.915	0.000
162	LOXL4	1.913	0.024
163	CACNA1C	1.906	0.001
164	LFNG	1.895	0.005
165	F2RL2	1.885	0.001
166	BMF	1.880	0.001
167	LRRC17	1.878	0.001
168	EFR3B	1.872	0.003
169	PCED1B	1.871	0.012
170	PPP1R3B	1.861	0.001
171	CMTM1	1.856	0.012
172	TLE3	1.853	0.000
173	AGPS	1.850	0.000
174	ACSL1	1.849	0.002
175	HBEGF	1.848	0.002
176	RHOBTB1	1.847	0.003
177	LMO1	1.847	0.013
178	VDR	1.843	0.000
179	KIAA1324	1.840	0.000
180	PRR16	1.839	0.033
181	CNIH3	1.839	0.002
182	SUSD6	1.831	0.001
183	GALNT10	1.816	0.000
184	PXDC1	1.811	0.000
185	MAF	1.810	0.003
186	MAPRE3	1.809	0.002
187	LIMS3L	1.807	0.012
188	HIC1	1.805	0.006
189	SH3BP4	1.786	0.002
190	UCN2	1.774	0.000
191	IL1RAP	1.773	0.008
192	SGK1	1.772	0.001

193	DOCK3	1.768	0.002
194	SLC24A3	1.760	0.002
195	GFRA2	1.753	0.000
196	ZNF827	1.752	0.012
197	PHLDB1	1.747	0.001
198	OSBPL10	1.745	0.001
199	TCAF2	1.739	0.003
200	HEY1	1.738	0.000
201	SH2B3	1.738	0.001
202	SKI	1.737	0.002
203	GPR183	1.734	0.002
204	ZNF521	1.730	0.001
205	GFPT2	1.728	0.000
206	SMURF2	1.717	0.009
207	SGCA	1.713	0.003
208	PODNL1	1.709	0.001
209	BHLHE41	1.708	0.001
210	TBC1D10C	1.696	0.005
211	ARL5C	1.680	0.031
212	EFR3A	1.677	0.001
213	EPHB2	1.673	0.000
214	ATP9A	1.668	0.000
215	CREB3L2	1.661	0.000
216	LIMS1	1.661	0.000
217	PPP1R13L	1.653	0.000
218	GBP1	1.649	0.000
219	SNAI1	1.649	0.000
220	SAMD5	1.645	0.004
221	GDNF	1.638	0.001
222	ITPRIP	1.635	0.005
223	KHDRBS3	1.611	0.001
224	PTPRU	1.606	0.000
225	TRIB1	1.602	0.000

226	SPON2	1.594	0.019
227	KCNN4	1.582	0.000
228	FOSB	1.575	0.013
229	NRBF2	1.541	0.000
230	HLF	0.165	0.001
231	KCNB1	0.180	0.000
232	FOXQ1	0.652	0.002
233	IMPA2	0.646	0.000
234	TBC1D8	0.644	0.001
235	CABLES1	0.630	0.000
236	GCLC	0.626	0.001
237	LRP5	0.625	0.000
238	NTN4	0.621	0.010
239	IFIT1	0.620	0.001
240	MINOS1-NBL1	0.619	0.000
241	NOV	0.615	0.000
242	METTL7A	0.614	0.000
243	ALDH3A1	0.613	0.000
244	PPAP2B	0.613	0.000
245	NEFH	0.611	0.001
246	C3	0.610	0.000
247	ARIH2OS	0.610	0.006
248	IFIH1	0.609	0.000
249	SLC47A2	0.609	0.004
250	PNPLA7	0.608	0.000
251	PDE5A	0.606	0.000
252	ALDH3A2	0.603	0.000
253	KCNC1	0.602	0.001
254	RGCC	0.599	0.000
255	OSGIN1	0.599	0.001
256	LIMCH1	0.597	0.002
257	SLC47A1	0.597	0.001
258	NRG2	0.595	0.001

259	PTPRJ	0.595	0.002
260	RAB26	0.594	0.000
261	PLEKHA6	0.593	0.001
262	MESP1	0.590	0.006
263	STEAP1	0.590	0.005
264	HIPK2	0.589	0.000
265	SIX1	0.584	0.000
266	CMTM8	0.583	0.000
267	ST3GAL1	0.582	0.001
268	SLPI	0.574	0.000
269	DAZL	0.573	0.000
270	HPGD	0.569	0.040
271	STEAP2	0.568	0.001
272	REP15	0.565	0.049
273	INHBB	0.564	0.001
274	FAM117B	0.563	0.001
275	DEPTOR	0.561	0.006
276	SLC12A7	0.561	0.000
277	EPHA4	0.559	0.000
278	SOBP	0.558	0.000
279	FMO3	0.557	0.003
280	DRD2	0.556	0.000
281	SOWAHD	0.555	0.001
282	GLCCI1	0.554	0.001
283	TMEM56	0.554	0.002
284	GUCY1A3	0.553	0.000
285	PAMR1	0.553	0.000
286	TLE1	0.552	0.000
287	ARL4D	0.552	0.001
288	SEMA3C	0.551	0.002
289	SEMA5A	0.547	0.001
290	ERMP1	0.542	0.005
291	TMEM64	0.542	0.000

292	ARHGAP9	0.539	0.001
293	AF119870	0.537	0.006
294	APOBEC3H	0.532	0.006
295	SLFNL1	0.532	0.001
296	CX3CL1	0.531	0.001
297	FOXF1	0.531	0.000
298	LRIG1	0.530	0.001
299	ARHGAP20	0.528	0.000
300	BIRC3	0.527	0.005
301	PPL	0.520	0.003
302	GHRLOS	0.520	0.011
303	JPH1	0.518	0.010
304	SLC51B	0.515	0.009
305	FAM49A	0.514	0.000
306	MYBPH	0.512	0.002
307	FLRT3	0.512	0.012
308	ADRB1	0.510	0.007
309	ADAMTS9	0.509	0.001
310	ITGA6	0.509	0.000
311	SLC12A2	0.503	0.008
312	EPAS1	0.501	0.000
313	ITGAM	0.500	0.010
314	MAP7D2	0.500	0.009
315	OSR1	0.496	0.006
316	PPM1E	0.496	0.027
317	NR6A1	0.493	0.001
318	SOCS1	0.492	0.001
319	RORC	0.490	0.018
320	KITLG	0.489	0.030
321	SLIT2	0.488	0.000
322	SCN4B	0.486	0.007
323	CLMN	0.483	0.003
324	FAM110B	0.482	0.000

325	TREM2	0.475	0.001
326	PLK2	0.471	0.000
327	ABCA3	0.469	0.001
328	ANKRD35	0.465	0.002
329	PIK3R5	0.463	0.010
330	TIMP3	0.462	0.000
331	PI15	0.460	0.001
332	PTGS2	0.459	0.004
333	DBP	0.458	0.001
334	KIAA1462	0.457	0.000
335	CA12	0.455	0.001
336	EDNRA	0.454	0.002
337	AK002210	0.450	0.024
338	ADAMTS5	0.444	0.001
339	TMEM38A	0.444	0.001
340	HS6ST3	0.443	0.035
341	TNFRSF11B	0.442	0.000
342	TXNIP	0.438	0.000
343	EXOC3L2	0.436	0.031
344	CEACAM1	0.436	0.047
345	RANBP3L	0.434	0.000
346	ADRB2	0.432	0.001
347	HS6ST1	0.426	0.000
348	FA2H	0.425	0.001
349	NPAS3	0.423	0.000
350	RAB17	0.421	0.000
351	NOG	0.415	0.004
352	LMO2	0.410	0.000
353	PAX9	0.409	0.000
354	FOXA1	0.407	0.004
355	KCNE4	0.407	0.001
356	PARD6B	0.405	0.000
357	SIPA1L2	0.404	0.000

358	TMEM132B	0.402	0.000
359	NFE2	0.399	0.000
360	CLEC1A	0.396	0.001
361	BHLHE22	0.394	0.003
362	TGFBR3	0.390	0.000
363	PRM2	0.390	0.020
364	NSG1	0.389	0.004
365	ENTPD8	0.388	0.003
366	CD86	0.365	0.046
367	LRRTM4	0.365	0.011
368	RNF43	0.363	0.014
369	WDR86	0.357	0.004
370	ETNPPL	0.347	0.010
371	APCDD1	0.344	0.000
372	SLC4A4	0.342	0.024
373	FAM65C	0.342	0.000
374	CXCL3	0.340	0.003
375	CD22	0.336	0.021
376	CABYR	0.323	0.000
377	FAM91A1	0.322	0.001
378	PAPPA	0.317	0.000
379	NXPH3	0.313	0.000
380	BMP1	0.312	0.000
381	SLCO4A1	0.312	0.001
382	ADH1A	0.310	0.009
383	TMEM100	0.291	0.000
384	RASL11A	0.290	0.001
385	CFTR	0.263	0.000
386	KLRC4	0.262	0.009
387	SUSD4	0.257	0.006
388	PTGS1	0.253	0.000
389	HCAR3	0.248	0.009
390	FGG	0.247	0.000

391	PODXL	0.244	0.000
392	CBLN2	0.234	0.000
393	KIT	0.227	0.034
394	GPR182	0.219	0.043
395	ADH1C	0.198	0.000
396	CHRM1	0.198	0.000
397	PTPLB	0.198	0.000

List of publications

1. **Shree B**, Tripathi S, Sharma V. Transforming Growth Factor-Beta-Regulated LncRNA-MUF Promotes Invasion by Modulating the miR-34a Snail1 Axis in Glioblastoma Multiforme. *Front Oncol.* 2022 Feb 8;11:788755. PMID: 35223453; PMCID: PMC8865078. https://doi.org/10.3389/fonc.2021.788755 **(Impact factor: 5.738)**

2. **Shree B**, Sengar S, Tripathi S, Sharma V. LINC01711 promotes transforming growth factor-beta (TGF-β) induced invasion in glioblastoma multiforme (GBM) by acting as a competing endogenous RNA for miR-34a and promoting ZEB1 expression. *Neurosci Lett.* 2023 Jan 1;792:136937. PMID: 36341927. https://doi.org/10.1016/j.neulet.2022.136937 **(Impact factor: 3.197)**

3. **Shree B***, Das K, Sharma V*. The emerging role of transforming growth factor-β-regulated long non-coding RNAs in prostate cancer pathogenesis. *Cancer Pathogenesis and Therapy*. 2022 Dec 24. https://doi.org/10.1016/j.cpt.2022.12.003. (*Corresponding Authors)

4. Khetmalis YM, **Shree B**, Kumar BV, Schweipert M, Debarnot C, Ashna F, Sankaranarayanan M, Trinath J, Sharma V, Meyer-Almes FJ, Sekhar KV. Design, Synthesis, and Biological Evaluation of Tetrahydroisoquinoline Based Hydroxamate Derivatives as HDAC 6 Inhibitors For Cancer Therapy. *Journal of Molecular Structure.* 2023 Jan 11:134952.; https://doi.org/10.1016/j.molstruc.2023.134952 **(Impact factor: 3.841)**

5. Tripathi S*, **Shree B***, Mohapatra S, Swati, Basu A, Sharma V. The Expanding Regulatory Mechanisms and Cellular Functions of Long Non-coding RNAs (lncRNAs) in Neuroinflammation. *Mol Neurobiol*. 2021 Jun; 58(6):2916-2939. PMID: 33555549. https://doi.org/10.1007/s12035-020-02268-8 **(Impact factor: 5.686)** (*Co-first Author)

6. **Shree B** and Sharma V. Linc‘ing’ RNA to DNA Repair. *Proc Indian Natn Sci Acad.* 2 2018 June; 84 (2): 521-529. DOI:10.16943/PTINSA/2018/49332 **(Impact factor: 0.62)**

List of conferences

- Poster presentation at 41st Annual Conference of the Indian Association for Cancer Research (IACR), Amity Institute of Molecular Medicine and Stem Cell Research, Amity University, Noida, from 2nd to 5th March 2022
- Poster presentation at the 90th Annual meeting of the Society of Biological Chemists organized by Amity Institute of Biotechnology & Amity Institute of Integrative Sciences and Health, Gurugram, India, December 2021
- Oral presentation in 3rd National Biomedical Research Competition NBRCOM 2021, Society of Young Biomedical Scientists, India, December 2021
- Poster presentation in Keystone Symposia eSymposia meeting on Non-Coding RNAs: Biology and Applications in May 2021
- Poster presentation at 34th Annual conference of Society for Neurochemistry, India, December 2020, organized by the University of Hyderabad, Hyderabad
- Poster presentation at the 88th Annual Meeting of the Society of Biological Chemists, India (SBCI-2019) and Conference on Advances at the Interface of Biology & Chemistry on November 1- 3, 2019, organized by Bio-Science Group, Bhabha Atomic Research Centre, Mumbai, and presented a poster

Brief Biography of the Candidate

Ms. Bakhya Shree pursued her Master's in Biological Sciences at the Vellore Institute of Technology. She is a doctoral student in the Laboratory of molecular medicine, Department of Biological Sciences, Birla Institute of Technology and Science, Pilani, Hyderabad Campus, under the supervision of Dr. Vivek Sharma. Her research topic is 'Identification and characterization of long non-coding RNAs (lncRNAs) involved in transforming growth factor-beta (TGF-β) pathway in Glioblastoma multiforme (GBM).' She is the recipient of the ICMR-Senior Research Fellowship (SRF). She attended several national and international symposiums during her Ph.D. work. She won the best poster presentation award at the 34th Annual conference of the Society for Neurochemistry, India, in December 2020. She received a global scholarship for attending the Keystone Symposia eSymposia meeting Non-Coding RNAs: Biology and Applications and presented a poster in May 2021. She was also awarded 2nd prize in oral presentation in the 3rd National Biomedical Research Competition NBRCOM 2021, Society of Young Biomedical Scientists, in December 2021. During her Ph.D., she characterized the role of TGF-β-regulated lncRNAs in GBM. She is well-versed in molecular biology techniques, such as RNA isolation, qRT-PCR, western blotting, Chromatin immunoprecipitation, RNA immunoprecipitation, and cell culture-based assays. She has 5 publications during her Ph.D. in peer-reviewed International journals (ORCID: 0000-0002-0008-6575). Her research interests are molecular mechanisms underlying cancer progression, cellular signaling pathways, and their perturbations driving cancer pathogenesis.

Brief Biography of the supervisor

Dr. Vivek Sharma has been working as an Assitant Professor in the Department of Biological Sciences at BITS Pilani Hyderabad since 2017. He did his pre-doctoral studies in Biotechnology at GGSIPU, Delhi. During his doctoral studies at NBRC, Manesar he elucidated the role of inflammation in Glioblastoma and outlined several novel treatment approaches for glioma. During his post-doctoral training at OICR, Toronto, he demonstrated oxidative stress as the primary cause of reprogramming-induced genomic aberrations in (iPS) cells. As a post-doctoral fellow in the laboratory of Tom Misteli at NIH, USA, he characterized the role of a novel lncRNA named DDSR1 in DNA damage and repair. He is a recipient of several awards, such as the Khorana Nirenberg Fellowship, NCI Director's innovation award, DBT Ramalingaswamy fellowship, and OPERA award from BITS Pilani. He has authored more than 30 publications. At BITS Pilani Hyderabad campus, he leads a research group working to elucidate the role of non-coding RNAs in Cancer and Neuroinflammation. He also serves as an Associate Editor for the RNA section of the journals Frontiers in Genetics and Frontiers in Molecular Biosciences. He also serves on the journal Brain Sciences (Neurooncology Section) editorial board.