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REST REGULATORY CIRCUIT CONTROLS DISTINCT ONCOGENIC PROPERTIES OF GLIOBLASTOMA STEM CELLS THROUGH SPECIFIC MICRORNAs

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A

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DOCTOR OF PHILOSOPHY

By

Anantha Marisetty, M.S.

Houston, Texas

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Dedication

To my parents and sister for all the love and support they have provided

To my husband, thank you for believing in me, for your patience, and for letting me fly

Acknowledgements

A project work of this magnitude is not possible without the help of several people either directly or indirectly. I take this opportunity to thank all those magnanimous persons who rendered their full support to my work.

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they have given me, I have achieved what I am today and I am thankful to all. I thank my husband Anil for his unfailing support, encouragement and putting up with me. I also thank my in laws for their constant support and encouragement.

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REST regulatory circuit controls distinct oncogenic properties of glioblastoma stem cells through specific microRNAs

Anantha Marisetty, M.S.

Advisory Professor: Sadhan Majumder, Ph.D

Glioblastoma Multiforme (GBM) is the most common and aggressive primary malignant brain tumor in adults. With an average survival of only 12-16 months the prognosis for GBM patients remains dismal, with less than 5% of patients surviving 5 years. New mechanism-based approaches are necessary for the management of patients with GBM. Many GBM tumors are believed to be caused by self-renewing, glioblastoma-derived stem-like cells (GSCs). These GSCs are resistant to chemo- and radiation therapies, and are believed to be responsible for tumor recurrence. In a recent paper from our lab we have shown that REST, RE1-silencing transcription factor, regulates oncogenic properties such as proliferation, invasion, and apoptosis in GSCs. However, the mechanism by which REST regulates oncogenic properties of GSCs is not clearly understood. Thus, the overall aim of this project is to delineate the mechanism by which REST mediates oncogenic properties of GSCs. Using genome-wide expression analysis followed by biochemical validations, we show that REST targets two microRNAs, miR-124 and miR-203 in High REST GSCs (HR-GSCs). Independent studies were carried out to determine the role of these microRNAs in HR-GSC derived brain tumors. Gain of function of either miR-124 or miR-203 in HR-GSCs leads to increased survival when tumor cells are transplanted into mice. Importantly, the increased survival of tumor-bearing mice caused by knockdown of Rest in HR-GSCs

can be reversed by double knockdown of Rest and miR-124 or miR-203, indicating that the REST-miR-124/miR-203 axis controls tumorigenesis. We further show that the REST-miR-124 axis regulates proliferation, invasion and apoptosis of GSCs both *in vitro* and *in vivo*, while the REST-miR-203 axis specifically regulates invasion and not proliferation or apoptosis. Our results indicate that invasion is a major hallmark of HR-GSC tumors and that the REST-miR-124/203 axis is critical in this process. These results also suggest that the REST-miR-124/203 axis could potentially be targeted in therapeutic approaches to block invasion in REST-stratified GBM tumors.

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Chapter 1: Introduction

1.1 Overview

Glioblastoma (GBM; Glioblastoma Multiforme) is the most common and aggressive form of adult brain tumors with a mean survival of 12-16 months (*1-5*). The current therapies for GBM patients involve surgical resection followed by radiation and chemotherapy or a combination of both (*2, 3, 6, 7*). Many GBM tumors are believed to be caused by self-renewing, glioblastoma-derived stem-like cells (GSCs) that are highly proliferative, invasive and resistant to chemo- and radiation therapies, and are believed to be responsible for tumor recurrence (*8-16*). Recent studies from three different groups (*17-19*), including ours, have shown that RE1 silencing transcriptional factor (REST) regulates self-renewal and oncogenic properties like proliferation, apoptosis and invasion in a class of GSCs. However, the mechanistic understanding of the REST driven self-renewal and oncogenesis needs further elucidation. The purpose of this study was to determine the mechanism by which REST regulates these oncogenic properties (proliferation, apoptosis and invasion) of GSCs and this will produce new mechanisms that can potentially be used for novel mechanism-based targeted therapy in GBM.

1.2 Glioblastoma (GBM)

Glioblastoma is one of the most lethal human cancers, accounting 82% of the malignant gliomas (*16, 20*). Gliomas are more common in adults ages 45-65 and affect men more than woman (American brain tumor association/ABTA). Based on the

histology of the tumor, the World Health Organization (WHO) has classified glioblastoma as grade IV tumor of the Central nervous system (*20-22*). These tumors are heterogeneous and characterized by high mitotic activity, microvascular proliferation and necrotic areas. Clinical, histological and radiologic evidences suggest that the primary GBM arise *de novo* whereas the secondary GBM arise from the progression of pre –existing lower grade gliomas (*23*). Primary tumors most commonly arise quickly and are very aggressive. These tumors account for the majority of glioblastoma and occur in persons age 55 or older. Secondary tumors are usually found in persons age 45 or younger, normally start as low grade glioma and ultimately transform into malignant rapidly growing gliomas.

These tumors are highly invasive and the tumor cells invade into the surrounding brain parenchyma but do not metastasize (*20, 22*). As the tumor cells invade the surrounding brain, complete surgical resection of the tumor is not possible. The standard care for patients who have been newly diagnosed with GBM includes surgical resection followed by concurrent adjuvant radiotherapy in combination with the chemotherapeutic agent Temozolomide (TMZ, alkylating agent); but still less than 5% of the patients survive less than 5 years (*24-27*). TMZ have the ability to methylate DNA, leading to DNA damage which triggers the death of tumor cells. However, some cells have an ability to repair this type of DNA damage by expressing a protein O^6-alkylguanine DNA alkyltransferase (AGT) encoded in humans by the O-6-methylguanine-DNA methyltransferase (*MGMT*) gene. In some tumors, MGMT is epigenetically silenced preventing the synthesis of this enzyme and thereby making the tumors more sensitive to TMZ. A recent study has suggested that methylation of the promoter of O^6-methguanine-DNA methyl transferase (MGMT) has a survival advantage upon chemotherapy with TMZ when compared to the unmethylated

promoter status of MGMT (*27*). This study has established that MGMT promoter methylation can be used as a strong predictive marker. There are currently three molecular markers that are being used routinely in the clinic which include mutations in the isocitrate dehydrogenase 1 and 2 (IDH1/2), codeletion of 1p and 19q chromosomes and MGMT promoter methylation (*10, 28, 29*). These markers have diagnostic, prognostic and predictive value and have gained significant clinical relevance. A better understanding of the cellular origin and the molecular pathways that regulate these tumors needs to be elucidated.

The recurrence of the disease is mainly due to invasive cells that are radio and chemo-resistant, especially the cancer stem cells (CSCs). Studies have shown that heterogeneous tumors are composed of tumor cells and a small percentage of cancer stem cells. Cancer stem cells constitute around 2%-3% of the tumor mass. The CSCs are highly tumorigenic and have self-renewal potential (*30*). CSCs are phenotypically similar to the normal stem cells, expressing the genes that are the characteristic features of neural stem cells and express CD133 gene. The $CD133^{+}$ cells have a higher DNA repair capacity when compared to normal cells as the gene down regulates autophagy genes (*31, 32*). Studies have also shown that as few as 100 CD133+ cells when implanted into the brain of immunodeficient (SCID) mice can reproduce tumors but a million $CD133^{-}$ cells cannot reproduce the same tumor (*33*). Usually CSCs are in the quiescent state, but upon surgery, radiation or chemotherapy, they are stimulated and proliferate exponentially and are responsible for tumor recurrence.

In an effort to better understand the genomic changes that occur in glioblastoma, the cancer genome atlas research network (TCGA) compiled molecular profiles on GBM patient samples and found that when looking at gene expression and mutation data,

that GBM could be classified into four subclasses: proneural, neural, classical and mesenchymal (*8, 21, 34, 35*). The proneural subclass is characterized by the amplification of platelet derived growth factor receptor -α (PDGFRα) and also expresses several proneural development genes, the neural subclass is characterized by the presence of neural markers, classical group by the amplification of Epidermal growth factor receptor (EGFR) and cdkn2a deletion, and mesenchymal group by the loss of neurofibromin (NF1)(*8, 21, 34, 36, 37*). This classification has helped to understand the molecular signature of GBM but did not provide any prognostic tool or survival advantage.

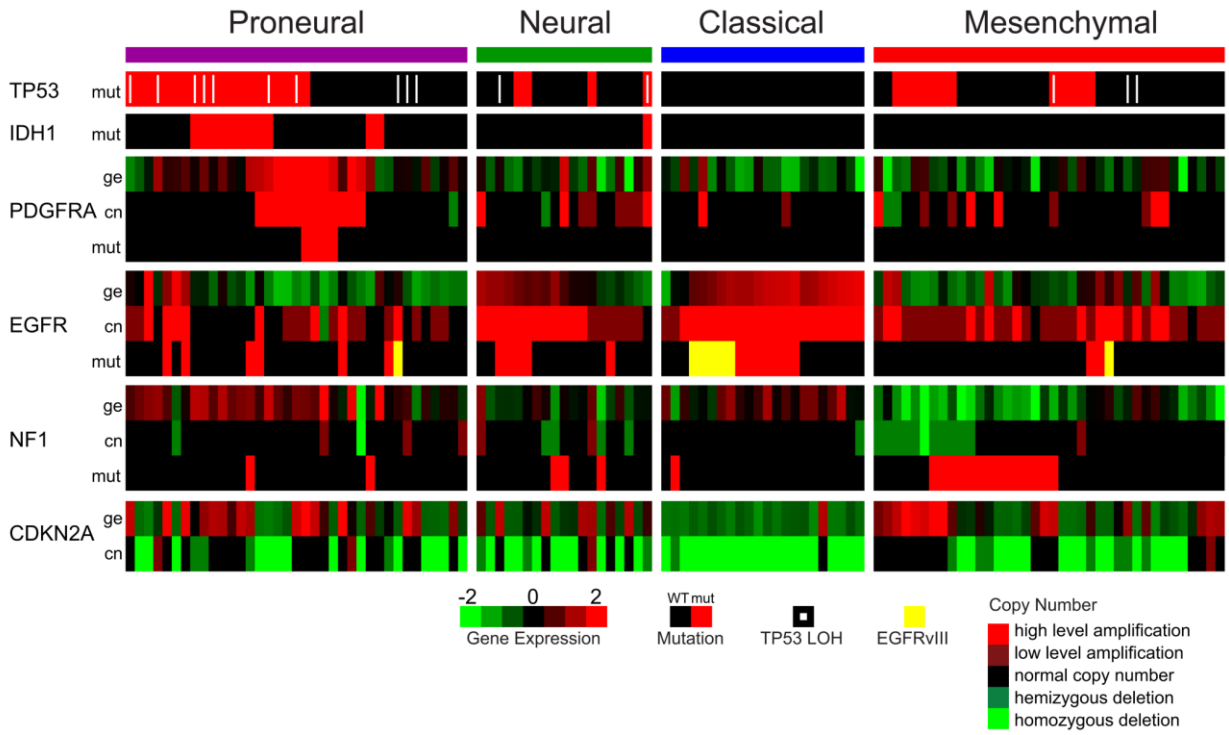


Figure 1: Molecular subtypes of GBM. In an effort to better understand the genomic changes that occur in glioblastoma, TCGA did molecular profiles on GBM patient samples. Based on the gene expression and mutation data GBM could be classified into four subclasses. The four subtypes are Proneural, Neural, Classical and Mesenchymal. Figure is taken from reference (*38*), with the following license number 3626010907812.

1.3 REST

The Repressor Element-1 (RE-1) silencing transcriptional factor (REST), also known as neuron restrictive silencing factor (NRSF), is a major transcriptional repressor of neurogenesis in neural cells and neuronal differentiation in non-neural cells (*39*). REST is expressed at higher levels in neural stem cells (NSC) and embryonic stem (ES) cells and thereby prevents them from neuronal differentiation (*40, 41*). In mature neurons, REST is expressed at very low levels. REST expression is preserved in NSCs and ESCs to ensure these cells maintain stem cell properties (*39, 42, 43*). Studies have shown that REST is important for normal brain development, and homozygous deletion of REST resulted in embryonic lethality at E11.5 (*44*). Most of the studies to date have shown the role of REST in embryonic development and neurogenesis. In addition, dysfunction and abnormal expression of REST has been found in Downs syndrome, Huntington's disease and medulloblastoma(*42*).

REST is a 116 kilo Dalton (kDa) kruppel type zinc finger transcriptional factor that contains a central DNA binding domain (Zn-DBD) consisting of eight zinc finger motifs, that binds to the consensus RE1 (or NRSE) sequence on the target genes regulatory regions(*43, 45, 46*). It contains two distinct repressor domains (RD): one at the N-terminus (RD1), and the other within the zinc finger motif at the C-terminus (RD2)(*43, 45*). The central DNA binding domain can recognize two types of RE-1 motifs on the target genes; a) canonical RE-1 motif and b) non-canonical RE-1 motif (*47, 48*). The canonical and the non-canonical motif differ in the length of the insertion between two conserved sequences. The former is characterized by the presence of a single nucleotide that separates the conserved sequences while the

later has variable length of non-conserved nucleotides. The canonical motif has a higher binding affinity to REST suggesting tissue specific functions. The repressor domain RD1 interacts with mSin3A while RD2 interacts with Co-REST (*43, 49*). Both RD1 and RD2 repressor domains recruit the histone deacetylase (HDACs) silencing complex to remodel chromatin and represses its target gene expression (*43, 49*) . A recent study using a combination of in silico and biochemical approaches has identified 1,892 human, 1,894 mouse, and 554 *Fugu* RE-1 sites on target genes (*47*).

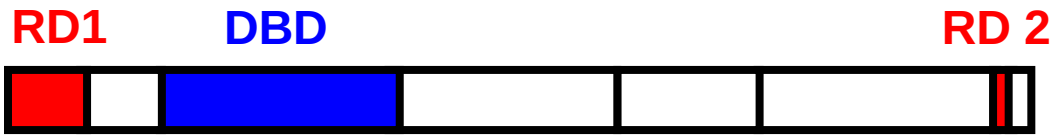


Figure 2: Structure of REST protein. REST contains a DNA binding domain (DBD) with eight zinc finger domains and two repressor domains (RD1 and RD2). REST recognizes RE1 consensus sequence on the target gene regulatory elements. (Adapted from (*39, 45*))

REST is regulated by ubiquitin mediated proteosomal degradation, β-Transducin repeat containing protein (β-TRCP) an E3 ligase, promotes REST degradation during the G2 phase of cell cycle in ES cells and non-neural cells(*50, 51*). Failure to degrade REST attenuates the neuronal differentiation process in neural cells. Studies have also identified the ability of USP7 (herpes virus-associated ubiquitin specific protease) to compensate REST ubiquitination which prevent differentiation through deubiquitination. Deubiquitination mediated by USP7 and the ubiquitination by β-TRCP together regulate the REST protein levels and its function(*52*).

Depending on the cellular context, REST can function either as a tumor suppressor or oncogene. REST is expressed in non-neural tissues such as lung, breast and colon epithelium and there by represses neurogenesis (*53-56*). Inhibition of REST in epithelial cells has increased the capability for transformation. A mutant form of REST, which lacks the c-terminus acts as a dominant negative isoform, when transfected into colon cells promotes anchorage - independent growth. Most of the non-small cell lung cancers (non- SCLC) have normal expression levels of REST at protein and RNA level. However, around 10% of the cancers have loss of SWI/SNF complex, a cofactor required for REST activity. Loss of function of REST is also observed in other cancers like lung, prostate and breast and successive increase in the neuroendocrine genes (*53, 54*). In non-neural tissues dysfunction or loss of REST leads to neuroendocrine carcinomas suggesting REST contributes to tumor development. In non-neural cancers REST acts as a tumor suppressor by regulating cell proliferation and apoptosis. In human mammary epithelial cells (HMECs) knock down of REST enhances Akt phosphorylation and inhibition of PI3K signaling and there by reduces the transformation capability of the cells (*50*). Studies have shown that in lung and breast cancers loss of function of REST enhances cell proliferation and survival and up regulation of BCL-2 gene expression(*42*). Most of the studies to date suggested that in non-neural cells or tissues REST has a tumor suppressor function and inhibits growth. However a recent study has shown that conditional deletion of REST in colon crypts increased the expression of REST-target genes but no significant effect on tumor development is observed(*57*).

As REST plays an important role in maintenance of NSCs, functional abnormality of REST is found in the development of many brain tumors. Interestingly,

elevated levels of REST protein have been observed in neuroblastoma and medulloblastoma tissues when compared to the surrounding normal tissue. The higher expression levels of REST in medulloblastoma and neuroblastoma correlate with poor patient survival (*42, 56, 58*). REST mainly acts as a oncogenic promoter in brain tumors. Studies have shown that ectopic expression of mutant REST, REST-VP16 in medulloblastoma cells have significantly reduced the tumorigenic potential of these cells (*59*). Inhibition of REST in medulloblastoma leads to apoptosis. A recent study has shown that REST regulates hedgehog signaling during embryonic development. Hedgehog signaling regulates the proliferation and differentiation of neural stem cells. Also, during medulloblastoma development there is an up regulation in the hedgehog signaling. Studies have also shown that Wnt signaling pathway that controls properties of neural stem cells also regulate the expression of REST. Thus a cross talk between these 3 signaling cascades plays an important role in medulloblastoma tumorigenesis. REST has very tissue specific function. Inhibition of REST in epithelial cells leads to increased proliferation while in medulloblastoma cells leads to increased apoptosis (*59-61*). Studies have shown that REST requires additional partners or oncogenes to promote tumorigenesis of medulloblastoma. Over expression of REST alone doesn't promote medulloblastoma tumor formation. Rather co-expression of c-myc is required to promote the formation of medulloblastoma tumors (*62*). Studies have clearly show that REST has opposing functions in different tissues; it acts as a tumor suppressor in epithelial cells and as an oncogene in brain tumors. The tissue specific roles of REST might be attributed to forming different repressor complexes with its co activators. As REST plays a crucial role in maintenance of neural stem cells and also development

of brain tumors, it is critical to develop a novel therapeutic approach specifically targeting REST in cancer cells.

1.4 MicroRNAs

MicroRNAs are small molecules of 20-22 nucleotides endogenous noncoding functional RNAs (*63-65*). They regulate gene expression either by regulating mRNA translation or degradation of specific mRNAs that control cellular processes. Computation and experimental approaches have discovered that a single microRNA can target more than 100 genes mRNA. Studies have shown that 60% of the human protein coding genes are predicted to contain miRNA binding sites in their 3`- untranslated region (UTR ) (*64*). MicroRNAs activity is primarily through binding to the 3`UTR of messenger RNAs resulting in degradation and translational repression. microRNAs do not require perfect base pairing unlike other small RNAs but can regulate anetwork of specific genes. However studies have also reported binding and activity through the 5`-UTR .(*66*)

A high-throughput screen and functional studies in cancer has revealed that miRNAs play important roles in human disease. A small change in the expression levels of microRNA has significant effect on the mRNA targets. MicroRNAs were found to affect cellular proliferation, apoptosis, invasion, angiogenesis, and stemness of the malignant cells. MicroRNAs can be either tumor suppressors or oncogenes based on their expression in malignant tissue compared to the surrounding normal tissue.

Accumulating evidences has shown that miRNA expression can be used as a prognostic or diagnostic marker in cancers. It has been shown that classification of cancers based on their miRNA expression signature is more accurate than their mRNA based signature (*29, 67*). It is easy to modulate the expression of microRNAs either using antisense oligonucleotides or precursor or mimic sequences. The expression profiles of microRNAs differ between the normal tissue and tumor tissue and between tumor types. Studies have shown that aberrant microRNA expression can affect cell proliferation, apoptosis, invasion and epithelial to mesenchymal transition(*63*). Additionally, studies have identified correlations between microRNA expression and recurrence and survival.

Genome wide analysis and high throughput screenings have revealed that some MiRs have RE1 binding sites. Studies have also shown that miR-21 is expressed at higher levels in glioblastoma and has survival significance (*68*). Studies have shown that REST regulates the expression of miRs that promote neuronal differentiation including miR-124a, miR-9 and miR-132 (42).

Based on binding studies and expression analyses, miR-124a was one of the earliest identified miRs demonstrated to be a direct target of REST. Studies have shown that in NSCs and non-neural cells REST represses miR-124a expression, thereby preventing neurogenesis and expression of neuronal genes. Two independent studies have shown that treatment of GBM stem cells with miR-124 led to differentiation of these cells and decreased expression of stem cell maintenance proteins(*69*). This result suggests us that low expression level of miR-124 in the GBM patient's results in decreased differentiation and increased proliferation of GBM stem cells.

miR-124 is one of the abundant microRNAs in the brain and is often down regulated in GBM patients. Studies have shown that addition of miR-124 in the cells that lack them reduces the migratory and invasive potential (*69, 70*).

MicroRNA, miR-203 has been identified as a tumor suppressor microRNA in basal cell carcinomas and hepatocellular carcinoma (*71-74*). Studies have shown that miR-203 is epigenetically silenced in hepatocellular carcinoma tumors and leukemias (*72, 75*). Studies have also shown that it is over expressed in pancreatic adenocarcinoma and shows correlation with poor prognosis (*76*). MiR-203 has been shown to be up regulated upon UV radiation indicating its role in apoptosis (*77*). Studies have shown that role of microRNAs in malignancy is due to translocation of chromosomes or allelic deletions. Recent evidences have shown that the 14q chromosome harbors a multiple tumor suppressor genes and plays an important role in GBM pathogenesis. This region harbors several microRNAs including miR-203. Two independent studies have shown that there is a allelic deletion on chromosome 14q in 20%-40% patients (*78, 79*).

1.5 Glioblastoma Stem cells (GSCs)

The recent beginning of the “cancer stem cell” hypothesis has brought a new perspective to our understanding of GBM biology and therapy (*11, 14*). According to this hypothesis, GBM tumors contain stem-cell-like cells, known as glioblastoma stem cells (GSCs), which have the capacity for long-term self–renewal. GSCs are more efficient at forming tumors than non-stem cells and are highly resistant to chemo and radiation therapies and are believed to be responsible for tumor

recurrence. Several studies have demonstrated that GSCs promote tumor angiogenesis and invasion.

The glioblastoma stem cells (GSCs) used in the current study are derived from the patient tumors that are treated at MD Anderson Cancer Center, for GBM after getting patients consent. The tumors are washed and subjected to enzymatic dissociation and allowed to recover in a medium that promote only stem cell growth.

Our research suggests that REST is expressed at varying levels in these GSCs established from patient tumors and helps in maintaining their self-renewal and oncogenic properties like proliferation, apoptosis and invasion (*17*). This shows the heterogeneity of these established cell lines. These GSCs can recapitulate the human tumor when implanted into the mice brains.

Based on the REST protein expression levels our lab has classified the GSCs available as High REST (HR-GSC) and Low REST (LR-GSC) GSCs. We observed that GSCs with higher REST expression had a higher self-renewal capacity, sphere forming capacity and oncogenic properties when compared to GSCs with lower REST expression. Studies have suggested targeting REST for proteosomal degradation may disrupt the oncogenic potential of GSCs.

In order to study the role of REST in self-renewal and GBM tumorigenesis we knocked down REST in the HR-GSCs and performed self-renewal and tumorigenic assays. Our group and two other groups have observed that upon knocking down of REST there is a decrease in the self-renewal capacity, decreased proliferation, increased apoptosis and decreased invasion both *in vitro* and *in vivo*. When REST knock down GSCs were implanted into the brains of nude mice there is an increased survival of the mice when compared to the controls. The tumors formed by HR-GSCs are highly invasive but

upon REST knock down the tumors are more circumscribed. Our studies along with two other groups indicated that REST is involved in glioblastoma tumorigenesis (*17-19*). However, the mechanisms by which REST regulates these tumorigenic properties of GSCs are still unclear.

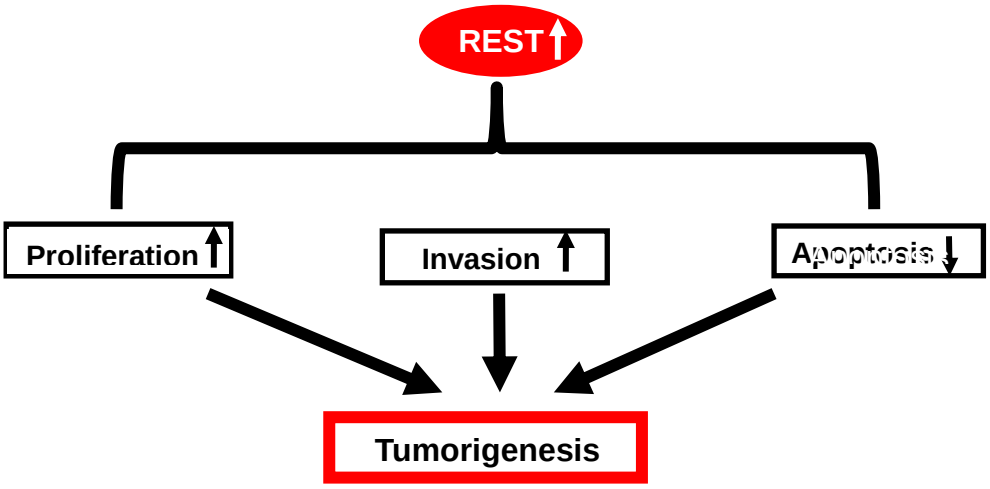


Figure 3: REST regulates oncogenic properties of Glioblastoma Stem cells (GSCs). When REST is expressed at higher levels there is an increase in level of proliferation and invasion and decreased apoptosis leading to increased tumorigenesis both *in vitro* and *in vivo*. When REST is expressed at lower levels there is decreased proliferation and invasion and increased apoptosis leading to decreased tumorigenesis both in vitro and in vivo.

Chapter 2: Material and Methods

2.1. Cell culture

GSCs used for this study were obtained from the Brain Tumor center core, and were established from human GBM surgical specimens from patients treated at The University of Texas MD Anderson Cancer Center, Houston, TX as described previously (*17*). The cells are maintained in Dulbecco's modified Eagle's medium (DMEM) F12 (Sigma) supplemented with B-27 (1:50, Gibco), L-glutamine (1:100, sigma), 20 ng/ml epidermal growth factor (Sigma), 20 ng/ml fibroblast growth factor, and Penstrep (1:100) as previously described (*17*). Cells were maintained at 37°C with 5% CO_2. These cells were grown as spheres, fed every 2 days, and split in half once the sphere size reached 100 µm with Accutase (Sigma) and seeded as single cells. The cells are frozen as single cells in 10% FBS, 20% DMSO and 70% basal medium with no growth factors.

2.2. siRNA transfections

All siRNA transfections were performed using Amaxa nucleofector technology (Lonza) .Four million cells were suspended in 90µl of amaxa buffer. 10µl of siRNA at a concentration of 20µg/µl is added to the amaxa buffer to make a final volume of 100µl. The samples are subjected for electroporation and immediately resuspended in 500µl fresh medium. siNT (control/non targeting)and siRest siRNAs are obtained from dharmacon (GE Health care).

2.3. Total RNA isolation

Total RNA was extracted from the GSCs using Trizol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Briefly the cell pellets are lysed using 1ml of Trizol reagent followed by chloroform extraction by centrifugation at 13,000rpm for 15 min at 4° C. The aqueous phase was collected and precipitated with equal volumes of isopropanol. Glycogen is added at 1mg/1ml at this step as it aids in precipitation. The samples were further centrifuged at 13,500 rpm for 15min at 4°C. The pellet were washed with 1ml of 70% RNAse free ethanol and centrifuged at 7500rpm for 5min at 4°C. The pellets are air dried and resuspended in RNAse free water. Concentration of RNA was determined by spectrophotometric analysis using Nanodrop (Thermo scientific, Rockford, IL). The samples are stored at -80°C for future use.

2.4. Genome-wide expression analysis of GSCs with loss-of-function of REST (Microarray)

Total RNA was extracted from the transient transfections of siRest and siNT cells. RNA purity was assessed using Nanodrop spectrophotometric measurement of optical density (OD) 260/280 ratio of greater than 1.85. miR microarray was performed (Affymetrix, U-133 plus 2.0 array) by labelling and hybridizing using five hundred nanograms of the total RNA according to manufacturer's protocol. Bioinformatic analysis was performed by using R (2.14.2) program to identify the miRs with a two fold increase upon REST knockdown with a significant p value ($p<0.05$)

2.5. Western blotting

Whole cell extracts were prepared by using lysis buffer ((150mM NaCl, 25mM Tris, p^{H} 7.5,5mM EDTa,1% Triton X,0.1% SDS,0.5% sodium deoxycholate) with 1X protease

inhibitors. Briefly, after the addition of lysis buffer the samples were kept on ice for 30 minutes with frequent vortexing every 5 minutes. The samples were centrifuged at 13,000rpm for 15 minutes. The supernatant was collected and quantified using a BCA protein assay reagent kit (Pierce Biotechnology, IL). Samples were resolved by loading 50 µg of the lysates onto gradient HEPES gels (Pierce Biotechnology, IL). The proteins are transferred to a nitrocellulose membrane and blocked with 5% milk followed by incubation with rabbit anti-REST antibody (Millipore, USA) overnight at 1:1000 dilution or mouse anti-actin (1:10,000) and then incubation with fluorescent-labeled secondary antibody for 1 hour. The membranes were scanned using the Licor program (Odyssey detection system) to visualize the protein complexes.

2.6. Reverse Transcriptase Polymerase chain Reaction (RT-PCR)

Complementary DNA (c-DNA) was synthesized by using 1µg of total RNA using the verso c-DNA synthesis kit (Thermo Scientific) according to manufacturer's instructions. Briefly, 4µl of 5X cDNA synthesis buffer, 2 µl of 10mMdNTPs, 1ul of primer (1:3 ratio of oligo dT and random hexamers), 1µl of RT enhancer and 1µl of verso enzyme and 1µg of total RNA diluted in water to make a total volume of 20µl. Reverse transcription was done at 42°C for 30min.

2.7. Quantitative real-time PCR

The c-DNA synthesized was diluted to bring to a working concentration of 5ng/µl. Quantitative PCR (qPCR) was done using SYBR green master mix (Applied biosystems,CA) and primers for the 7 microRNAs identified in the screen (miR-124 ,miR-136, miR-203,miR-518e,miR-545,miR-557,miR-942). Primer sequences are shown in the Table. Analysis was performed on ABI 7900 real time PCR system

(Applied biosciences). Relative miRNA measurements were done using delta Ct method as described previously(*80*). All the experiments were done in triplicates

List of miR Primers that are potential targets of REST

Table 1: Primers for validation of microarray results by qRT-PCR

Primer	Forward Primer	Reverse Primer
miR-124	5`-TCCGTGTTCACAGCGGAC-3’	5`-CATTCACCGCGTGCCTTA-3’
miR-136	5`-GGACTCCATTTGTTTTGATGATG-3’	5`-AGACTCATTTGAGACGATGATGG-3’
miR-203	5`-TCCAGTGGTTCTTAACAGTTCA-3’	5`- GGTCTAGTGGTCCTAAACATT-3’
miR-518e	5`- GTTTTCTCAGGCTGTGACC-3’	5`- GTGTGTTCTCAGGCTGTGAC-3’
miR-545	5`- TTGCCCAGCCTGGCACCAT-3’	5`- GTTTTTCCCAGCTGGCA-3’
miR-557	5`-GTAGAATGGGCAAATGAACAGT-3’	5`- TTGTTCATGCTAAGAAT-3’
miR-942	5`-GGATTAGGAGAGTATCTTCTCT-3’	5`- TGTGTGATTAGGAGAGTATC-3’

2.8. Taqman assays to measure miR-124 and miR-203 expression

Total RNA was extracted as described in total RNA isolation section of the methods. Mature microRNA expression levels of miR-124 and miR-203 were quantified using Taqman®microRNA (Applied Biosystems, CA). Taqman® MicroRNA reverse transcription kit (Applied Biosystems, CA) was used to synthesize cDNA using specific primers for miR-124, miR-203 and Sno135 according to manufacturer's protocol. The quantitative PCR was performed using Taqman® universal master mix with No Uracil N-Glycosylase(UNG) (Applied Biosystems, CA) on ABI 7900 real time PCR machine (Applied biosciences). Relative expression levels of miR-124 and miR-203 were done using Sno135 expression as a control and □□Ct method as described previously.

2.9. Chromatin Immunoprecipitation (ChIP) Assay

GSCS dissociated into single cells by accutase were cross-linked at room temperature for 10 minutes using 2%formaldehyde. Cross linking was stopped by the addition of 1/10 volume of 1.4M glycine and rocking for 5 minutes. The cells are washed in 5ml of 1XPBS by spinning at 1200rpm for 2 minutes. The pellets were rewashed in 10ml of fresh PBS with 100µl of protease inhibitor and sonication wash buffer (10mM HEPES, p^H 7.5, 200mM NAcl,1mM EDTA,0.5mM EDTA) . The pellets were resuspended in lysis buffer (150mM NaCl, 25mM Tris, p^H 7.5,5mM EDTA,1% Triton X,0.1% SDS,0.5% sodium deoxycholate) and left on ice. The samples were sonicated with a continuous pulse at 65 amplitude for 15 seconds for 13 cycles. A small portion of the sample is reverse cross-linked and DNA is recovered and size of the DNA was determined. This yielded us a higher concentration of DNA at 500bp with increasing amounts of smaller fragments which indicated further sonication is not necessary. The samples were

precleared for immunoprecipitation by incubating with protein A beads for 2 hours at 4° C. The beads were collected by centrifugation and the supernatant is transferred to a new tube. A small aliquot of the sample is saved as an input and the remaining sample is divided into 2 equal aliquots for immunoprecipitation using anti-REST, anti-IgG antibody. The samples were incubated overnight at 4 °C. Any residual debris was removed by centrifugation. To the supernatant fresh Protein A beads were added and incubated for an hour at 4 °C. Beads were recovered by centrifugation and the supernatant was stored at -80C for future use. Beads were further washed in RIPA buffer (50mM Tris pH 8.0,150mM Nacl,0.1% SDS,0.5% sodium deoxycholate ,1% NP-40,1mM EDTA) for 10 min and the supernatant was recovered by centrifugation. Further the beads were washed with 1X high salt solution(50mM Tris pH 8.0,500mM Nacl,0.1% SDS,0.5% sodium deoxycholate ,1% NP-40,1mM EDTA) to disrupt protein-protein interactions. In order to break the RNA fragments the beads are further washed with 1X LiCl wash (50mM Tris pH 8.0,250mM Licl,0.1% SDS,0.5% sodium deoxycholate ,1% NP-40,1mM EDTA). The beads are washed in TE buffer (10mM Tris pH 8.0,1mM EDTA) followed by proteinase K treatment to remove the bound proteins. The samples along with the inputs were subjected to reverse crosslinking overnight at 65°C. The samples were extracted twice using equal volumes of phenol /chloroform. The final aqueous phase is collected and the DNA is precipitated using 1/10 volume of 3M sodium acetate, 2.5 volumes of ethanol and glycogen (carrier molecule) and stored at -80C for 2 hrs. DNA is collected by spinning at 13,000 rpm for 20 minutes followed by washing with 70% ethanol. The pellets are air dried and resuspended in water and stored at -20°C. PCR was performed after a 1:5 dilution of the samples. qRT-PCR was

performed and the analysis was done by fold enrichment method as described previously.

2.10. Identification and validation of REST response elements in the promoter regions of miR-124/203

REST binding sites on the promoter region of miR-124 and miR-203 were determined using Mat Inspector (Genomatix software suite v3.3), a tool that identifies transcriptional factor binding sites. RT-PCR was performed by using primers specific for the indicated regions as shown in the following tables

Table 2: List of primers for potential RE1 binding sites on the gene chromatin of miR-124

Site	Forward Primer	Reverse Primer
RE1 site # 1 -2648bp	5`-CCGCATTTTCCTTGGCACAG-3’	5`-ACCAGCACACGTCATTCTCA-3’
RE1 site # 2 (Nonspecific) -300bp	5`-GGAAAAAGCCTGGATGCGAA-3’	5`-TCCCCCAATCACACAGACAAT-3’

Table 3: List of primers for potential RE1 binding sites on the gene chromatin of miR-203

Site	Forward Primer	Reverse Primer
RE1 site # 1 -223bp	5'- CGTCTAAGGCGTCCGGTA- 3'	5'- GAGCTGCGGAGAGAGGAG- 3'
RE1site #2 -512bp	5'- GCCCAGACGAGACGGTTC- 3'	5'- CCGCGACTGATCCTCCAC- 3'
RE1site #3 -762bp	5'- CACACCCACCGGAGAGCTA- 3'	5'- CCCGAACCGTCTCGTCTG-'3
RE1 site # 4 -1223bp	5'- CAACCCCATACAGACACACTAA- 3'	5'- TGTCCAGGCCTGACCAGT-'3
RE1site #5 nonspecific site (-2167)	5'- CCGTCCTCTCTCGTCAGT- 3'	5'-CTCCCAAAGTGCTGGATT- 3'

2.11. Dual Luciferase Assay

The reporter construct pRLTK along with the PGL3- basic vector or PGL-3 with RE-1 sites of miR-124 (site #1) or miR-203 (site #4), PGL3 with mutated RE-1 site on miR-124 or miR-203 were co transfected into High REST and REST knock down cell lines by Amaxa electroporation. The DNA fragment containing the REST binding site

identified by chip was amplified from genomic DNA of HR-GSC2 line by PCR with 5' XhoI and 3' HindIII restriction sites.

Forward Primer 5'-ATACTCGAGGGTGGCTGTGTTCTGGTCTG-3' and Reverse primer 5'- ATAAAGCTTGCTAGCTCTCCGGTGGGT-3'. The mutant version is generated using the quick change mutagenesis kit (Agilent technologies). 48 hours post transfection luciferase activity was measured with dual –luciferase reporter assay (Promega) according to manufacturer's protocol.

2.12. Cell Transductions

Gain of function of miR-124/203: HR-GSC shNT cells have higher expression of REST and lower expression levels of miR-124 and miR-203, so we used these cells to over express miRs. The cells were transduced with either shNT control or premiR-124/203 lentiviruses (Thermo scientific Fisher, USA). Transduction efficiency was initially checked by fluorescence microscopy, as the vectors have a GFP tag. Overexpression of the miRs was further confirmed by qRT-PCR.

Loss of function of miR-203: HR-GSC shREST cells have lower expression of REST and higher expression levels of miRs. Cells were transduced using viruses for control shNT or shmiR-203 (Genocopeia, USA). Knockdown was confirmed by qRT-PCR.

The generated and expression confirmed cell lines for both miR-124 and miR-203 overexpression and knockdown were used for assessing the cells tumorigenic properties by performing proliferation, invasion, and apoptosis assays in vitro.

2.13. In vitro Proliferation assay

Proliferation assays were performed on the control and miR-altered cell lines using a colorimetric BrdU assay kit (Roche, Germany). Briefly, 3000 cells per well were seeded 8 hours before the BrdU labeling. The cells were incubated with BrdU labeling reagent for 24 hours , fixed and DNA is denatured by the addition of fixadent (proprietary solution, Roche ,Germany) which makes the labelled BrdU more accessible to the antibody in the next step. The cells are incubated with anti-BrdU antibody for 2 hours. The substrate was added and upon color development the color measured at 492 nm. The intensity of the color developed is directly proportional to the amount of DNA being synthesized and there by number of proliferating cells. All experiments were done in triplicates.

2.14. In vitro cell death detection

The assays were performed using a cell death detection ELISA ^{PLUS} kit (Roche, Germany). The assay is based on the principle of using monoclonal antibodies directed against DNA and histones. Briefly the lysates are plated onto streptavidin coated plates and incubated with a mixture of anti-histone-biotin and anti-DNA-POD. The anti-histone antibody binds to the immunocomplexes and at the same time binds to the streptavidin coated on the plate with the cell lysate. The DNA POD antibody reacts with the DNA of the nucleosome. The amount of POD is determined by using ABTS substrate.

2.15. In vitro invasion assay

Invasion assays (BD biosciences) were performed on the control and miR-altered cell lines as described previously [17] . Briefly, 5000 cells were seeded in the upper

compartment of the control or invasion chambers. Ten percent fetal bovine serum was added to the lower chamber, which acts as a chemo-attractant. Cells were incubated for 24 hours at 37°C and 5% CO_2. The chambers were fixed with methanol and stained with crystal violet. Cells were counted under a microscope and compared to controls. All experiments were done in triplicates.

2.16. Mouse orthotopic GBM models

All the mouse experiments were carried out according to protocols approved by the Institutional Animal Care and Use Committee at The University of Texas MD Anderson Cancer Center. Based on the power analysis for each cell type, eight nude mice 4-5 weeks old (obtained from the Department of Experimental Radiation Oncology at MD Anderson Cancer Center) were used. The cells transduced with vector alone were used as controls. Brain orthotopic tumor models were generated as described previously (*17*). Briefly, 50,000 cells were implanted in a total volume of 5 µl into the right frontal lobes of the mice as shown in figure 3. Prism 6.01 [Graph pad] was used to generate the Kaplan Meir survival curves.

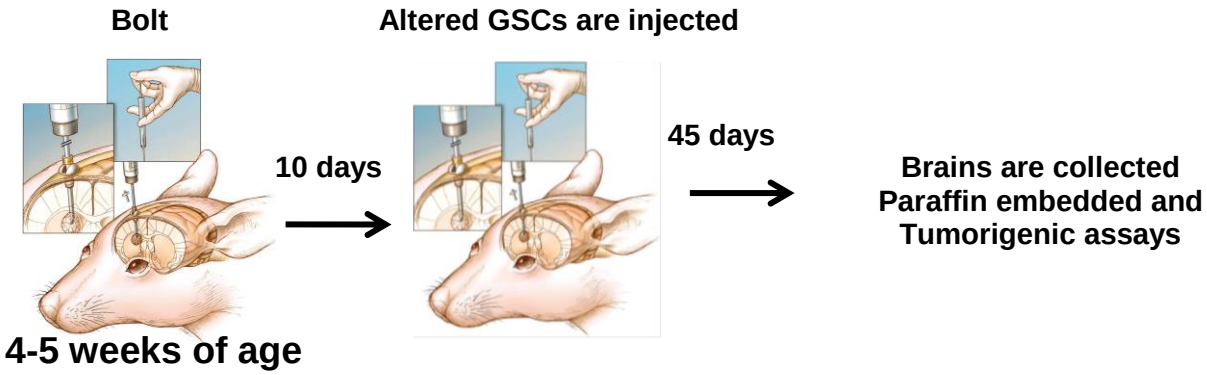


Figure 4: Implantable guide screw system for orthotopic brain tumor models. Male nude mice of 4-5 weeks old are bolted with either plastic or metallic bolts and allowed to recover for 10 days and altered GSCs are injected through the bolt. Tumor growth is monitored by MRI imaging and the brain tissue is collected after 45 days. In vivo assays are performed on the brain sections. Figure modified from (*81*)

2.17. In vivo proliferation assay

The paraffin sections were incubated at 60°C for 30 minutes. This allows the melting of paraffin and binding of the tissue to the glass slide. Following this, the sections were hydrated through alcohol grades after clearing with 3 times in xylene. Antigen retrieval was performed with citrate buffer in a steamer for 20 minutes and allowed to cool for 1 hour. Sections were blocked in 5% goat serum for 1 hour followed by incubation with human-specific NuMA antibody (1:100; Abcam) overnight at 4°C. After washing twice with 1X phosphate-buffered saline plus 0.1% Tween-20, the sections were incubated with Ki67 antibody (1:500, Dako Clone MB-1) for 1 hour and washed twice with phosphate-buffered saline followed by incubation with secondary antibodies. The slides were mounted with DAPI and double-positive cells (Ki67 and NuMA) were counted from 10 different fields with the fluorescent microscope.

2.18. In vivo apoptosis assay

In vivo TUNEL assay was performed according to the manufacturer's instructions (Roche, Germany). Briefly, after hydrating the paraffin sections through alcohol grades, antigen retrieval was performed and the slides were blocked for 1 hour in 3% bovine serum albumin and 20% fetal bovine serum. After washing, the slides are incubated with TUNEL reaction mixture at 37°C in a humidified atmosphere followed by mounting with DAPI. Positive cells were counted in 10 different fields.

2.19. In Vivo Invasion assay

Invasion potential of the cells was determined by staining with a NuMa antibody as described in the section in vivo proliferation assay..

2.20. Statistical analysis

An unpaired two-tailed Student`s t-test was performed to evaluate the differences between the control and treatment groups. All quantified data represent at least three independent experiments.

Chapter 3: Aim of the study

REST promotes oncogenic properties of GSCs both in vitro and in vivo. It affects the survival of the tumor bearing mice. Earlier studies have found that REST is overexpressed in class of medulloblastoma tumors causing the blockage of neuronal differentiation. Deregulation of normal expression of REST leads to tumor formation. The main goal of the project is to delineate the mechanism by which REST regulates oncogenic properties in GSCs. REST is predicted to regulate more than 1000 genes, it is crucial to identify the major downstream targets of REST that play an important role in regulating the oncogenic properties of GSCs. We considered the microRNA targets that are regulated by REST as they are fewer in number (1800) when compared the genes (21,000).

Aim 1: To identify and validate the potential miR-targets of REST that play role in GSC tumorigenesis

microRNA microarray was performed on siRest or siNT (control) transfected HR-GSCS. As REST is a transcriptional repressor we considered the microRNAs that are up regulated upon REST knock down. We identified two potential microRNAs, miR-124 and miR-203 that are regulated by REST. The identified potential REST targets were validated by taqman assays and REST binding to the gene chromatin of these microRNAs was confirmed by ChIP and luciferase assays.

Aim 2: To determine whether REST - miR-124 axis regulates oncogenic properties of GSCs *in vitro* and *in vivo*

miR-124 was identified as one of the potential targets of REST from our screen. miR-124 is the most abundant microRNA in adult and embryonic brain. Studies have shown that REST functions as a negative regulator of miR-124 in NSCs. In this aim we tested if miR-124 regulates oncogenic properties of GSCs and does the REST-miR-124 axis has any role in GSC tumorigenesis. We performed LOF and GOF of miR-124 by lentiviruses in GSC cells and performed in vitro and in vivo tumorigenic assays (proliferation, invasion and apoptosis). We observed that over expression of miR-124 leads to decreased proliferation and invasion and increased invasion both in vitro and in vivo. GOF of miR-124 has increased the survival in tumor bearing mice when compared to its controls.

Aim 3: To determine whether REST mediated control of miR-203 regulates oncogenic properties of GSCs *in vitro* and *in vivo*

Another novel microRNA target that has been identified in our screen was miR-203. This aim tests if REST-miR-203 axis regulates the oncogenic properties of GSCs both *in vitro* and *in vivo*. LOF and GOF of miR-203 GSCs were made by lentiviral transduction. Oncogenic potential of these cells was validated by proliferation, apoptosis and invasion assays. We observed either overexpression or knock down of miR-203 did not have any role in regulating the cell proliferation or apoptosis of these cells both *in vitro* and *in vivo*. But overexpression of miR-203 lead to decreased invasion potential of these cells both invitro and in vivo. Also, overexpression of the miR-203 in these cells led to increased survival of the tumor bearing mice.

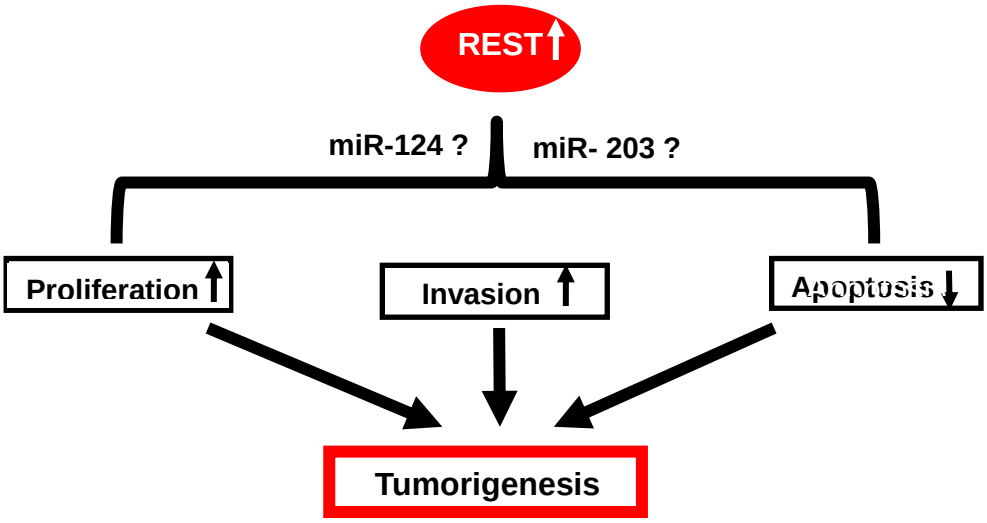


Figure 5: Schematic representation of REST regulatory circuit in tumorigenesis. REST might be regulating the tumorigenesis of GSCs either through either of the miRs or both the miRs.

Chapter 4: Identification and validation of REST-miR targets that may play a role in GSC tumorigenesis

Rationale

As reviewed in the introduction, our group and two other groups have shown that REST regulates the oncogenic properties of GSCs (*17, 18*). When REST is expressed at higher levels there is an increase in cell proliferation, invasion, self-renewal and decrease in apoptosis both *in vitro* and *in vivo*. GSCs with high REST(HR-GSCs) produced more infiltrative tumors while low REST GSCs (LR-GSCs) produced circumscribed tumors. A schematic representation of REST regulating oncogenic properties is shown in figure 3. REST regulates survival in orthotopic mouse tumor models. However, the mechanistic understanding of the REST driven self-renewal and oncogenesis needs further elucidation. REST is predicted to regulate more than 1000 genes, it is crucial to identify the major downstream targets of REST that play an important role in regulating the oncogenic properties of GSCs . Hence, the identification of mechanism(s) by which REST regulates oncogenic properties of GSCs will foster development of targeted therapy downstream of REST.

In this section, we identified the potential microRNA targets of REST that might play a role in GSC tumorigenesis by performing a microRNA microarray on REST loss of function cells. The identified microRNA targets were first validated by qRT-PCR to confirm the microRNA microarray results. Two potential microRNAs, miR-124 and miR-203 were identified and validated. Taq man assays were performed to find the expression levels of mature microRNAs in both loss and gain of function of REST cells.

REST binding to the gene chromatin of the miRs was further confirmed by ChiP assay and luciferase assay. Together our data suggest that miR-124 and miR-203 are the potential microRNA targets of REST and are differentially regulated with REST expression.

Results

REST directly targets miR-124 and miR-203 and forms the REST-miR-124/ REST-miR-203 axis

To determine potential miR targets of REST in GSCs, we performed genome wide profiling using microRNA microarray. To represent REST loss of function manipulations, we transiently transfected two high-REST (HR)-GSC cell lines (HR-GSC1 and HR-GSC2), with small interfering RNA Rest, (siRest) or a non-targeting control (siNT). A total of four cell lines: HR-GSC1/siNT, HR-GSC1/siRest, HR-GSC2/siNT, and HR-GSC2/siRest were generated. We used siRNA in these experiments to identify the potentially immediate direct targets of REST. We then performed a genome-wide miR expression analysis, As REST is a repressor transcriptional factor we looked into the microRNAs that are up regulated when REST is knocked down with a fold change of 2 or more with a significant p value of $p<0.05$. Whereas 128 miRs in HR-GSC1 and 43 miRs in HR-GSC2 had higher expression in the siRest-treated cells than in the siNT controls, only seven miRs were common between HR-GSC1 and HR-GSC2 in this differential analysis, as shown in (Fig 6). A tabular representation of the 7 microRNAs that are up regulated upon REST knock down, their fold changes in each cell line and chromosomal locations is shown in table 4.

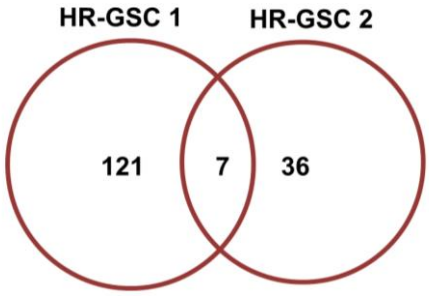


Figure 6: Venn diagrammatic representation of the microRNAs that are up regulated upon REST knock down in both the cell lines. In HR-GSC 1 and HR-GSc2 128 and 43 microRNAs are upregulated upon REST knock down. There are 7 microRNAs that are common in both the lines.

S.NO	MicroRNA	HR-GSC 1 (fold change)	HR-GSC 2 (fold change)	Chromosomal Location
1	miR-124	2.88	2.45	8
2	miR-136	2.7	2.62	11
3	miR-203	5.9299	3.13	14
4	miR-518e	2.189	2.59	19
5	miR-545	2.33	2.32	X
6	miR-557	2.7	2.12	1
7	miR-942	2.64	2.22	1

Table 4: Tabular representation of microRNAs that are up regulated upon REST knock down. Upon REST knock down in both HR-GSCs, 7 microRNAs are upregulated with a fold change of 2 or more.

We first validated the microRNA microarray data by performing qRT-PCR. We observed that microRNAs miR-124 and miR-203 were up regulated in both the HR-GSCS upon REST knock down with a significant p value ($p < 0.05$). On the other hand the other microRNAs are not up regulated in both the cell lines with a significant p value (Fig 7).

Interestingly, miR-124, a well-known target of REST with a critical function in neurogenesis was also identified in our screen. The capture of only a few miRs by this analysis suggested that most of the REST miR targets are cell-line dependent and that REST function is highly context dependent, as we found earlier.

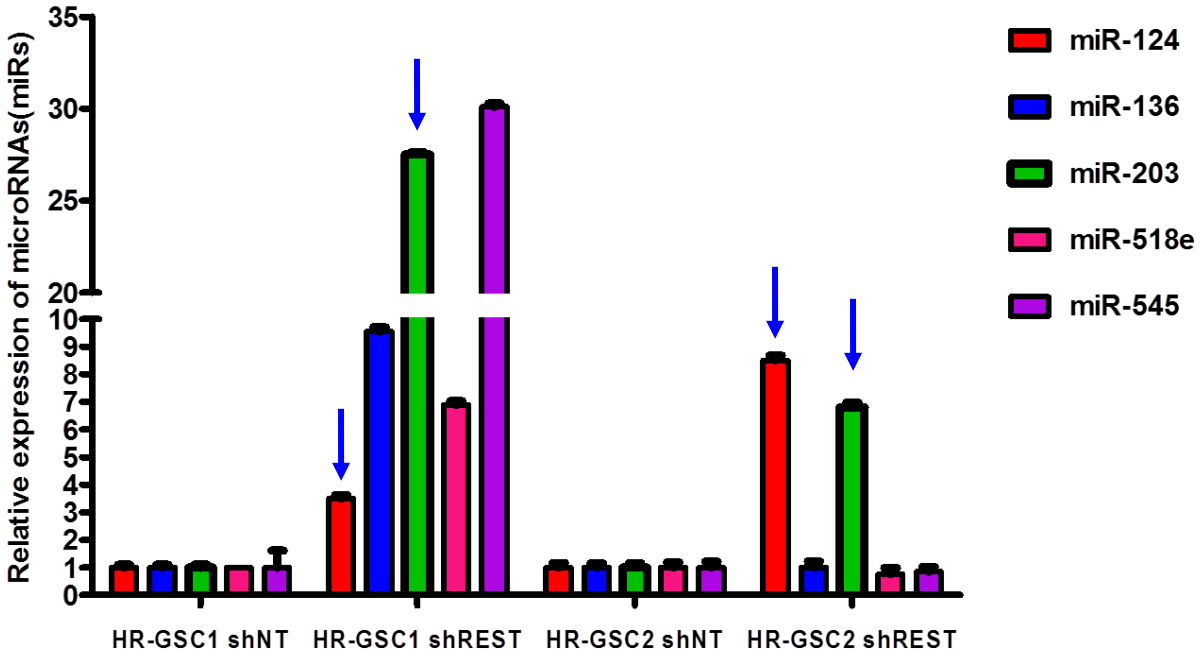


Figure 7: Validation of microRNA microarray by quantitative real time PCR (qRT-PCR). Seven microRNAs that are found be upregulated upon REST knock down were validated by qRT-PCR. miR-124 and miR-203 expression was significantly upregulated upon REST knock down in both the lines when compared to the others. Expression of miR-557 and miR-942 was not detected.

Two High REST cell lines are transduced with shRest or shNT lentiviruses (HR-GSC1shNT, HR-GSC1shRest, HR-GSC2 shNT, HR-GSC2shREST). REST expression was confirmed by western blotting in loss of function of Rest cells. From the same cells total RNA was extracted and the expression of miR-124 and miR-203 was confirmed by taq man assays. Both miR-124 and miR-203 were up regulated upon REST knockdown in both the GSCs as shown in Fig (8A & 8B)

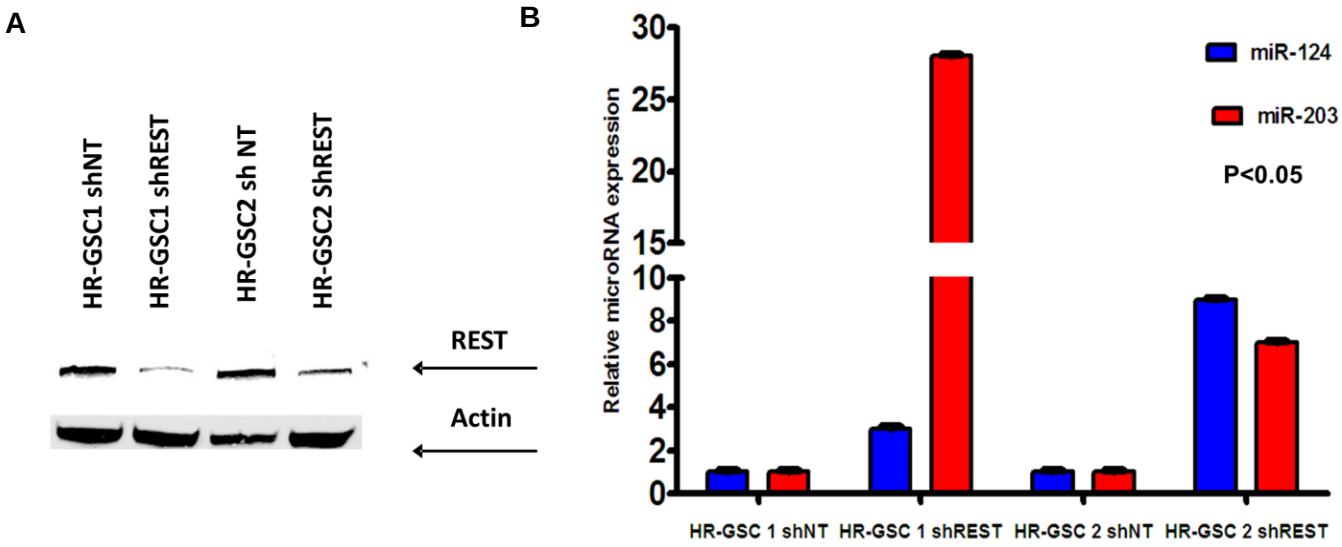


Figure 8: miR-124 and miR-203 are upregulated upon REST knock down.
A. Confirmation of REST knockdown in both the HR-GSC lines by western blotting. **B.** Upregulation of miR-124 and miR-203 is observed upon REST knockdown with a significant p value.

To further confirm the results obtained by REST loss-of-function manipulations, we performed REST gain-of-function manipulations in two low-REST (LR)-GSC lines by introducing either exogenous REST or the green fluorescence protein (GFP) control and confirmed the REST overexpression using Western blotting as shown in Figure 9. We determined the expression levels of miR-124 and miR-203 in these cells by taq

man assays. As shown, overexpression of REST consistently suppressed the expression of miR-124 and miR-203 in both the LR-GSC lines (Fig 9 A & 9B).

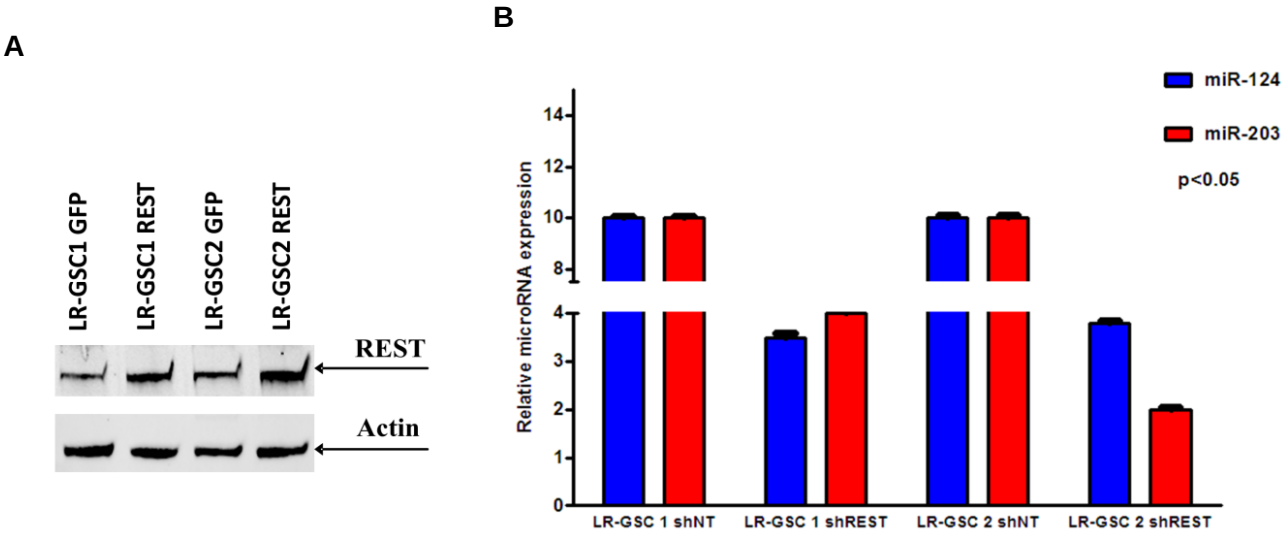


Figure 9: miR-124 and miR-203 are suppressed upon REST overexpression.
A. Immunoblot confirmation of REST expression in REST overexpression LR-GSCs. **B.** Taqman assays was performed to measure the relative expression levels of both the miRs. Both the microRNAs miR-124 and miR-203 were suppression upon REST overexpression.

To confirm whether miR-124 or miR-203 expression was suppressed by direct binding of REST on the gene chromatin, we performed bioinformatic analysis using matInspector (Genomatix software suite) to determine potential REST binding sites (RE1s) present on the miR-124 or miR-203 promoter elements 3000bp upstream and downstream of the miR-124 or miR-203 start site. We then performed chromatin immunoprecipitation (ChIP) analysis using either REST or IgG (control) antibody. qRT-PCR was performed using primers corresponding to sites present on the miR-124 promoter elements site # 1 (-2648) and site #2 (-300) (non-specific) upstream of the Transcriptional start site (TSS) and miR-203 promoter elements: site # 1 (-223), site #2 (-512) , site # 3 (-762), site # 4 (-1223) upstream of the TSS , the potential RE1 site and an additional random site, site #5. REST was found to bind to site #1 gene

chromatin of miR-124 and only at site #4 of miR-203 indicating specific REST binding sites.(Fig 10A & 10B)

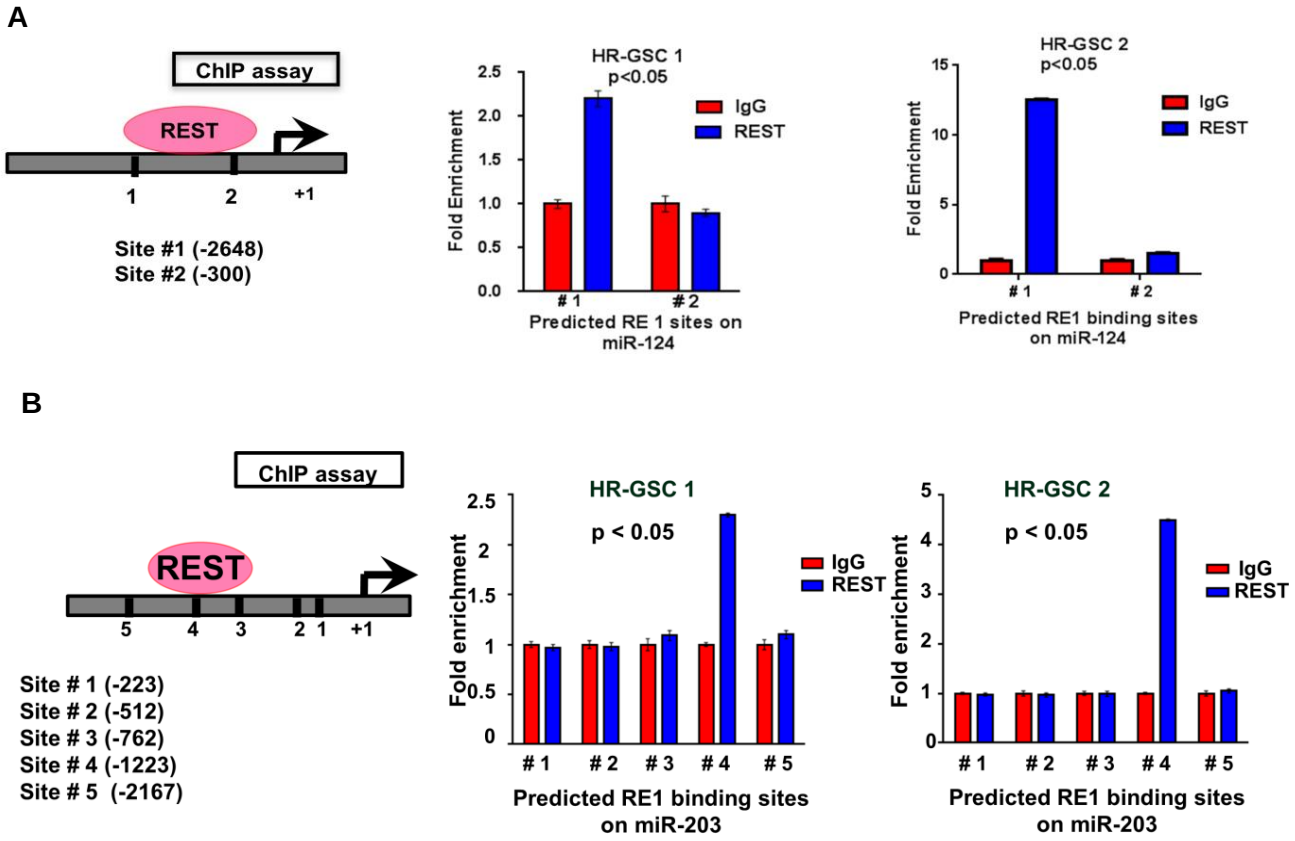
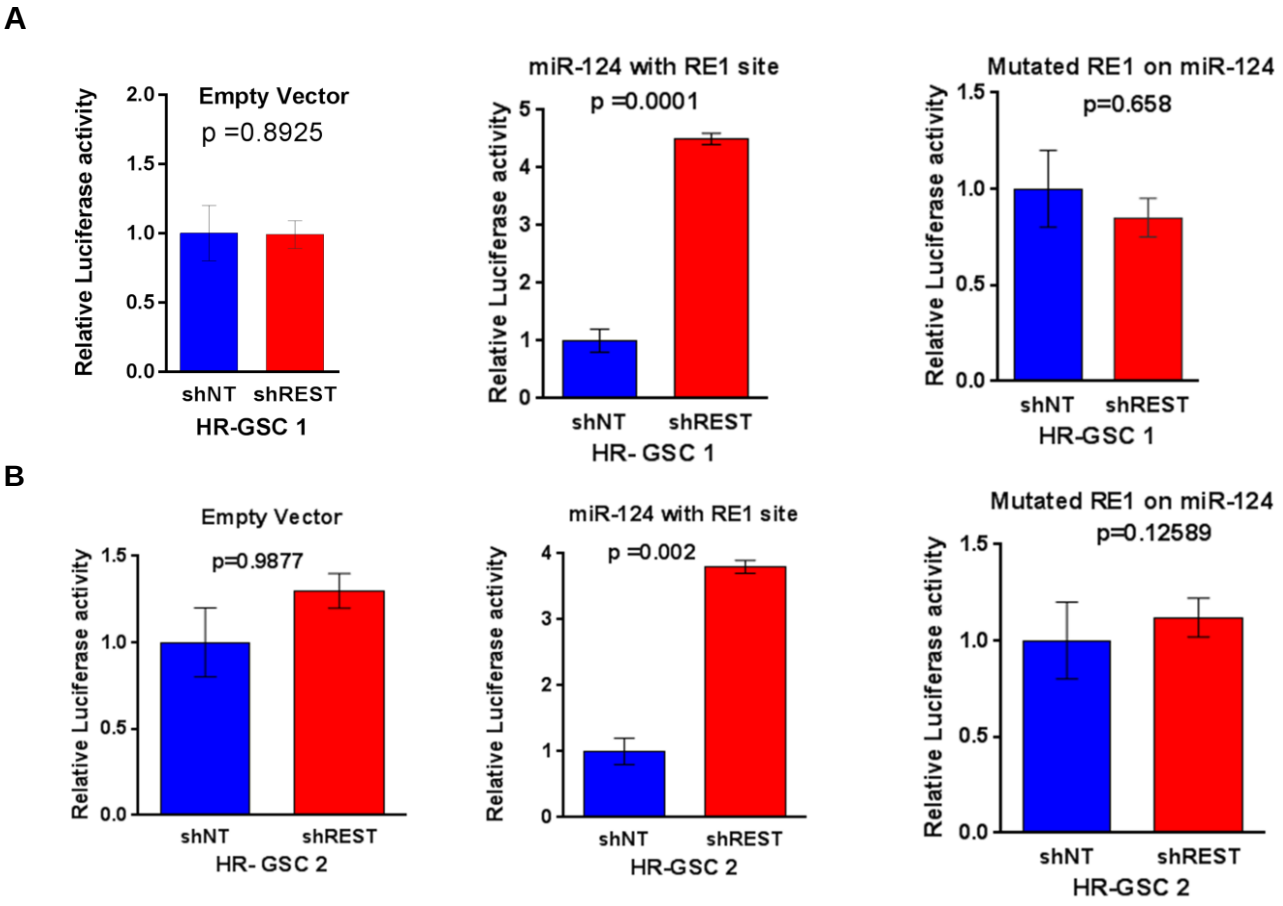


Figure 10: miR-124 and miR-203 are the transcriptional targets of REST. Predicted RE1 binding sites upstream of miR-124 and miR-203 TSS are represented **(A &B).** ChIP was performed in HR-GSC with anti-REST antibody or and IgG control followed by qPCR analysis using primers specific for the predicted RE1 sites upstream of miR-124 or miR-203. Student's t test was performed to determine the statistical significance.

To determine whether the single REST binding site present in the miR-124 or miR-203 gene chromatin directs REST-dependent expression, we sub cloned the site in front of a luciferase reporter gene. For comparison, we also used no site or a mutated version of the site. We then transfected the plasmids in HR-GSC1/2/shNT and HR-GSC1/2/shRest cells and determined luciferase activity in the resulting cells. Results

shown in Fig. 11 indicated that when no site was present or when the site was mutated, luciferase activity was similar in the shNT- and sh/Rest-expressing HR-GSC cells. In contrast, when the REST binding site was present, luciferase activity was higher in shRest-expressing cells than in shNT-expressing cells. Thus, taken together, these results indicated that REST directly targets miR-124 and miR-203 in GSCs.



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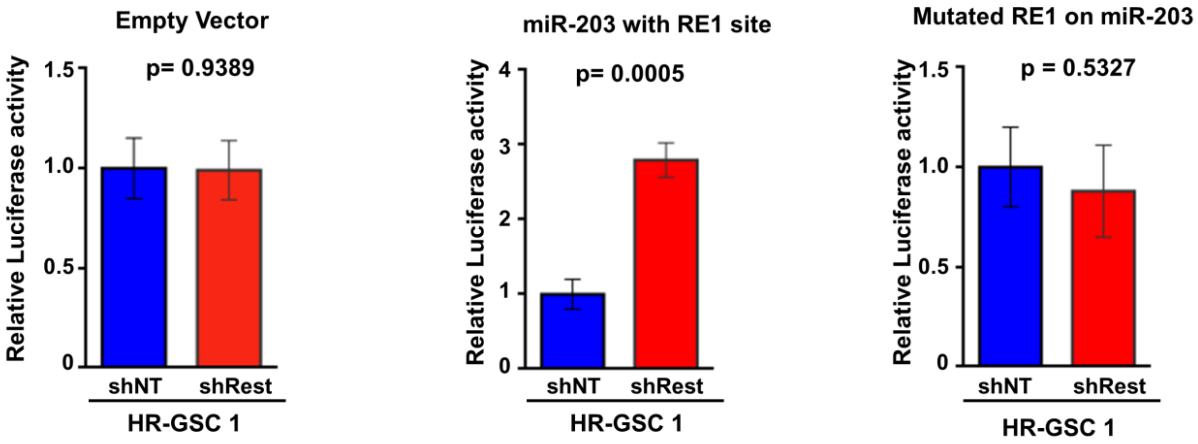

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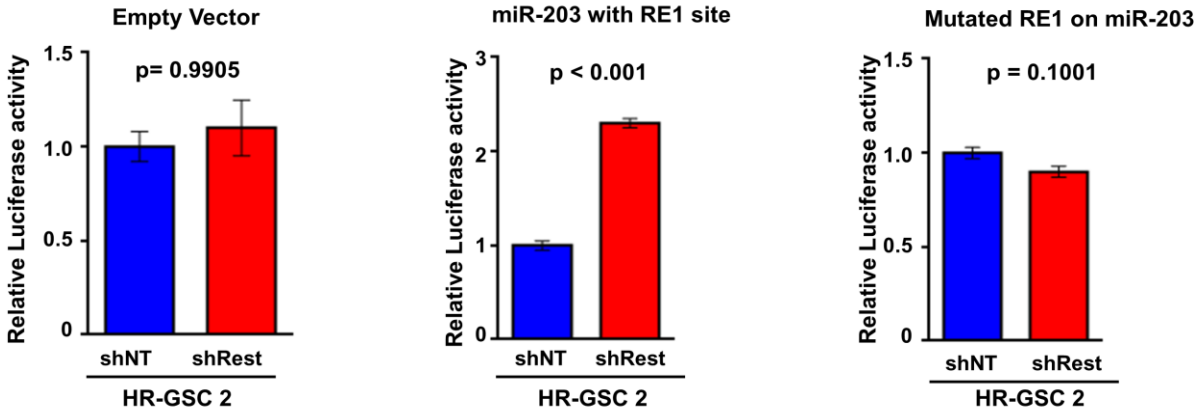


Figure 11: REST targets miR-124 and miR-203 gene expression through specific sites on gene chromatin. A & B: We performed reporter gene analysis using a plasmid containing luciferase gene downstream of wither the specific REST binding site present on the miR-124 gene chromatin, a mutated version of the site or no site transfected into HR-GSC1sh NT,HR-GSC 1 shREST **(A),** HR-GSC2 shNT,HR-GSC2 shREST **(B)** cells and measured the luciferase activity. Similar experiment was performed with the miR-203 gene chromatin binding region **(C &D**). Luciferase activity remained unaltered when an empty vector was expressed in either shNT or shREST expressing cells, it increased in shREST when compared to shNT when the plasmid contained the REST binding site. The increase of the activity is further reversed when the plasmid contained the mutated binding site.

Both the microRNAs miR-124 and miR-203 are identified as potential targets of REST that might play a role in GSC tumorigenesis. Both these microRNAs are identified as tumor suppressor microRNAs in other cancers. Studies have shown that both these

microRNAs are highly repressed in GBM tumors when compared to normal brain. Studies have also shown that miR-124 plays a important role in migration and invasion in glioblastoma. Loss of miR-124 leads to stem cell like traits and more invasiveness of these cells. miR-124 is a known target of REST in NSCs and ES cells. Studies have shown that miR-124 targets cyclin dependent kinase 6(CDK6) and there by regulates the cell cycle. miR-203 is found to be highly repressed in hepatocellular carcinomas and hematological malignancies. Ectopic expression of these microRNAs miR-124 and 203 in hepatocellular carcinomas resulted in inhibition of cell proliferation.

The microRNA miR-124 is already a known target of REST in NSCs and ES cells but its role in GSCs is not known. MiR-203 is a novel target of REST identified in our screen. So we decided to investigate independently on the two axes REST-miR-124 and REST-203 have any role on GSC tumorigenesis.

Summary

Our data suggest that upon REST knock down there is an up regulation in the expression levels of miR-124 and miR-203 in GSCs. Also, upon REST overexpression there is a down regulation of both these microRNAs. We have also shown that REST binds to the RE-1 sites located at 2.6kb and 1.2kb upstream of the TSS for miR-124 and miR-203 respectively. Our findings from luciferase assay further confirmed that there is an increase in the luciferase activity in the presence of REST binding site in shREST cells when compared to its controls (shNT). In contrast, when no binding site was present or when it was mutated there was no difference in the luciferase activity in REST knock down cells (HR-GSC shRest) and its controls (HR-GSC shNT). These results indicate that REST directly targets miR-124 and miR-203 in GSCs.

Chapter 5: REST-miR-124 axis regulates oncogenic properties of GSCs *in vitro* and *in vivo*

Rationale

In the previous section, we identified miR-124 as a potential target of REST. We observed a reciprocal relationship in the expression of the microRNA miR-124 and REST. We further determined that REST binds to the RE 1 sites located at 2.6kb upstream of the TSS of miR-124 gene chromatin. This was further confirmed by luciferase assays. The microRNA, miR-124 mature sequences are highly conserved and are the most abundant miRNA in adult and embryonic brain. Based on binding and expression studies, miR-124 was identified as one of the earliest miRs to be a direct target of REST. Studies have shown that miR-124 is a part of REST regulatory network in neural and non-neural cells where REST represses miR-124 expression, thereby prevents neurogenesis and allows expression of non –neural genes. Studies have shown that miR-124 is up regulated during differentiation of NSCs. miR-124 is up regulated during neuronal differentiation and promotes cell cycle exit by inhibiting cyclin D, a cell cycle regulator. A miR microarray study by Sibler et al and a immunohistochemical study by Fowler has shown that miR-124 is down regulated in gliomas relative to the normal brain (*82, 83*). Furthermore, down regulation of miR-124 correlates with poor survival in colorectal cancers.

In this section we investigated if the REST-miR-124 regulatory axis regulates oncogenic properties of GSCs both *in vitro* and *in vivo.* We studied this hypothesis by generating cell lines (2 independent cell lines) with loss and gain-of-function (LOF/GOF) of miR-124 gain by lentiviral transductions. Expression levels of miR-124

were confirmed by taqman assays and tumorigenic assays were performed both *in vitro* and *in vivo*. We performed proliferation, apoptosis and invasion assays *in vitro* on LOF and GOF miR-124 cells. After characterizing the cells *in vitro* we implanted the cells into the brains of nude mice and performed Kaplan Meir survival analyses. Further, we performed proliferation, apoptosis and invasion assays on tumor sections.

Confirmation of over expression or knock down of miR-124 expression

Two independent HR-GSCs and their respective REST knock down cells were obtained and cultured. A schematic representation of experimental design is shown in figure 12.

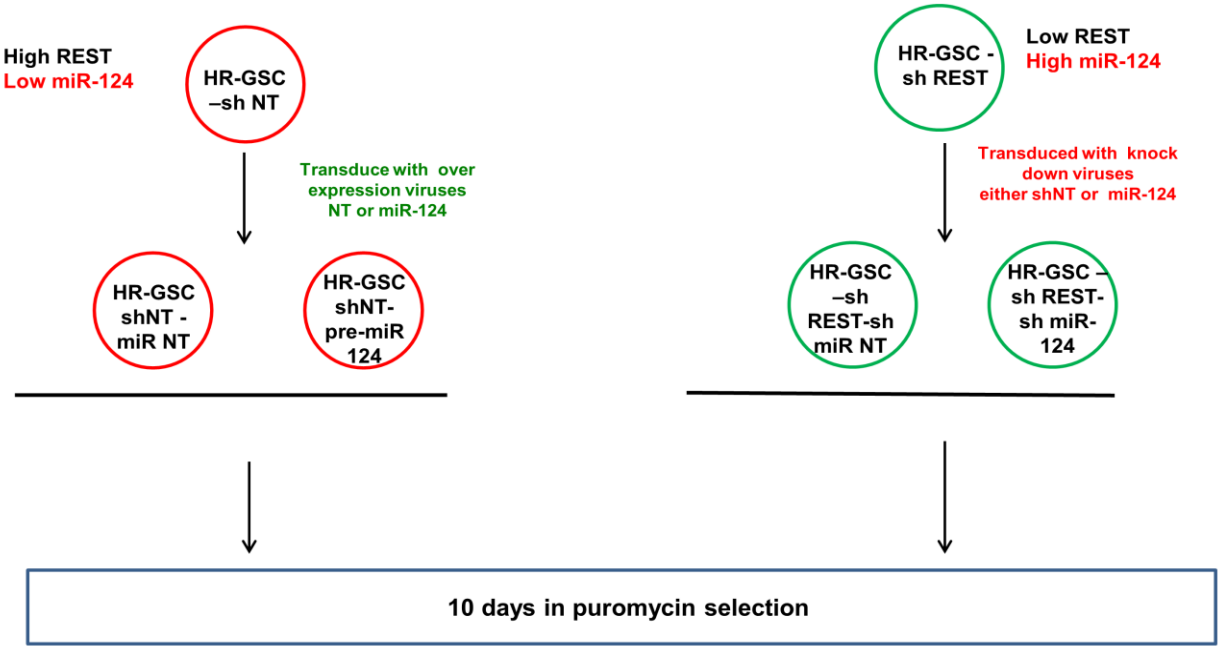


Figure 12: Schematic representation of experimental design. High REST GSCs have higher expression levels of REST and low expression of miR-124. We used this line to overexpress the miRs using lentiviruses. The REST knock down lines have lower expression of REST and are high levels of miR-124. Lentiviral knock downs of the miR-124 were performed in REST knock down lines.

Gain of Function: HR-GSCs have higher expression levels of REST and lower expression of miR-124. We transduced two HR-GSC with lentiviruses containing either the control vector (V) or pre-miR-124. The generated stable lines (HR-GSC1.shNT/V and HR-GSC1.shNT/ pre-miR-124; HR-GSC2.shNT/V and HR-GSC2.shNT/ pre-miR-124) were selected in puromycin and the overexpression of miR-124 was confirmed by taqman assays.

Loss of Function: Rest knock down cells have lower expression of REST and higher expression of miR-124. We transduced two REST knock down lines (HR-GSC shREST) with lentiviruses containing either the control vector (V) or shmiR-124. The generated double knock down (shREST and shmiR-124) stable lines (HR-GSC1.shREST/V and HR-GSC1.shREST/ shmiR-124; HR-GSC2.shNT/V and HR-GSC2.shNT/ pre-miR-124) are selected in puromycin and the knock down of miR-124 was confirmed by qRT-PCR.

We observed that upon gain or loss of function of miR-124 there is an increase and decrease in the expression of miR-124 respectively.

REST-miR-124 axis regulates cell proliferation, apoptosis and invasion in GSCs in vitro

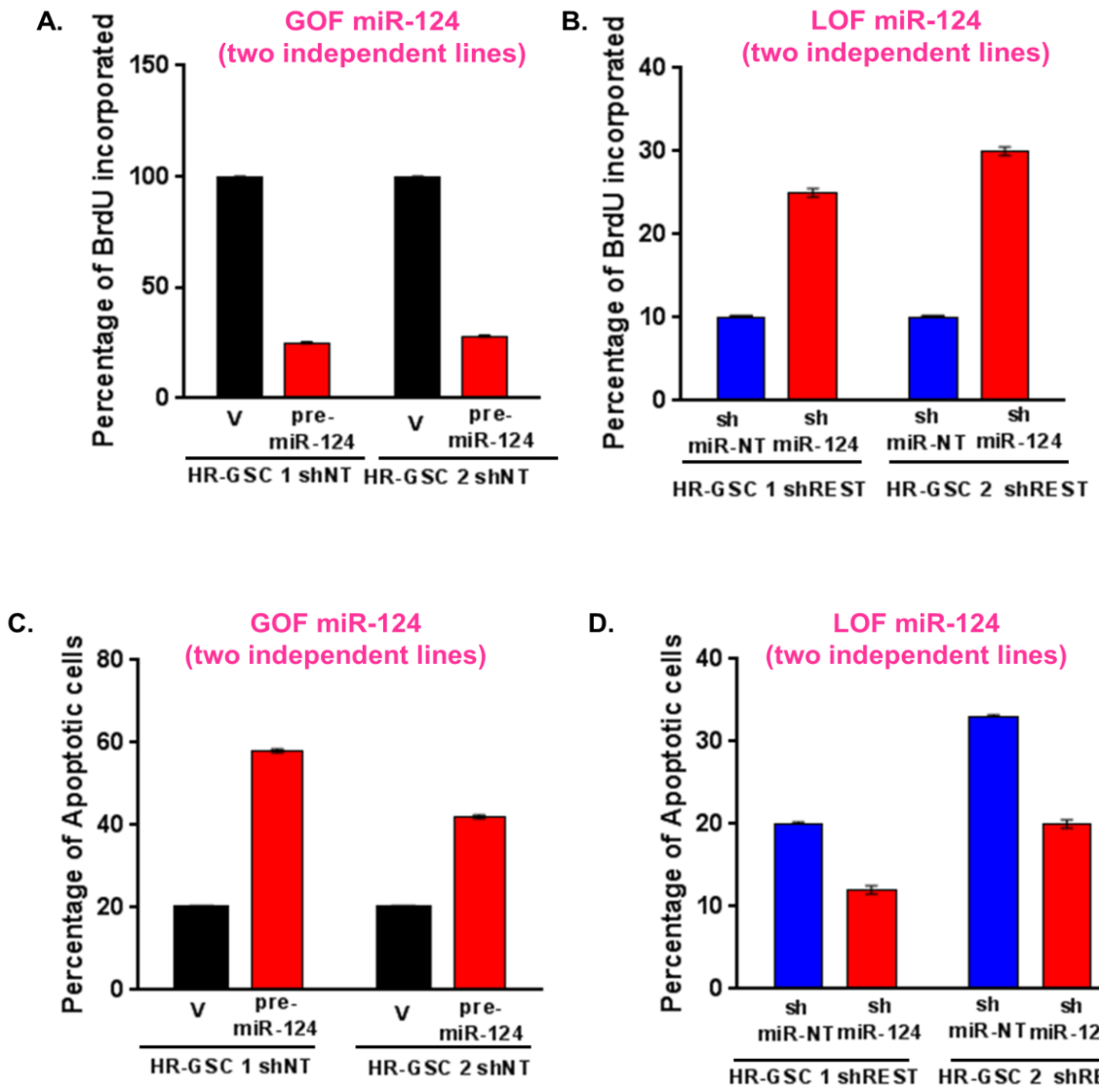
To understand the mechanism by which REST-miR-124 axis regulates the oncogenic properties of GSCs we used the established LOF and GOF miR-124 cells and performed tumorigenisis assays.

We performed a BrdU proliferation assay on the miR-124 GOF and LOF cells to determine the proliferative capacity of these cells. We observed that upon over expression of miR-124 (GOF) in two independent cells there is decrease in the cell proliferation when compared to its controls as shown in fig (13 A) .Upon knock down of miR-124 there is an increase in the percentage of BrdU incorporated cells indicating these cells are dividing at a higher rate when compared to its controls Fig (13 B)

We also performed apoptosis assay, to determine the DNA fragmentation that results from apoptosis. We observed that upon overexpression of miR-124 there is an increase in the percentage of apoptotic cells when compared to its control. Similarly upon knocking down of miR-124 we observed that there is a decrease in the apoptosis (Fig 13 C & D)

To determine the role of miR-124 in invasion, we subjected the GOF of miR-124 cells for invasion assay. Upon over expression of miR-124 we observed that there is a decrease in the percentage of invading cells when compared to its controls. Next we wanted to know if REST-miR-124 axis has any role in regulating invasion of GSCs. We knock down miR-124 in REST knock down cells (shREST/shmiR-124) and performed the invasion assay. We observed that there is an increase in the invasive potential of

these double knock down cells (shREST/shmiR-124) when compared to its controls (shREST/shmiRNT). (Fig 13 E & F)



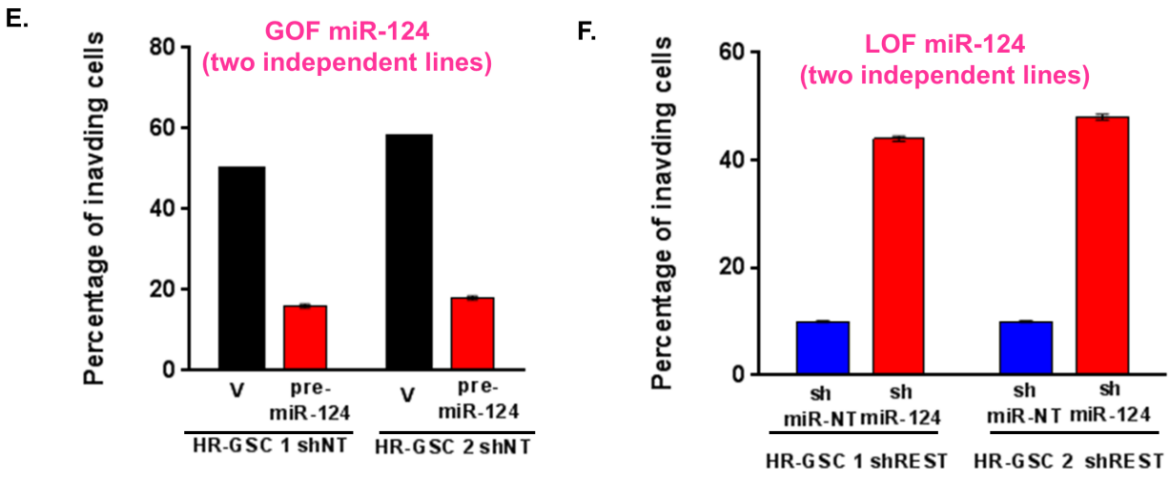


Figure 13: REST-miR-124 axis regulates cell proliferation, apoptosis and invasion in GSCs in vitro. A & B: We performed a BrdU Proliferation assay on GOF and LOF of miR-124 cells. We observed that upon over expression of miR-124 there is a decrease in the percentage of proliferating cells. **C & D:** Apoptotic assay was performed on the LOF and GOF miR-124 cells. We observed that upon overexpression of miR-124 there is an increase in the apoptotic cells. **E &F:** We performed an invasion assay. Upon knocking down of miR-124 there is an increase in the percentage of invading cells.

We have observed that over expression of miR-124 in the GSCs resulted in decreased cell proliferation, increased apoptosis and decreased invasion. Upon knocking down of miR-124 in the REST knock down cells lead to increased cell proliferation and invasion and decreased apoptosis. From this we can conclude that REST-miR-124 axis regulates the oncogenic properties of GSCs.

MiR-124 regulates survival of mice harboring GSC-derived brain tumors

REST-miR-124 axis regulates the oncogenic properties of GSCs in vitro. We wanted to test if miR-124 has any role in regulating the tumorigenesis and affect the survival of the tumor bearing mice. Two independent GOF of miR-124 (HR-GSC1.shNT/V and HR-GSC1.shNT/ pre-miR-124; HR-GSC2.shNT/V and HR-GSC2.shNT/ pre-miR-124) cell lines were generated and these are implanted into the brains of the nude mice and Kaplan-Meier survival analyses is performed. Over expression of miR-124 in two independent HR-GSC lines increased the survival of tumor bearing mice, indicating miR-124 has tumor suppressor functions (fig 14).

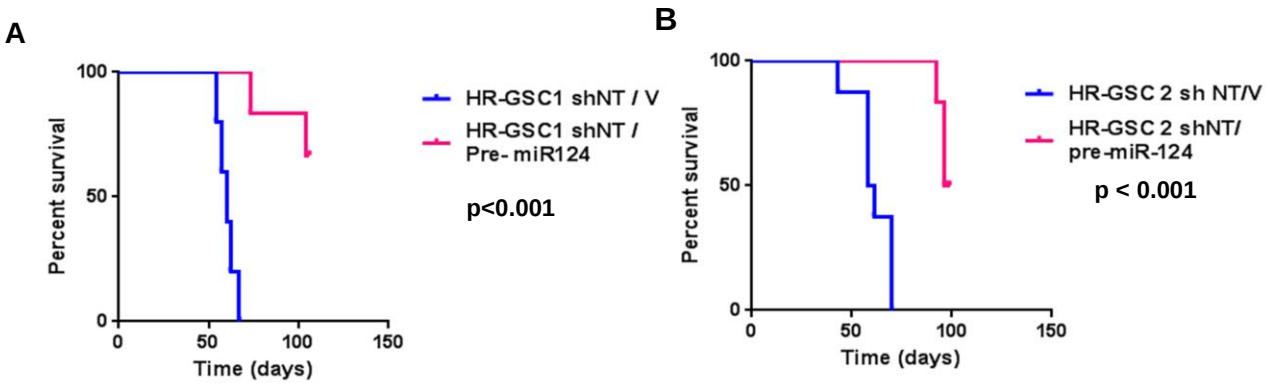


Figure 14: mir-124 regulates survival of the tumor bearing mice. Both the HR-GSCs over expressing miR-124 when implanted into the brains of nude mice increased the survival of tumor bearing mice.

REST-miR-124 axis regulates survival of mice harboring GSC-derived brain tumors

We observed that over expression of miR-124 increased survival of tumor bearing mice. We wanted to know if REST-miR-124 axis has any role in the survival of mice. A previous study has shown that REST knock down cells (shRest) when implanted into the brain of nude mice have a longer survival than controls (shNT). We implanted the double knock down cells (shREST/shmiR-124) and its control (shREST/shmiR-NT) into the mice and performed the survival analysis. We observed that upon double knock down (shREST/shmiR-124) down there is a decrease in the survival of tumor bearing mice when compared to its control (fig 15). These results indicate that REST-miR-124 axis regulates the survival of mice harboring GSC derived tumors.

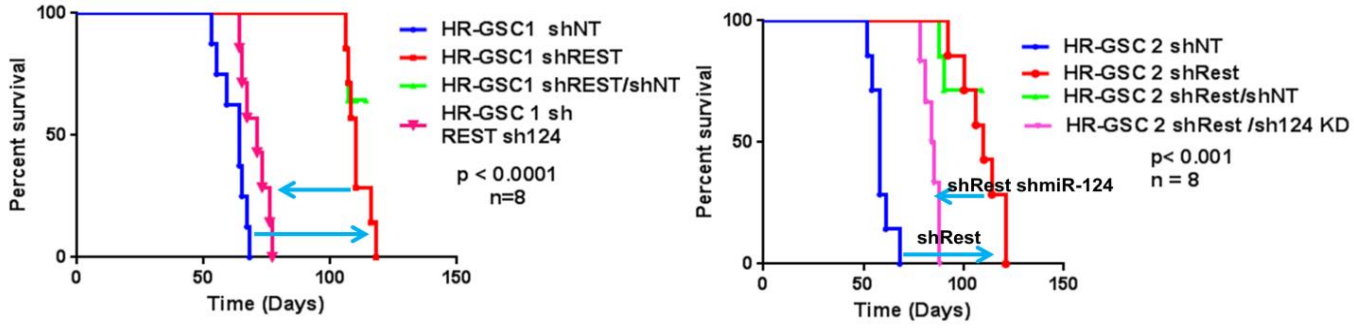


Figure 15: REST-mir-124 regulates survival of the tumor bearing mice. Upon REST knock down the mice survived for longer. Double knock down of REST and miR-124 decreased the survival of tumor bearing mice.

REST-miR-124 axis regulates proliferation, apoptosis and invasion in GSC-derived tumors in mouse brains

We observed that REST-miR-124 axis regulates proliferation, apoptosis and invasion *in vitro*. We also found that REST-miR-124 axis regulates the survival of mice. We wanted to determine if REST-miR-124 axis holds true *in vivo*. GSCs with either GOF or LOF of miR-124 are implanted into the brain of nude mice. Mice were euthanized around 40 days of time and the brains were paraffin embedded and tumorigenic assays were performed on the mouse brain tumor sections. To distinguish human cells from mouse cells, the tumor sections are stained using anti-NuMa antibody that specifically stains only human cells.

To measure the proliferative index in the tumor sections , the tumor sections are double labelled with NuMa and Ki67(proliferative marker). Over expression of miR-124 led to decreased cell proliferation in the tumor sections. In the double knock down tumor sections (shREST/shmiR-124) there is an increase in the proliferating cells when compared to its control.(fig 16)

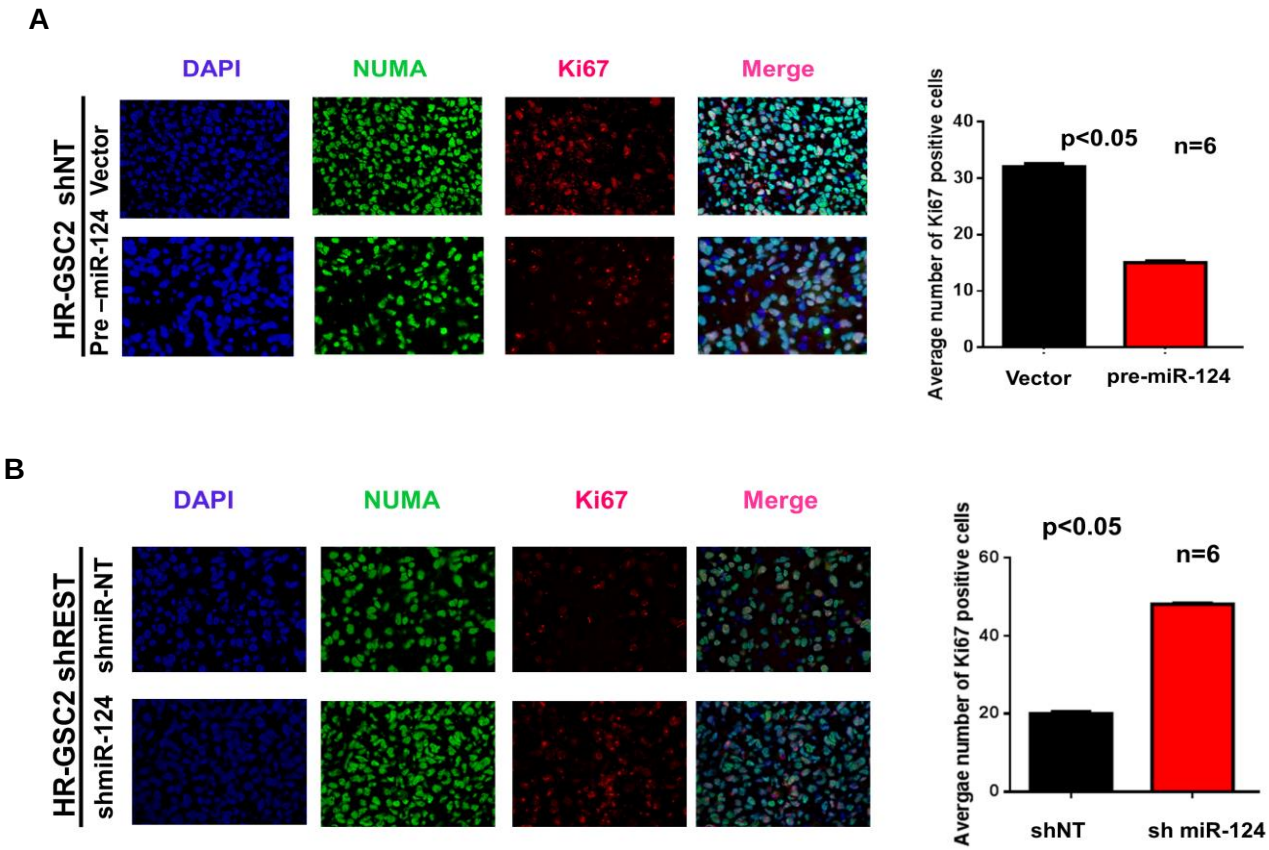


Figure 16: Involvement of REST-miR-124 in cell proliferation. The tumor sections are stained with human specific NuMa antibody (Green). Ki67 (red) a proliferative marker stains the proliferating cells and Dapi (blue) stains all the cells of the tissue. **A.** Over expression of miR-124 decreases the ki67 positive cells. **B**. Double knock down of REST and miR-124 resulted in increase in cell proliferation. A quantitative representation of the same is shown in a bar graph.

We performed TUNEL assay on the tumor sections. Upon over expression of miR-124 we observed that there is an increase in the percentage of apoptotic cells when compared to controls. (Fig 17)

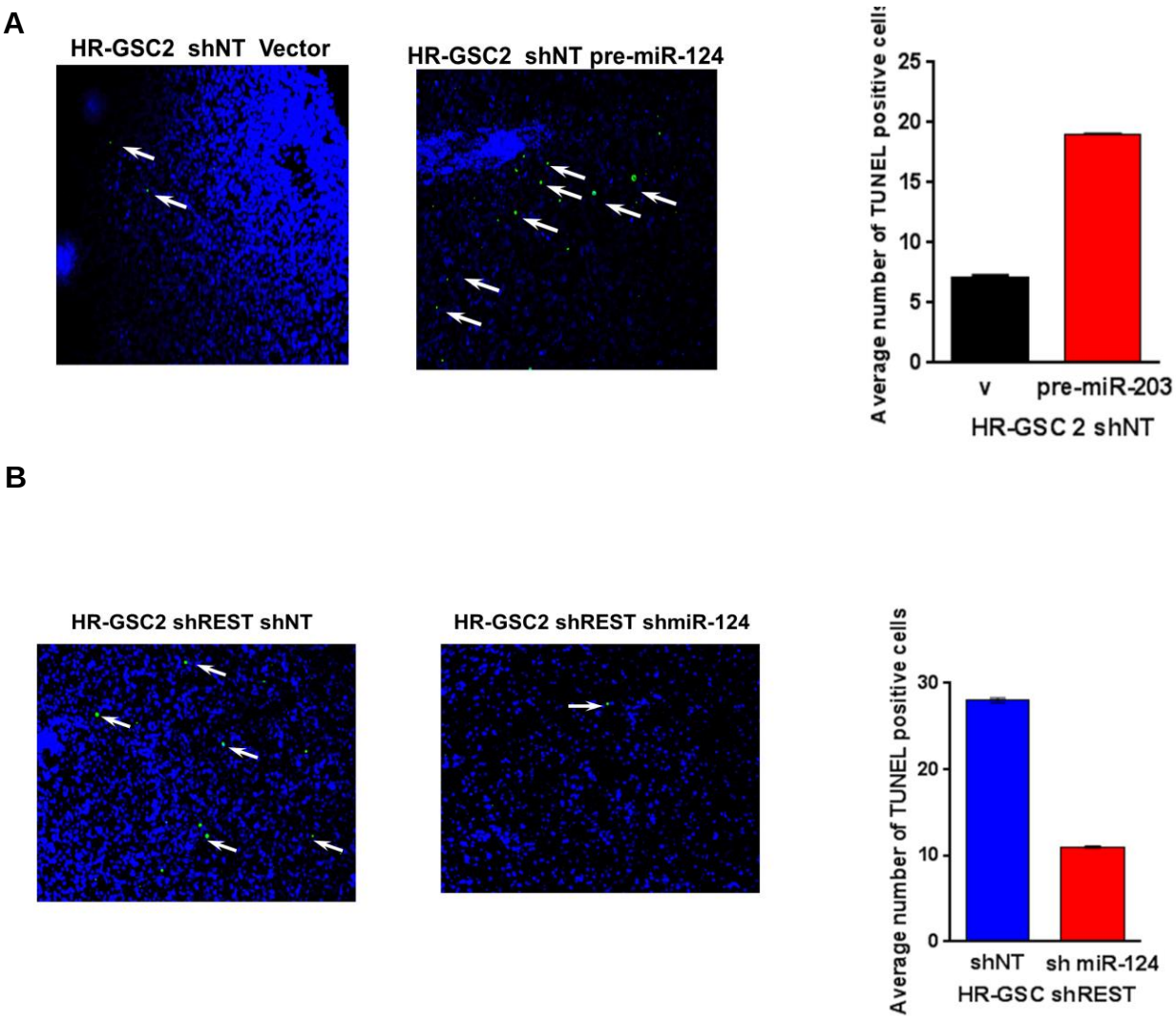


Figure 17: REST-miR124 axis role in apoptosis *in vivo*. Tumor sections are subjected to TUNEL labelling assay. Upon over expression of miR-124 there is an increase in the number of TUNEL positive cells when compared to its controls **(A).** Knock down of 124 in REST knock down cells decrease the number of apoptotic cells **(B).**

Tumors formed by both HR-GSCs were found to be more invasive as we observed tumor cells invading from the core of tumor to the pial surface of the brain. But upon overexpression of miR-124, we observed there is decreased tumor invasion and tumors formed are circumscribed (fig 18A). We wanted to check whether REST-miR-

124 axis has any role in GSC tumor invasion. Double knock down of REST and miR-124 (shREST/shmiR-124) resulted in highly invasive tumors when compared to controls (fig 18B). These results indicate that REST-miR-124 axis regulates cell proliferation, apoptosis and invasion in GSC tumors.

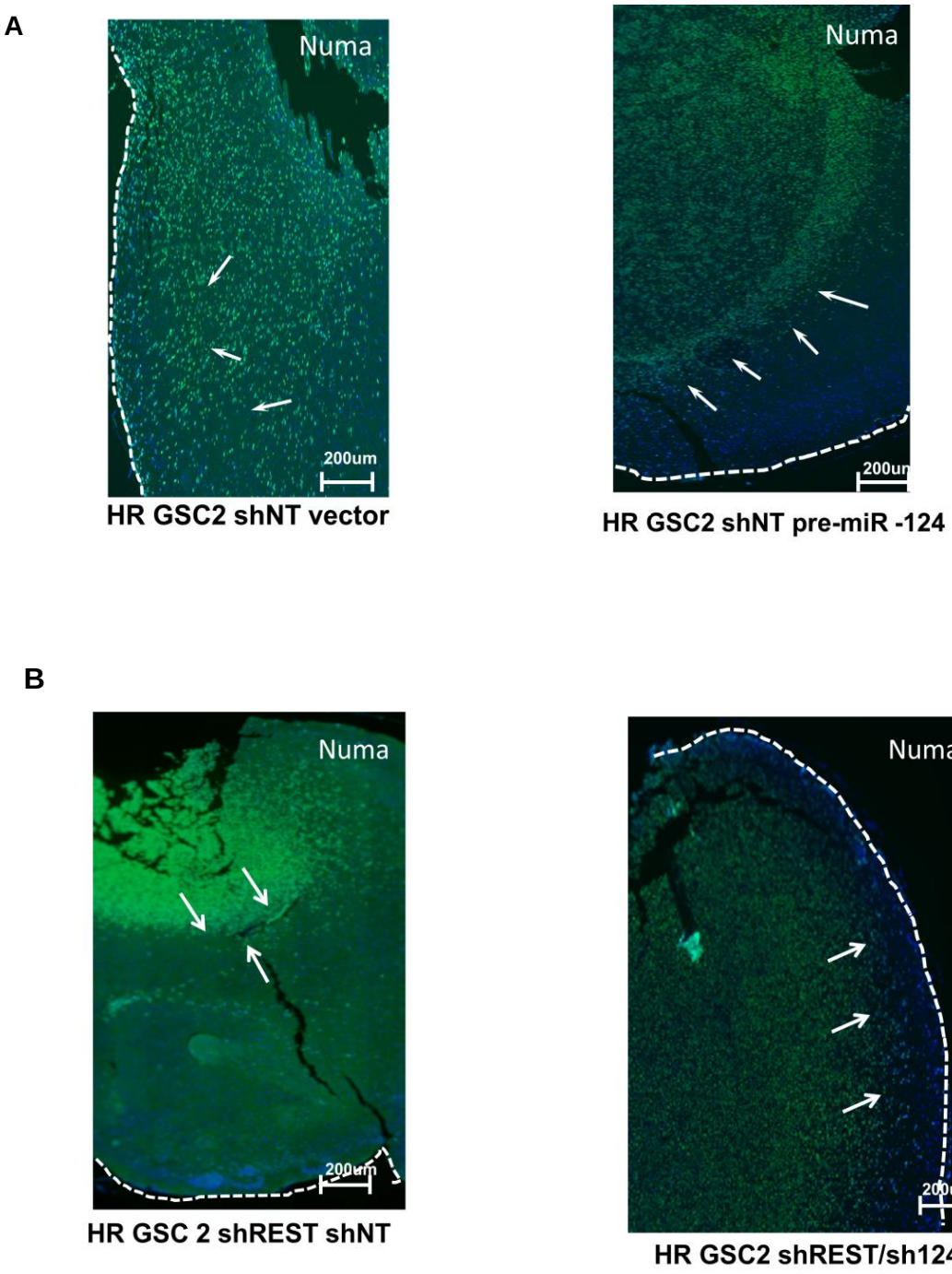


Figure18: Decreased cell invasion caused by shRest is reversed by knockdown of miR-124 in mouse tumors. Upon over expression of miR-124 there is a decrease in the invasion which is shown by staining the human cells implanted in the mouse brain with NuMa. Knock down of miR-124 led to the formation of more invasive tumors.

Summary

In this section we investigated the mechanism by which the REST-miR-124 axis regulates the oncogenic properties of GSCs. We observed that upon over expression of miR-124 there is a decrease in the proliferation of the cells, increased apoptosis and decreased invasion in vitro. Implantation of the GSCs with GOF of miR-124 in the brains of nude mice led to increased survival of the tumor bearing mice. Knock down of miR-124 in REST knock down cells (double knock downs) led to decreased survival of the tumor bearing mice indicating miR-124, is a tumor suppressor gene. *In vivo* tumorigenic assays were performed on the brain sections, we observed increase in the number of proliferative (ki67 positive) cells and invading cells and decrease in apoptotic cells in the mice implanted with double knock down cells (shRest/shmiR-124).

Our finding concludes that miR-124, is a target of REST and they act as an axis that regulates the oncogenic properties of GSCs.

Chapter 6. REST-miR-203 axis regulates invasion but not proliferation or apoptosis of GSCs

Rationale

Genome wide analyses have identified two microRNAs miR-124 and miR-203 that might play a role in regulating GSC tumorigenesis. In chapter 3, we established that a reciprocal relationship exists between REST (protein) expression and miR-124/203 expression. Further, REST binding to the RE1 sites upstream of TSS was confirmed by ChIP and luciferase assays. Additionally, In chapter 4 we have identified that REST regulates miR-124 and there by regulates cell proliferation, apoptosis and invasive potential of the GSCs both *in vitro* and *in vivo*. In the current chapter we investigated the role of the REST-miR-203 axis in regulating oncogenic properties of GSCs. miR-203 has been identified as a skin-specific microRNA and promotes epidermal differentiation by inducing cell cycle exit. Expression of miR-203 has been found to be dysregulated in diseases like psoriasis, rheumatoid arthritis and cancers. Studies have shown that miR-203 is expressed at lower levels in glioma tissue when compared to the normal brain. Studies have shown that miR-203 regulates cell proliferation, invasion and apoptosis of established glioma cell lines U251. The current study mainly focuses on the mechanism by which REST-miR-203 axis regulates the oncogenic properties of GSCs. Either LOF or GOF of miR-203 cells were generated using lentiviruses as described in chapter 1. After confirming the expression levels of miR-203 the cells are subjected to *in vitro* tumorigenic assays (proliferation, apoptosis and invasion). Further the LOF and GOF cells are implanted in the nude mice and survival of the tumor bearing mice is analyzed. The tumor sections were evaluated for proliferation, apoptosis and invasive potential.

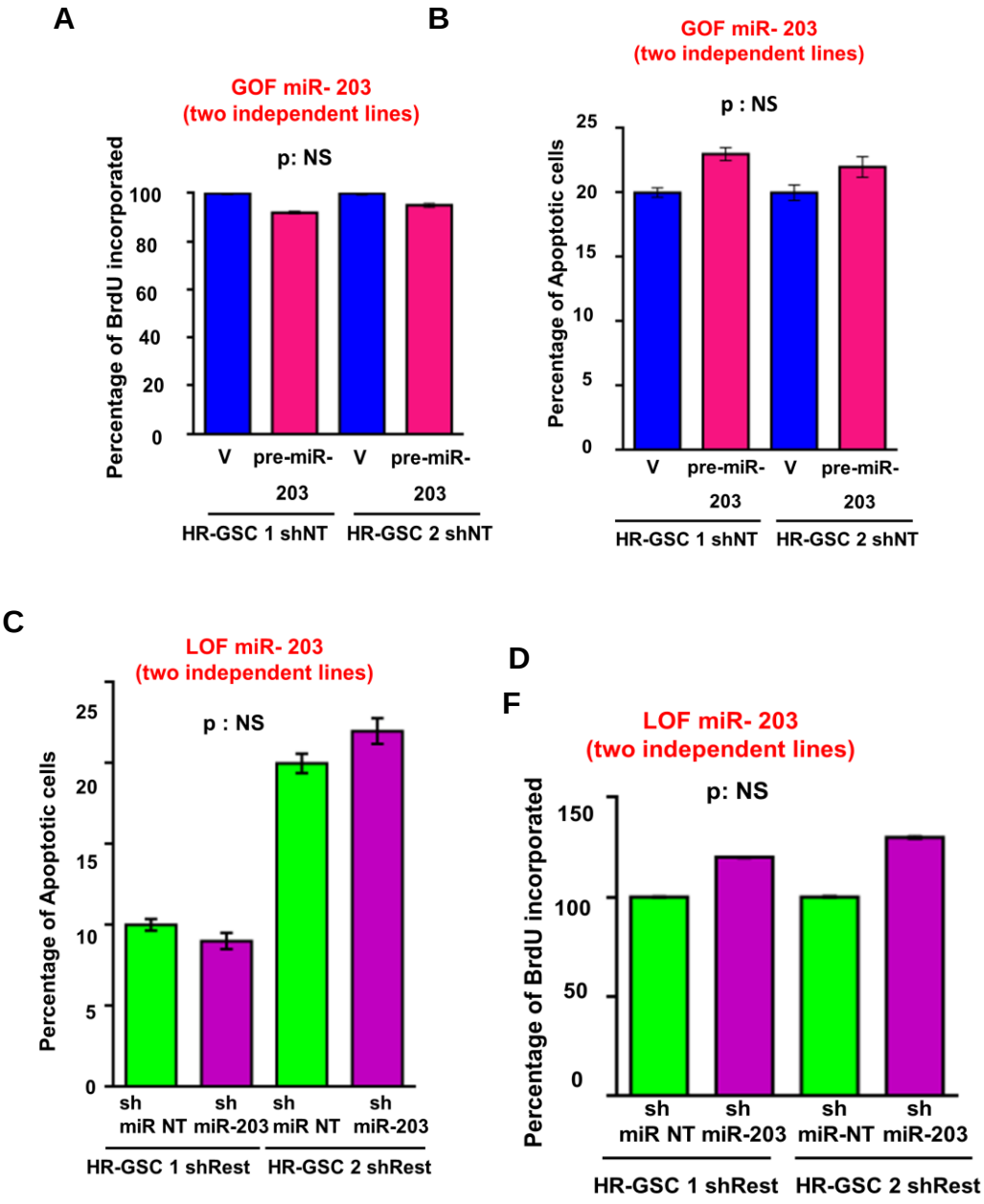
REST-miR-203 axis regulates invasion, but not cell proliferation and apoptosis, in GSCs in vitro

To determine the roles of miR-203 in REST-mediated tumorigenicity, we first determined whether its manipulation in GSCs affected cell proliferation and apoptosis in vitro. We first used the miR-203 gain-of-function cells (HR-GSC1.shNT/V and HR-GSC1.shNT/ pre-miR-203 HR-GSC2.shNT/V and HR-GSC2.shNT/ pre-miR-203) and subjected them to bromodeoxyuridine (BrdU)-labeling assay as shown in Figure 19 .We observed that over expression of miR-203 in two independent GSCs did not affect the cell proliferation. To determine the apoptotic status of these cells, we performed an apoptosis assay and found that over expression of miR-203 did not alter the apoptosis in these cells (Figure 19).

We then determined whether the REST-miR-203 axis impacted cell proliferation or apoptosis. We found earlier REST knock down cells (shREST) cells showed decreased proliferation rates and higher apoptosis when compared to the control cells (shNT). We wanted to determine if knock down of miR-203 in REST knock down cells (double knockdown shRESTshmiR-203) has any impact on cell proliferation or apoptosis.miR-203 had no effect on shREST mediated cell proliferation and apoptosis in both the HR-GSCs *in vitro* as shown in figure 19 (A-D).

To determine the role of miR-203 in invasion in vitro, we took the miR-203 loss- and gain-of-function cell lines described in the preceding paragraph and determined the impact of these manipulations on cellular invasion using invasion chamber assays. The addition of exogenous miR-203 in either HR-GSC1 or HR-GSC2 cells decreased invasion when compared with the control vector, indicating that miR-203 negatively

regulates the invasion as shown in figure 19 .To determine whether the REST-miR-203 axis regulates cellular invasion, we subjected both the HR-GSC1 and HR-GSC2 cell types to shRest/shmiR-203 double knockdown. Results showed that the decreased cellular invasion caused by shRest could be reversed by the addition of shmiR-203 as shown in Fig 19. Thus, the REST-miR-203 axis controls cellular invasion in GSCs in vitro. (Fig 19 E &F)



E

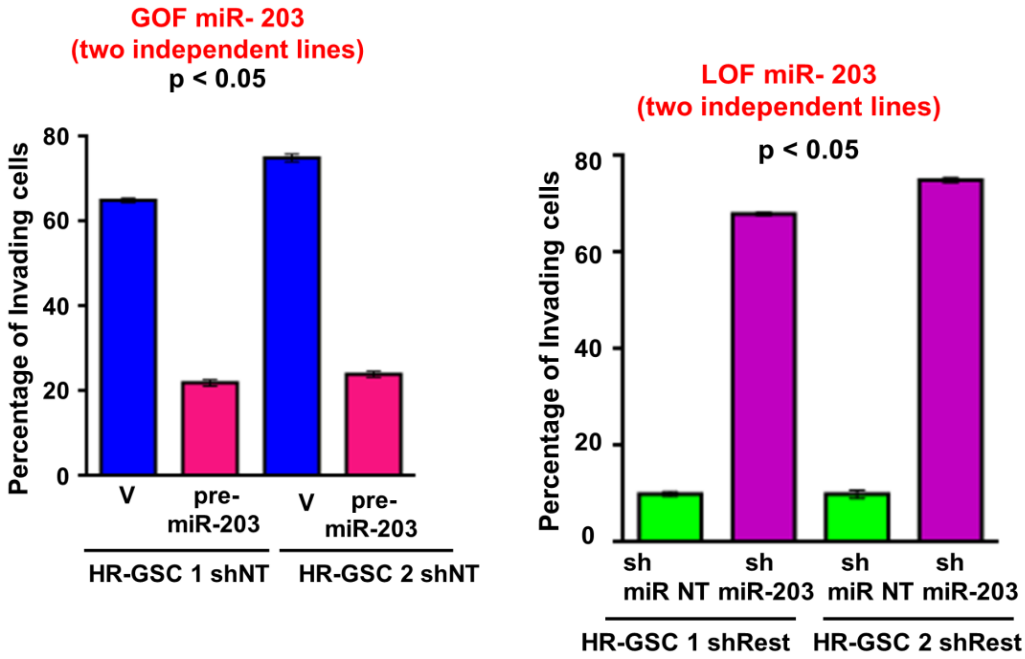


Figure 19: REST-miR-203 axis regulates invasion but not cell proliferation or apoptosis in GSCs. miR-203 gain-of-function cells HR-GSC1.shNT/ pre-miR-203 and HR-GSC2.shNT/ pre-miR-203 were subjected to in vitro BrdU-labeling assays to determine their cell proliferation properties (A) and TUNEL-labeling assays to determine their apoptotic status (B) compared with their corresponding HR-GSC1.shNT/V and HR-GSC2.shNT/V controls. As shown, none of the miR-203 manipulations made a significant difference either in their cell proliferation or apoptotic properties in either of the two HR-GSC lines. Previous studies indicated that the expression of shRest in both HR-GSC1 and HR-GSC2 cells results in decreased cell proliferation and increased apoptosis as compared with shNT-expressing cells (*17*). Additional loss-of-function manipulations with shmiR-203 in these cells (resulting in double knockdown shRest/shmiR-203) did not significantly alter either cell proliferation (C) or apoptosis (D). In contrast, when the same cells were subjected to invasion chamber assays, gain of function of miR-203 in both HR-GSC1 and HR-GSC2 cells decreased invasion when compared with the control vector (E). Similarly, when double knockdown of shRest/shmiR-203 was performed in HR-GSC1 and HR-GSC2 cells, the decreased cellular invasion caused by shRest was reversed by the addition of shmiR-203 (F).

MiR-203 regulates survival of mice harboring GSC-derived brain tumors

To determine whether miR-203 is relevant in the regulation of tumorigenesis, we determined whether its manipulation in GSCs affected the survival of mice bearing brain tumors derived from the altered GSCs. We performed miR gain-of-function experiments, in which we took the two HR-GSC control lines transduced with shNT that we characterized previously. Both lines express high REST and low miR-203. We transduced both these lines with lentiviruses containing either the vector (V) control or premiR-203, selected cells expressing the virus-encoded drug resistance, and confirmed the overexpression of miR-203 by taqman assays in the stable cell lines (HR-GSC1.shNT/V and HR-GSC1.shNT/ pre-miR-203; HR-GSC2.shNT/V and HR-GSC2.shNT/ pre-miR-203). We then transplanted these cells into the brains of nude mice using a screw-guided system we had utilized before and performed Kaplan Meier survival analyses. Overexpression of miR-203 in either of the independent HR-GSC lines increased the survival of tumor-bearing mice, indicating that miR-203 has a tumor-suppressor function in GSCs (Fig 20).

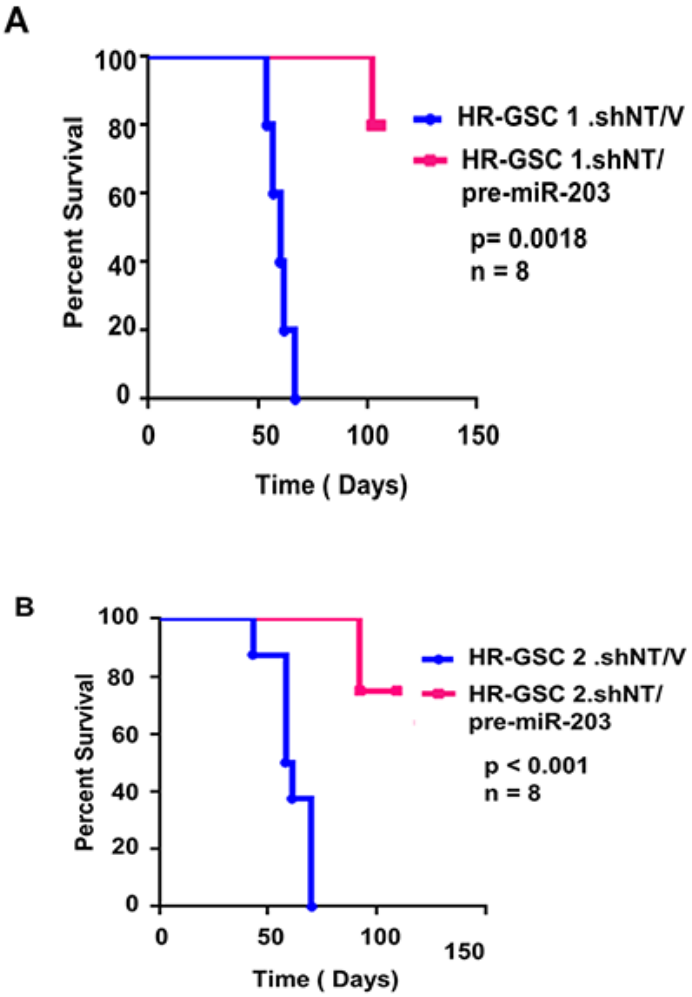
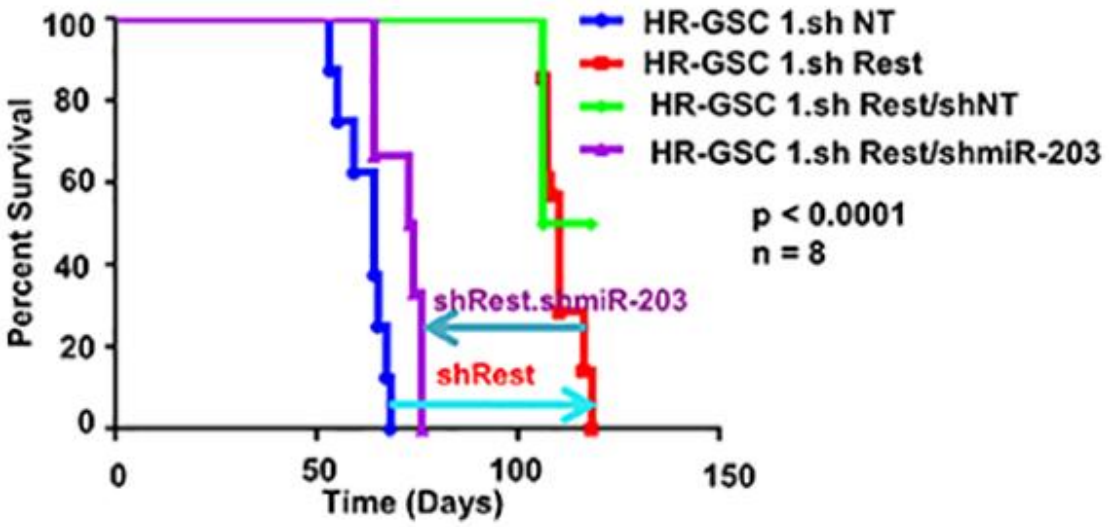


Figure 20: MiR-203 regulates survival of mice harboring brain tumors derived from both HR-GSC1 and HR-GSC2 cells. Kaplan-Meier survival plots of mice harboring (A) HR- HR-GSC1.shNT/V and HR-GSC1.shNT/pre-miR-203 and (B) HR-GSC2.shNT/V and HR-GSC2.shNT/ pre-miR-203 cells show that overexpression of miR-203 in either HR-GSC1 or HR-GSC2 cells increased the survival of tumor-bearing mice, indicating that miR-203 has a tumor-suppressor function in GSCs.

REST-miR-203 axis regulates survival of mice harboring GSC-derived brain tumors

We then determined whether the tumor suppressor function of miR-203 is mechanistically connected to REST. We took the two previously studied HR-GSC stable lines that were transduced with shRest: HR-GSC1/shRest and HR-GSC2/shRest (expressing low REST and high miR-203) .As described before, REST knockdown with shRest in these cells causes longer survival in tumor-bearing mice. To determine whether miR-203 can rescue these effects of shRest in GSCs, we performed shRest/shmiR-203 double knockdown in these cells. We transduced each of the GSC lines with lentiviruses containing shNT control, shRest, shRest/shNT, or shRest/shmiR-203; selected cells expressing the virus-encoded drug resistance; and confirmed the knockdown of miR-203 by taqman assays followed by qRT-PCR analyses. We then transplanted these cells into the brains of nude mice as described in the preceding paragraph and performed Kaplan Meier survival analyses. The mouse survival was longer in the shRest-expressing HR-GSC1 cells than in the shNT controls, as expected. Also, as expected, expression of additional shNT in the shRest-expressing cells (shRest/shNT: as a control for the expression of shmiRs) did not alter survival significantly. However, the double knockdown of shRest/shmiR-203 attenuated the increase in survival caused by shRest in these cells. The double knockdown of shRest/shmiR-203 caused decreased survival when compared to the control shRest/shNT cells. These results indicated that the REST-miR-203 axis regulates the tumorigenesis of GSCs.

A



B

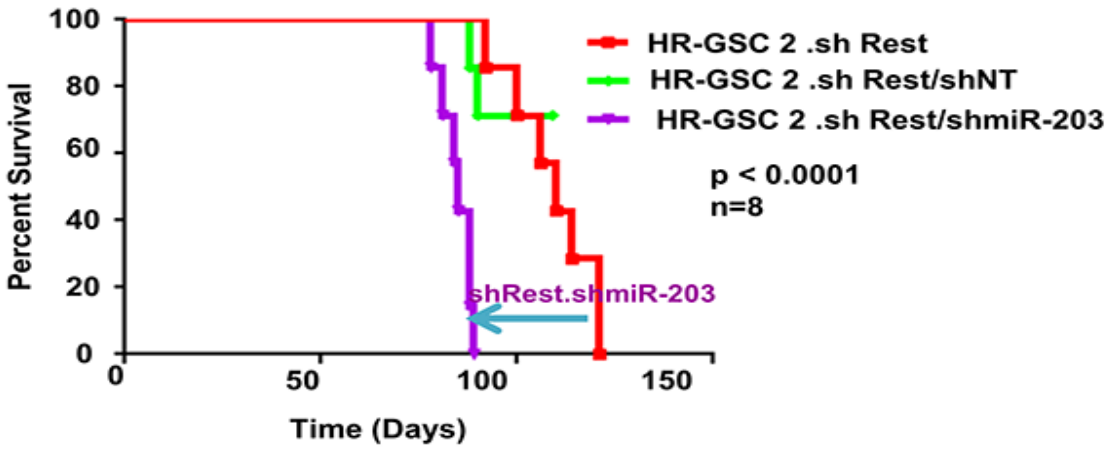
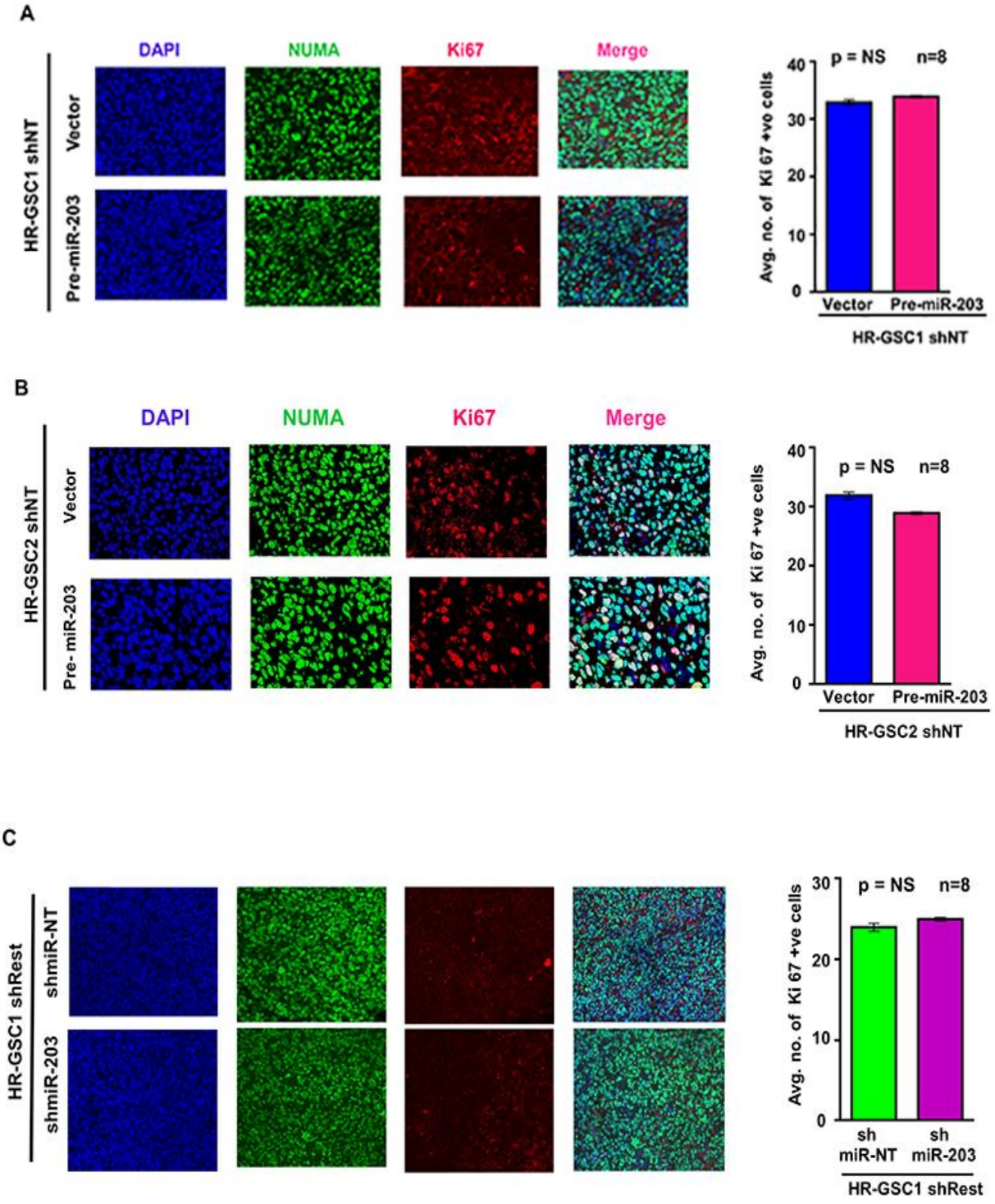


Figure 21: REST-miR-203 axis regulates survival of mice harboring tumors derived from both HR-GSC1 and HR-GSC2 cells. Kaplan-Meier survival plots of mice harboring (A) HR-GSC1.shNT, HR-GSC1.shRest, HR-GSC1.shRest/shNT and HR GSC1.shRest/shmiR-203 and (B) HR-GSC2.shRest, HR-GSC2.shRest/shNT and HR-GSC2.shRest/shmiR-203. Knockdown of REST by shRest in HR-GSC1 and HR-GSC2 cells caused increased survival when compared with their shNT controls, as expected[27]. Additional expression of shNT in these shRest-expressing cells did not significantly alter survival. In contrast, the double-knockdown shRest/shmiR-203 cells reversed the increased survival caused by single shRest in both.

REST-miR-203 axis regulates invasion, but not cell proliferation and apoptosis, in GSC-derived tumors in mouse brains

As described above, we found the REST-miR-203 axis regulates survival in mice harboring GSC tumors. To determine whether the properties of the REST-miR-203 axis seen in in vitro assays were also present in mouse brain tumors, we took the GSCs with miR-203 and REST manipulations described in the preceding sections, transplanted them into the brains of nude mice, waited for 40 days, euthanized all the mice, and examined their brain sections using immunofluorescence analysis. To differentiate the human GSC cells from the mouse brain cells, we stained the tumor sections with anti-NuMA antibody that selectively stains human cells. Double labeling of NuMA and the proliferation marker Ki67 showed that overexpression of miR-203 in the HR-GSC tumors or knockdown of miR-203 in the HR-GSC/shRest tumors did not significantly alter tumor cell proliferation. (Fig 22)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Merge
HR-GSC1 shNT
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30
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10
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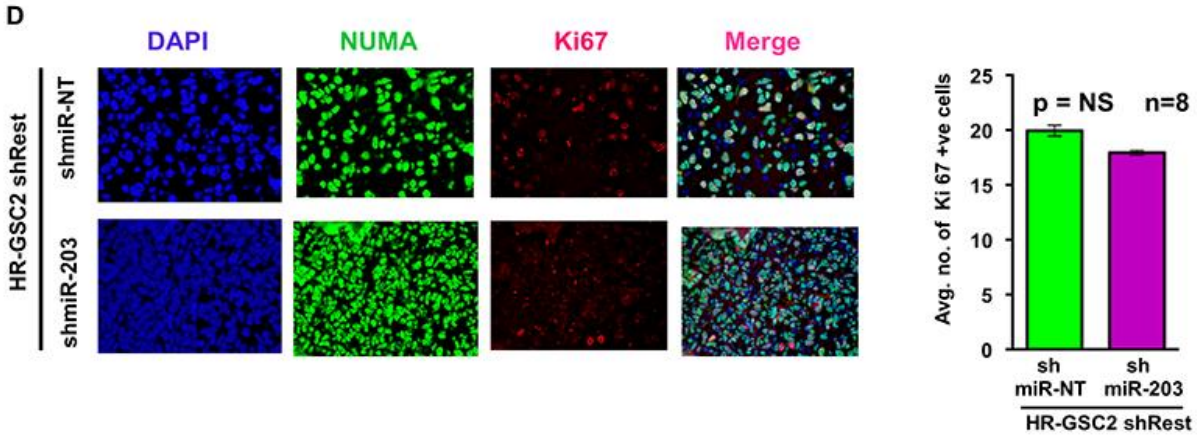
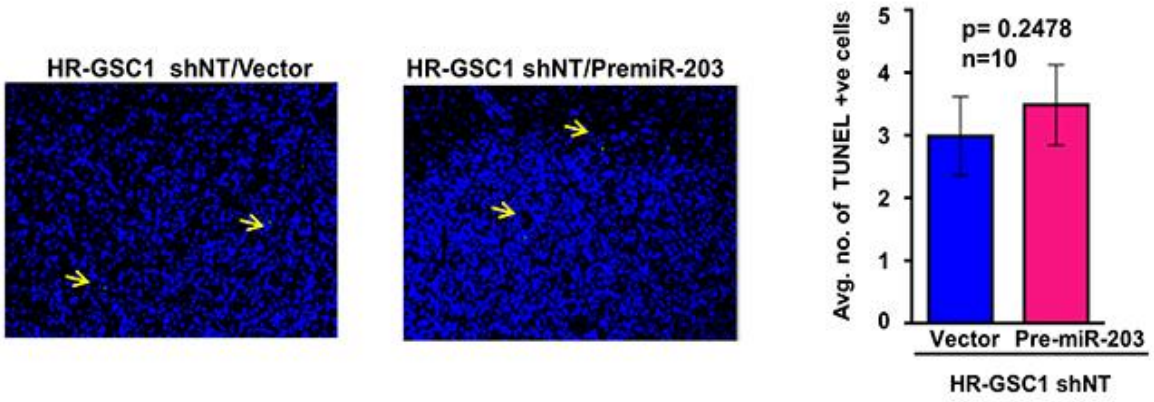


Figure 22: REST-miR-203 axis does not regulate proliferation in GSC –derived tumors in mouse brain. HR-GSC1 and HR-GSC2 cells with various REST and miR-203 manipulations described were transplanted into mouse brains. All mice were euthanized at day 40, and mouse brain sections were labeled with antibodies against NuMA (to detect human cells present among the mouse brain cells) and Ki67 (to detect cell proliferation).Brain sections were then examined using immunofluorescence analysis. Double labeling of brain sections with NuMA and Ki67 showed that overexpression of miR-203 in either of the HR-GSC tumors (A & B) or double knockdown of miR-203 in either the HR-GSC1/shRest or HR-GSC2/shRest tumors (C & D) did not significantly alter tumor cell proliferation.

TUNEL assays also showed that neither the overexpression of miR-203 in the two HR-GSC tumors nor the knockdown of miR-203 in the two HR-GSC/shRest tumors significantly affected apoptosis. (Fig 23)

A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HR-GSC1 shNT/PremiR-203
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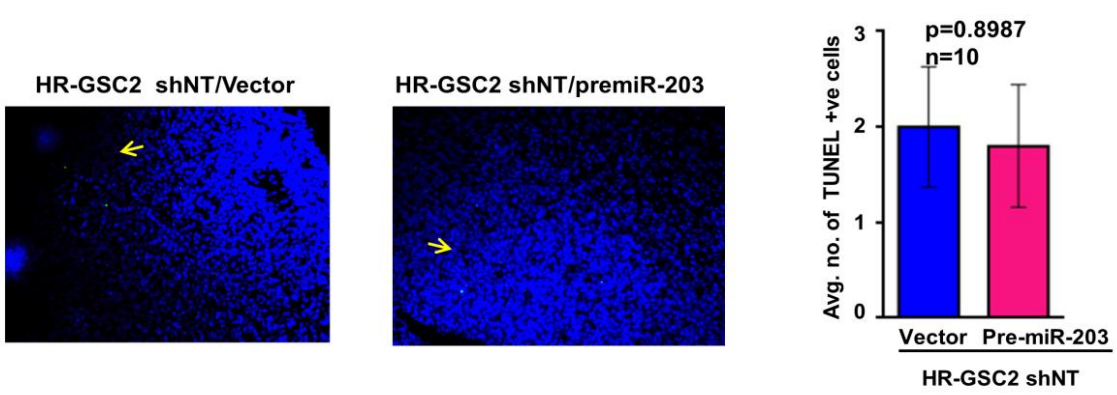
4
3
2
1
0
Vector
Pre-miR-203
HR-GSC1 shNT

B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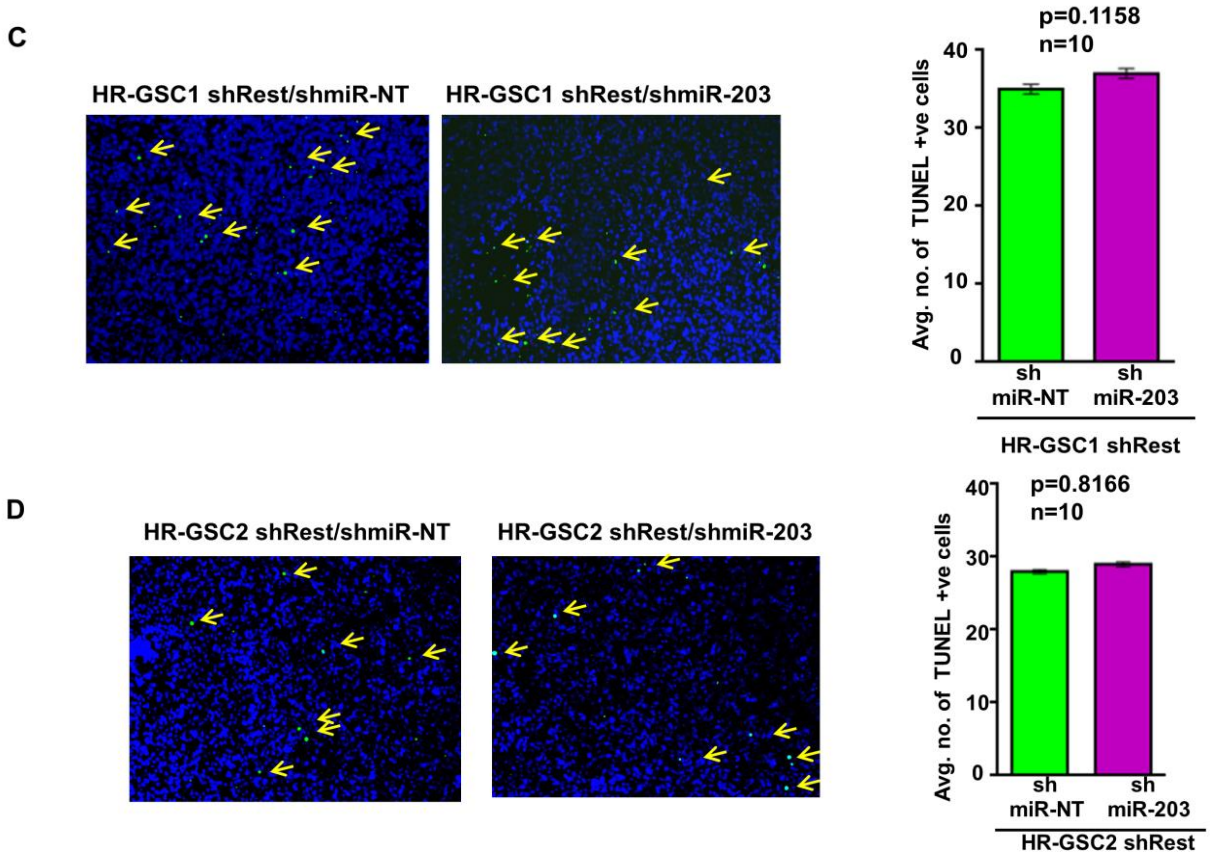
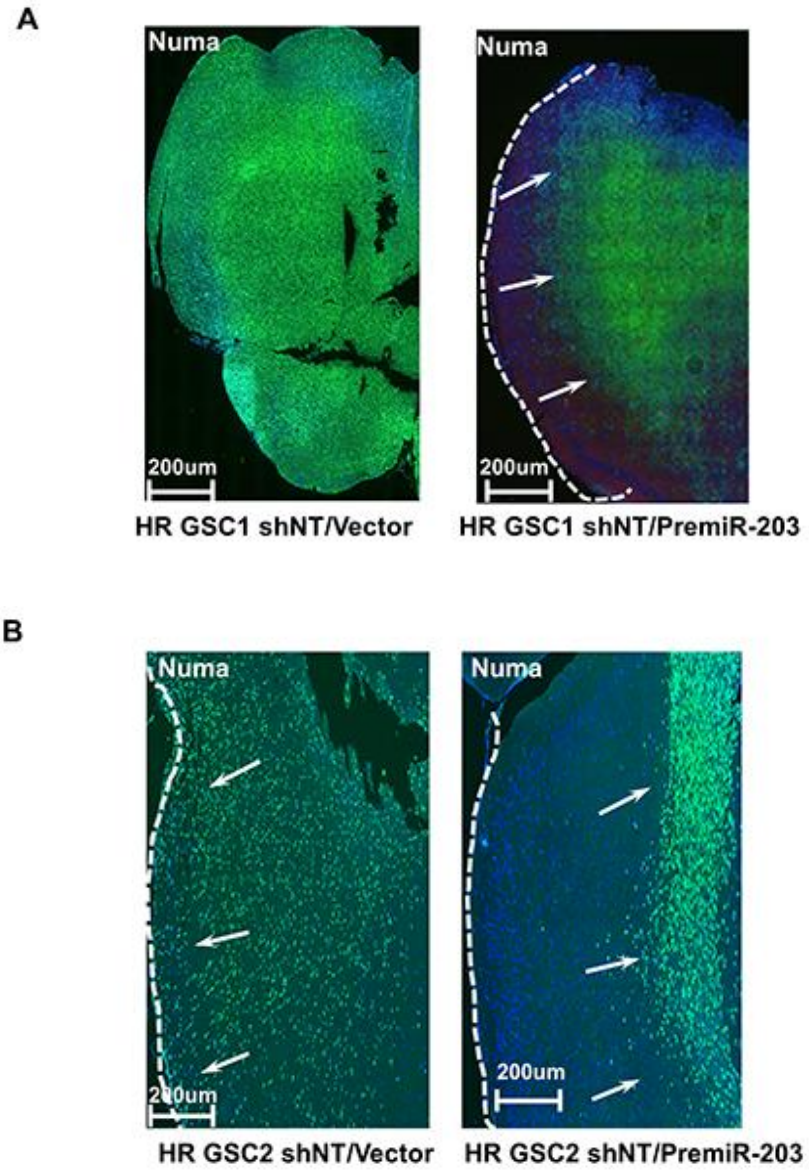
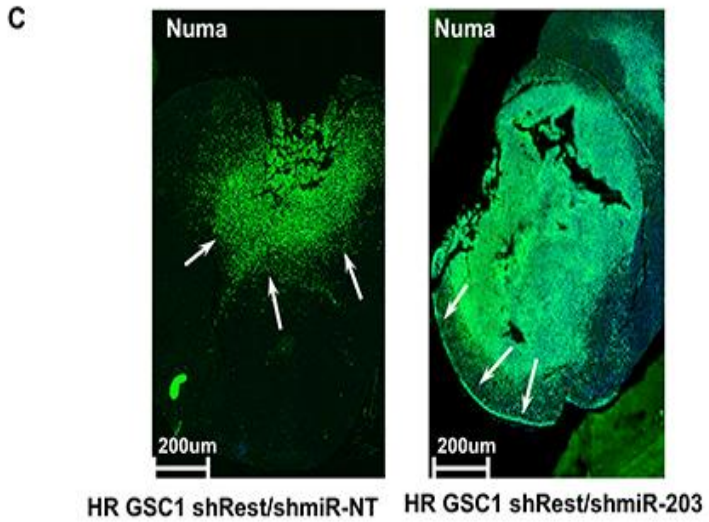


Figure 23: REST-miR-203 axis does not regulate apoptosis in GSC –derived tumors in mouse brains. TUNEL assay was performed on the mice brain sections. Either over expression of miR-203 in the two HR-GSC tumors (A & B) or knockdown of miR-203 in the HR-GSC/shREST tumors(C & D) did not show any significant alteration in apoptosis.

In contrast, HR-GSC1 and HR-GSC2 tumors, in which the cells were found to show a very high degree of migration causing cell invasion from the core of the tumor to the pial surface, were suppressed by overexpression of miR-203, which resulted in decreased cellular migration and distinct circumscribed tumors as shown in figure 24. The role of the REST-miR-203 axis in blocking tumor invasiveness was further illustrated when the decreased tumor invasion caused by knockdown of REST was reversed by the additional knockdown of miR-203, which resulted in a highly invasive phenotype in both HR-GSC1 and HR-GSC2 tumors as shown in figure. Thus, these

results indicated that the REST-miR-203 axis regulates invasion but not cell proliferation or apoptosis in GSC tumors. (Fig 24)





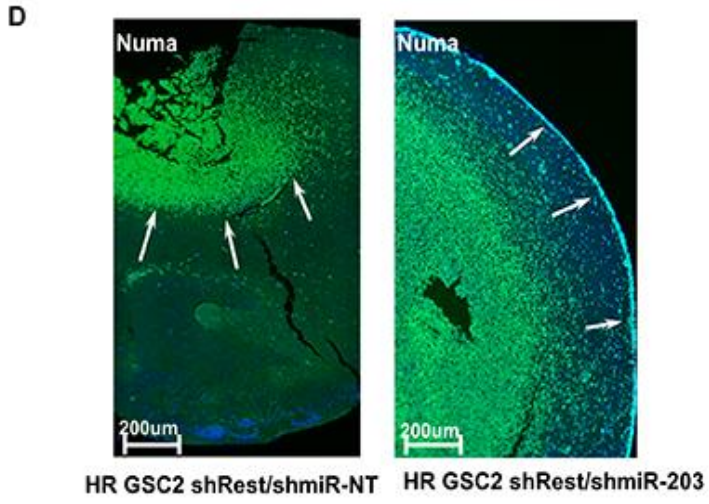


Figure 24: REST-miR-203 axis regulates the invasion in GSC-derived tumors in mouse brains. Overexpression of miR-203 in either of the HR-GSC tumors (A & B)) resulted in the blockade of invasion and the formation of circumscribed tumors. Similarly, the decreased invasion seen in either of the HR-GSC tumors expressing shRest was reversed in shRest/shmiR-203 double-knockdown tumors (C & D).

Chapter 7: Discussion

Glioblastoma is the most common malignant adult brain tumor with an average life expectancy of 9-12 months (*4, 22*). Despite decades of basic science and clinical research, there is no widely used prognostic criterion for GBM patients. The mechanisms that initiate different tumors still remains unknown, however several evidences point towards role of impaired stem cell development in tumor initiation. GBM tumors are believed to be caused by self-renewing, glioblastoma-derived stem-like cells (GSCs). These GSCs are resistant to chemo- and radiation therapies, and are believed to be responsible for tumor recurrence (*1, 27, 84, 85*). REST, a transcriptional repressor of neuronal differentiation and a known regulator of self-renewal in neural stem cells, has been recently identified in our laboratory and others to regulate tumorigenesis in GSCs. However, understanding the mechanism by which REST regulates oncogenic properties of GSCs is critical to developing therapeutic approaches. Here we report the potential mechanisms by which REST regulates oncogenic properties of GSCs.

Recent evidences suggest that microRNA can be therapeutic targets for various cancers(*63*). To understand the candidate miRNAs regulated by REST we altered REST levels in GSCs and performed microRNA microarray. Our miRNA profiling identified two tumor suppressor miRNAs miR-124 and miR-203. Studies have shown that both these microRNAs are expressed at lower levels in the glioma tumors when compared to the surrounding normal tissue. We also observed a negative correlation between the expression levels of REST and both these microRNAs in the cancer genome atlas (TCGA) datasets of GBM patients. Our results indicated that upon REST knock down both the microRNAs are up regulated and vice versa. REST binding to the

gene chromatin of miR-124/203 was confirmed by chromatin immunoprecipitation (ChIP) and luciferase assays. In the current study role of REST and miR-124 an/miR-203 axis was studied independently.

Here, we show that REST-miR-124 axis regulates the cell proliferation, apoptosis and invasion in HR-GSC tumors. Over expression of miR-124 led to increased survival in tumor bearing mice and the tumors formed are more circumscribed. We are the first ones to show that REST-miR-124 axis regulates the oncogenic properties in GSCs derived from the patient tumors. Studies have shown that miR-124 is the most abundant microRNA in adult and embryonic central nervous system (CNS)(*42, 70, 82*). Studies have also shown that down regulation of miR-124 is correlated with poor survival in GBM patients (*67, 86*). Previous studies have shown that overexpression of miR-124, induced differentiation in neural stem cells, mouse ES cells and mouse embryonal carcinoma cells. miR-124 also promoted cell cycle arrest in established GBM cells that are deprived of growth factors. Growth factor signaling (EGF, FGF, and PDGF) and epigenetic modification of the transcriptional regulatory sequences of the genes that encode miR-124 are the two possible mechanisms that it is suppressed in GBM tumors(*70, 82*). Previous studies indicated that the ability of miR-124 to induce differentiation depends on the cell type, developmental timing and other factors. Abrogation of the growth factor signaling along with the overexpression of miR-124 might enhance the cell cycle arrest and differentiation of cells. Studies have shown that miR-124 targets cell cycle regulators like CDK6 and its downstream targets like phosphorylated retinoblastoma (RB) (*87*) . Targeted delivery of miR-124 to the tumor cells may be therapeutically valuable for GBM disease treatment. Targeted in vivo delivery faces many challenges including the limited stability of miRNA, rapid blood

clearance, off target effects and poor cellular uptake. Stable and chemically modified nucleotides (locked nucleic acids, 2`-O-methylation) can be made for delivery which can be delivered at the site and also can be used to augment the specific binding of miRNA to the miRISC complex.(*88*). miRNAs can also be delivered as a precursor by a plasmid through viral vectors. As both mir-124 and miR-203 are suppressed in tumor tissue when compared to the surrounding brain tissue we need to overexpress both these microRNAs. Lower expression levels of the microRNAs should be supplemented with oligonucleotide mimics containing the same sequence as endogenous miRNA, known as microRNA mimics. These mimics should have an ability to enter the RISC complex and affect miRNA target mRNAs. Double stranded mimics composed of passenger strand (complementary sequence to mature miRNA) and guide strand (sequence identical to mature miRNA) should be used for delivery as they have a higher efficiency when compared to single stranded RNA. Synthetic conjugates such as cholesterol can be used for the delivery of mimics (*89*). Currently, there are several lipid –based delivery systems available for targeted delivery such as polycationic liposome-hyaluronic acid (LPH) nanoparticles. In addition to synthetic polymer materials, naturally occurring polymers such as chitosan, protamine and atellocollagen and peptides derived from protein translocation domains can be used for delivery. The application of natural compounds is limited by immunogenicity. Atellocollagen exhibits least immunogenic response when compared to other natural polymers. Studies have shown that miR-34a/atellocollagen complex when delivered intratumorally in lung cancers suppressed tumor growth (*90*) .

Our results indicated that REST-miR-203 axis regulates only invasion of GSCs both *in vitro* and *in vivo* but not cell proliferation and apoptosis. The tumors formed by the knockdown of miR-203 are highly invasive when compared to their controls and the mice die much faster. One of the principal reasons of our failure to provide better therapeutic approaches for GBM patients is due to the invasive nature of these tumors. Previously, it was found that REST regulates invasion of HR-GSCs (*17, 18*). However, the mechanism of this process was unclear. Here, we show that a novel REST-miR-203 axis regulates invasion in HR-GSC tumors, with REST suppressing miR-203 gene expression and miR-203 functioning as a tumor suppressor. Earlier ChIP-Seq assays using a mouse kidney cell line had indeed found miR-203 to be a potential target of REST in those cells (*91*). The finding that REST also represses miR-203 in HR-GSCs indicates that this regulatory axis likely operates in many different cell types. Recent observation that miR-203 expression was significantly lower in a large number of high-grade GBM tumor tissues than in low-grade glioma tissues or normal brain tissues (*92, 93*), would support miR-203's role as a tumor suppressor in GBM. In addition, miR-203 is also known to act as a tumor suppressor in other cancers (*94*) .

Interestingly, the publications on GBM cited above also indicated that miR-203 positively regulated invasion as well as cell proliferation in some glioma cell lines (*92, 93*) . However, our studies indicate that the REST-miR-203 axis specifically regulates invasion but not cell proliferation or apoptosis in HR-GSCs. It is unclear whether this difference in activity is due to the use of GSCs derived from primary GBM tumors in the

current study rather than the glioma lines used in the other studies, as cell culture conditions that can affect various signaling pathways (*41*), or a special property of the HR class of GSCs.

Recent studies have discovered a six-miR gene signature, which includes miR-203, in the serum of colorectal cancer patients that can be used as a biomarker and prognostic indicator (*95*). While two of the miR levels were elevated in colorectal cancer patient serums, levels of the other four, including miR-203, were lowered. Although lowered miR-203 levels were also seen in the HR-GSCs in our study, it is unknown whether such a miR signature exists in GBM. This needs to be addressed in future studies. Our study does suggest, however, that because GBM patients can now potentially be stratified based on the REST gene signature (28), the REST-miR-203 mechanism could potentially be manipulated in therapeutic approaches to block GBM invasion.

The downstream effector molecules of the REST-miR-124/miR-203 axis are still unknown. There are many known targets of miR-124/miR-203 but whether any of them are relevant in the context of HR-GSCs is unclear. To identify relevant targets, we performed a genome-wide mRNA expression analysis of HR-GSC1 and HR-GSC2 cells expressing either shNT or shREST (Stable lines). Using prediction programs we predicted the target genes of for microRNAs. We first filtered the genes whose expression was down regulated when REST was knocked down with a fold change of 2 or more and selected only those genes that contained potential miR-124 or miR-203 binding site(s) in their 3' untranslated region. Sorting for the potential targets that were common to both HR-GSC lines resulted in a short list of 25 and 18 genes for REST-

miR-124 and REST-miR-203 respectively. Ingenuity pathway analysis (IPA) of the potential target genes of the REST-miR-124 indicated that these genes are involved in metabolism of amino acids, lipids and nucleic acids. Similarly, upon analysis of the REST-miR-203 axis target genes, the top gene ontology was found to be involved in cellular movement. A network map of these genes obtained by performing an Ingenuity Pathway Analysis indicates a large network composed of two major hubs: one composed of genes involved in inflammation, angiogenesis, cell growth and apoptosis; the other composed of genes involved in ubiquitin and GTP binding proteins. A canonical pathway analysis of the 18 genes indicated that they are involved in various signaling, such as axonal signaling, ERK5 and p53 signaling, growth hormone signaling and melanocyte development. Efforts are under way in our laboratory to validate the actual targets of the REST-miR-124/203 axis using mouse orthotopic tumor models.

Conclusion/Summary: The REST-miR-124 axis regulates cell proliferation, apoptosis and invasion of the GSCs both invitro and in vivo. The REST-miR-203 axis specifically regulates the invasion potential of glioblastoma stem cells but not cell proliferation or apoptosis both in vitro and in vivo. Since invasion is a major hallmark of high REST GSC tumors and the REST-miR-203 axis could be potentially targeted to block the invasion.

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Vita

Anantha Marisetty was born in a town called Bapatla in Andhra Pradesh, India. She obtained bachelors in Biotechnology from Nagarjuna University in 2006. Upon completion of bachelors worked in JK Agri Genetics before she moved to United States of America for obtaining her Masters at University of Massachusetts, Lowell in spring 2007. She studied a novel Ca2+/ Calmodulin –dependent protein kinase cascade that regulates blood stem cell growth and development. Along with academic research she was also involved in industrial training at Acambis Inc. a subsidiary of Sanofi Pasteur where she was involved in generating a peptide library and validation of humoral response for a lead vaccine candidate. After completion of her masters in summer 2009 she joined the PhD program in cancer biology at the Graduate school of biosciences at UT MD Anderson cancer center. She joined the laboratory of Dr. Sadhan Majumder and currently studying the mechanism by which REST regulates tumorigenesis s in adult brain tumors.