Identification and characterization of miR-148a as a new oncogenic and prognostic microRNA in glioblastoma

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ABSTRACT

Great interest persists in useful prognostic and therapeutic targets in glioblastoma (GBM), the most common and most deadly human brain tumor. In this study, we report the identification and characterization of microRNA-148a (miR-148a) as a novel prognostic oncomiR in GBM. We show that miR-148a expression is elevated in human GBM specimens, cell lines, and stem cells (GSC) compared with normal human brain and astrocytes. High levels of miR-148a were a risk indicator for GBM patient survival. Functionally, miR-148a expression increased cell growth, survival, migration, and invasion in glioblastoma cells and GSCs and promoted GSC neurosphere formation. We identified two direct targets of miR-148a, the EGF receptor (EGFR) regulator MIG6 and the apoptosis regulator BIM. Rescue experiments showed that MIG6 and BIM were essential to mediate the oncogenic activity of miR-148a. By inhibiting MIG6 expression, miR-148a reduced EGFR trafficking to Rab7- expressing compartments, which includes late endosomes and lysosomes. This process coincided with reduced degradation and elevated expression and activation of EGFR. Finally, inhibition of miR-148a strongly suppressed GSC and glioblastoma xenograft growth in vivo. Taken together, our findings provide a comprehensive analysis of the prognostic value and oncogenic function of miR-148a in glioblastoma, further defining it as a potential target for glioblastoma therapy.

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Chapter 1: INTRODUCTION

I. Glioblastoma (GBM)

Glioblastoma (GBM) (WHO grade IV glioma) is a fast-growing and lethal malignant brain tumor. It is the most common brain tumor in adults. More than 22,000 Americans are diagnosed with and ~13,000 die from brain and other nervous system cancers each year. GBM accounts for about 15 percent of all brain tumors and occurs mostly in adults between the ages of 45 to 70 years. Patients with GBM have a poor prognosis and usually survive less than 15 months following diagnosis. Currently there are no effective long-term treatments for this disease.

i. Epidemiology

The annual incidence of malignant gliomas is approximately 5.05 cases per 100,000 people-years for children 0-19 years of age (4.92 per 100,000 person–years for children less than 15 years) and 25.86 per 100,000 person–years for adults (20+ years) [1]. Each year, more than 14,000 new cases are diagnosed in the United States [2]. Gliomas account for more than 81% of malignant GBM tumors, and of these, grade IV glioblastoma (GBM) is the most frequent and malignant histologic type, based on the Central Brain Tumor Registry of the United States (CBTRUS) (Diagram 1). Malignant gliomas are twice as common in whites as in blacks and 40% more common in men than in women [1]. CBTRUS indicates that the incidence of primary brain tumors has increased over a 5-year period from 2004 to 2008 [3, 4]. Some of this increase may be due to the advent of better imaging techniques, particularly magnetic resonance imaging (MRI), although other factors that have yet to be identified may be playing a role [5]. The median age of patients at the time of diagnosis is 64 years in the

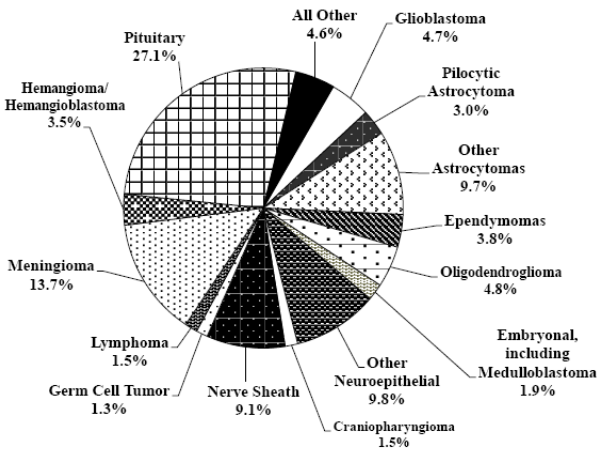


Diagram 1: Distribution of All Primary Brain and CNS Tumors by Histology (N=295,986) from [3, 4]

case of GBM and 45 years in the case of anaplastic gliomas (WHO grade III) [6]. Epidemiological studies have revealed increased brain tumor risks associated with certain occupations. The cause of the majority of malignant gliomas is unknown. Therapeutic X-irradiation constitutes the only environmental factor that is established as a risk factor for brain tumors [6]. Evidence for chemical exposure, diet, smoking and mobile phone use as risk factors are inconclusive to date. It is been reported that about 5% of patients with malignant gliomas have a family history of the disease. Some of these familial cases are associated with rare genetic syndromes such as Li-Fraumeni Syndrome, Neurofibromatosis Type 1, Neurofibromatosis Type 2, and Turcot Syndrome. Li-Fraumeni Syndrome is a cancer predisposition syndrome that can affect both children and adults and that is characterized by germ-line p53 mutations [7]. Neurofibromatosis Type 1 is an autosomal dominant disorder characterized by multiple neurofibromas, malignant peripheral nerve sheath tumors, optic nerve gliomas and other gliomas. The majority of gliomas in NF1 patients are pilocytic astrocytomas (WHO grade I) of the optic nerve [8].

ii. Pathology

According to The World Health Organization (WHO) classification, there are three main histopathological subtypes of gliomas based on the resemblance of tumor cells to normal brain cells: astrocytomas contain cells with morphological features of astrocytes, oligodendrogliomas have cells that resemble oligodendrocytes, and oligoastrocytomas have cells with features of both astrocytes and oligodendrocytes [9]. Furthermore, gliomas are classified on the basis of histologic features into four prognostic grades: pilocytic astrocytoma (Grade I), diffuse astrocytoma (Grade II), anaplastic astrocytoma (Grade III), and glioblastoma (Grade IV). Grades III -IV tumors are high-grade and typically contain both neoplastic and stromal tissues, which contribute to their histologic heterogeneity and variable outcome [10]. Grade I pilocytic astrocytoma is considered benign (a disease with limited

long-term consequences). This astrocytoma is rare in adults and, because it grows very slowly, it is often associated with long survival. Grade IV GBM account for more than 70% of all brain tumors in adults and are invariably fatal. These tumors grow fast, invade nearby healthy tissues, are mitotically very active and appear histologically abnormal. The 5-year survival rate of GBM patients is less than 3% in the USA [11]. These tumors partially resemble glial cells, but their cell of origin is controversial. GBM can be separated into two main categories on the basis of their diagnostic history: primary and secondary GBM [9, 12]. Primary GBMs are cancers of the CNS that appear *de novo*, without any preexisting sign of progression from lower grade gliomas. They typically occur in patients older than 50 years of age and are characterized by EGFR amplification and mutation, loss of heterozygosity of chromosome 10q, deletion of phosphatase and tensin homologue on chromosome 10 (PTEN), and p16 deletions. Secondary glioblastomas are tumors of the CNS that progress from lower grade gliomas and tend to occur in younger patients. These tumors are characterized by mutations in the p53 tumor suppressor gene, overexpression of the platelet derived growth factor receptor (PDGFR), abnormalities in the p16 and retinoblastoma (Rb) pathway, and loss of heterozygosity of chromosome 10q [9, 13, 14]. One remarkable alteration of secondary GBMs is Isocitrate Dehydrogenase (IDH) mutations [15]. The mutation of IDH1 in exon 4 results in reduced enzymatic activity [16]. Koichi et al. showed that IDH1 mutations were found in 54% of astrocytomas and 65% of oligodendroglial tumors, and secondary glioblastomas but not in primary GBM [16].

iii. **Molecular Basis**

Cancers including glioblastoma are a disease of genetic alterations such as DNA sequence changes, copy number aberrations, chromosomal rearrangements and modification in DNA methylation. These genetic alterations are thought to cause the development and progression of human malignancies. Therefore, the standard treatment of most cancers consisting of a

combination of surgery, radiation and chemotherapy, is not adequate to cure genetically altered cancer. It has been suggested that molecular targeting could transform cancer therapy. Targeting gene products and molecular signaling pathways that promote tumor formation and growth is therefore a promising new strategy for cancer therapy [17]. To better identify molecular targets in GBM advanced technologies such as whole genome sequencing, Sanger targeted, and exome sequencing have been used [18, 19]. Recently, the Cancer Genome Atlas (TCGA) used such technologies to analyze ~ 600 human GBM tumors. The findings classified genetic and molecular deregulations of GBM as belonging to three main pathways: Receptor tyrosine kinase (RTK) pathways, p53 pathways and Rb pathways (Diagram 2) [18].

iii.i. RTK pathways in GBM

The receptor tyrosine kinase (RTK) pathways are frequently deregulated in cancer and GBM. RTKs are a major type of cell-surface receptors and are high-affinity receptors for various polypeptide growth factors, cytokines, and hormones [20]. Binding of ligands to RTKs stimulates the receptor's intrinsic protein-tyrosine kinase activity, which subsequently stimulates a signal-transduction cascade leading to changes in cellular response and patterns of gene expression. Whole-exome and transcriptomal sequencing data performed by TCGA confirmed alterations in RTK signaling pathways affecting the receptors Epidermal growth factor receptor (EGFR), Platelet-derived growth factor receptor (PDGFR), MET and Fibroblast growth factor receptors (FGFR) 2/3 [19]. Among RTKs, the epidermal growth factor receptor (EGFR) is the most commonly altered [21]. It is mutated and/or amplified in 40% and overexpressed in > 60% of tumors regardless of amplification status [22, 23]. EGFR and its ligands, epidermal growth factor (EGF), transforming growth factor (TGF)-α, and heparin-binding EGF (HB-EGF), are key determinants in brain tumor malignancy [21, 24]. Activation of EGFR leads to the

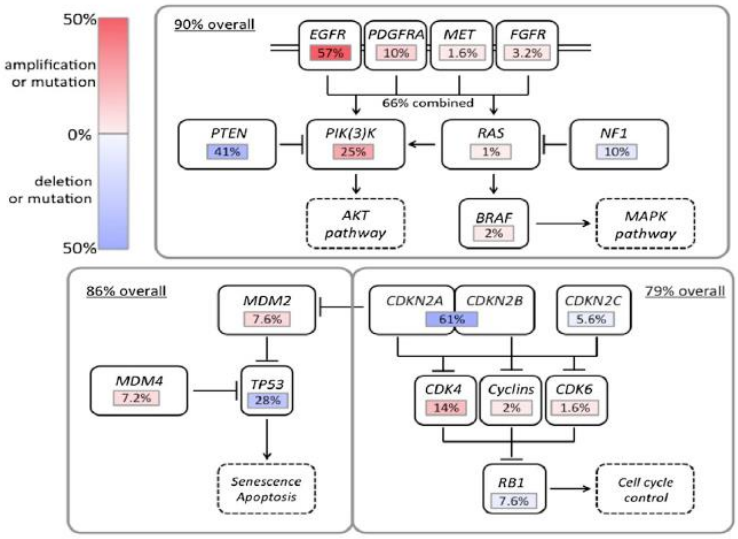


Diagram 2: Landscape of pathway Alterations in GBM from [19]

induction of tumor cell proliferation, migration, and invasion, as well as resistance to chemotherapy and radiation [25-27]. Besides the RTK receptors, alterations in components of RTK downstream signaling pathways are also frequent in GBM. For instance, PI3-kinase mutations are found in 25.1 % of GBM and frequent deletion and mutations of the PTEN tumor suppressor gene are found in 45% of GBM [19]. The neurofibromin 1 (NF1) gene is deleted or mutated in 10% of cases. Overall 90% of GBM samples harbor at least one genomic event in the RTK/PI3K pathway [18, 19].

iii.ii.p53 pathway in GBM

The p53 pathway is deregulated in 85.3% of GBM through mutation or deletion of TP53 (27.9%), amplification of MDM1/2/4 (15.1%) and/or deletion of cyclin-dependent kinase inhibitor 2A (CDKN2A) (57.8%) [19]. p53 is a tumor suppressor and transcription factor that plays critical roles in regulating the cell cycle, apoptosis and genomic integrity [28]. 27.9 % of GBM tumors show TP53 gene mutations or homozygous deletions leading to inactivation of wild-type p53. Inactivation of the p53 pathway also occurs in the form of CDKN2A deletion. Through the use of shared coding regions and alternative reading frames, the CDKN2A gene produces 2 major proteins: p16 (INK4), which is a cyclin-dependent kinase inhibitor and critical regulator in the RB signaling pathway, and p14 (ARF), which can bind the p53-stabilizing protein MDM2 to induce inactivation of p53 signaling [29]. Inactivated p53 signaling can also be due to amplification of the p53 E3 ubiquitin ligase MDM2/4 [18, 19].

iii.iii. RB pathways in GBM

Based on TCGA analysis, 79% of GBM samples harbor RB pathway aberrations. The retinoblastoma protein (RB) is a tumor suppressor protein that is mutated and dysfunctional in several major cancers [30]. One of the important functions of RB is

controlling cell growth through cell cycle inhibition. Interaction of RB with the E2F family of transcription factors represses gene transcription required for G1-S phase transition [31]. The most common events are the deletion of the CDKN2A/CDKN2B locus on chromosome 9p21and CDK4 locus amplification [19]. Homozygous deletion or mutation of CDKN2A/B occurs in 61%, homozygous deletion of CDKN2C in 5.6%, amplification of CDK4 in 14%, amplification of Cyclins in 2%, amplification of CDK6 in 1.6%, and homozygous deletion and/or mutation of RB1 itself in 11% of GBM [19]. These critical genetic alterations lead to decontrolled cell-cycle progression.

iv. Therapeutic tools and resistance

GBM is one of the most aggressive and treatment-resistant cancers. The standard therapy for newly diagnosed GBM consists of surgical resection, radiotherapy, and chemotherapy [10]. The currently most prevalent standard-of-care for newly diagnosed GBM was first described by Stupp, et al [32]. This regimen consists of at least six weeks of daily temozolomide together with five days per week of fractionated radiotherapy. Thereafter, temozolomide is administered five days out of 28 for six months. The combined treatment strategy results in a median survival increase compared to previous regimens from 12.1 to 14.6 months [32], leaving a great deal of room for improvement.

Factors responsible for GBM malignancy and poor prognosis include rapid cell proliferation, resistance against apoptosis, distant invasion of the surrounding brain, as well as the hypothesized existence of therapy resistant stem cells and tumor heterogeneity [10, 33-35]. The ability of glioblastoma cells to diffusely invade adjacent brain tissue and

spinal cord prevents surgical cure and is a major clinical obstacle to achieving disease control. Invading tumor cells appear to be especially resistant to cytotoxic therapy [9].

Another potential contributor to the therapeutic resistance of GBM is the existence within the tumor of a subpopulation of undifferentiated and therapy resistant stem-like cells (GSC). GSCs are characterized by high tumorigencity, self-renewal, pluripotency, neurosphere formation, and the expression of certain cell surface markers. Several molecules, including CD133 [36], CD15 [37], and A2B5 [38] have been identified as cell surface marker for GSCs. These GSCs could be highly relevant clinically due to the potential for intrinsic therapeutic resistance [39]. Comparative analysis of histological sections of patients, before or after radiation-therapy, shows the proportion of CD133+ cells are dramatically increased after intensive radiation therapy compared to the patient sections from patients having no radiation therapy [40]. Recently, it was also shown that GSCs can also give rise to vascular pericytes to support vessel function and tumor growth [41].

One of the biggest challenges for GBM therapy is intratumoral heterogeneity [35]. Remarkably, the intercellular genetic diversity and complexity of GBM includes DNA sequence changes, copy number aberration, chromosomal rearrangements and modification in DNA methylation and other molecular dysfunctions [42]. It has been recently shown that biopsies from the same tumor can exhibit completely different dysfunction profiles [35].

Resistance to cell death and apoptosis is another cause of therapy resistance in GBM. GBM as well as other cancers overcomes apoptosis initiated either at the cell surface (death receptor pathways) or intrinsically by intracellular signals such as extensive DNA

damage by inactivating proapoptotic pathways or activating genes that promote cell survival [43, 44].

In addition to the above, oncogenic signaling redundancy and compensatory mechanisms can play important roles in the development of therapy resistance. Signaling redundancy could be theoretically overcome simultaneous by targeting multiple growth signaling pathways. However, such combination therapies with targeted molecular drugs often lead to an exponential increase in toxicity, which may limit the doses that patients can tolerate [45]. For example, a previous study demonstrated the limitation of combining EGFR inhibitors and mTOR inhibitors due to high toxicity [45]. Thus discovery of new therapeutic strategies to target multiple genes at the same time is necessary. My thesis hypothesizes that microRNAs that target multiple important genes in glioblastoma could be potentially used for the above purpose.

v. Cancer stem cells

The cancer stem cell theory stipulates that there is a subset of undifferentiated stem-like cells that are highly tumorigenic and that are responsible for tumor initiation, recurrence and resistance to therapy. GSCs possess stem cell characteristics and are highly tumorigenic, pluripotent, and can self-renew. The self-renewal capacity is speculated to be responsible for tumor recurrence and the emergences of tumor resistance. In support of the stem cell theory, scientists have been able to isolate rare subpopulations of cells with flow cytometry using specific stem cell markers and show that small numbers of these cells are capable of forming tumors upon implantation in immunocompromised mice [46, 47]. Biomarkers for cancer stem cells tend to differ by the cancer, such as $CD34^{+}CD38^{+}$ for acute myeloid leukemia (AML) [46], $CD44^{+}/CD24^{-/low}$ for breast cancer [48] and $CD133^{+}$ for GBM [49] . However, the cancer stem cell theory is not universally accepted.

Opponents of the theory criticize the growth conditions used for stem cells, which usually consists of either media supplemented with growth factors or immunocompromised mice [50]. Additionally, the markers frequently used to isolate cancer stem cells are controversial. For example, the cancer stem cell marker CD133 is used to isolate and define GBM stem cells [49]. However, some researchers have found that both $CD133^{+}$ and $CD133^{-}$ populations have tumorigenic ability [49, 51, 52]. Moreover, other research shows that CD133+ cells are nonexistent in lower grade gliomas, thus arguing against the definition of these cells as tumor initiating cells [53]. Nontheless, in our hands, GSCs and GSC-derived xenografts display molecular and pathologic characteristics that better resemble human tumors. Therefore, in my thesis I also use GSCs and GSC-derived xenografts in addition to established GBM cell lines and xenografts.

II. microRNAs in Cancer

microRNAs are approximately 17-22 nucleotide long small noncoding regulatory RNA molecules that exert a profound impact on a wide array of biological and pathological processes [54]. miRNAs are powerful regulators of gene expression. They exert their effects by targeting the 3' untranslated regions (3'UTR) of mRNA leading to mRNA degradation or inhibition of translation. miRNAs were discovered in the early 1990s by Ambros and colleagues [55]. The human genome is estimated to encode approximately more like 1,500-2000 microRNAs that are predicted to regulate the expression of more than 60% of human genes.

i. microRNA biogenesis

Canonical microRNA biogenesis is shown in Diagram 3. Most miRNA genes are located in introns or in intergenic regions of protein-coding genes, or as polycistronic clusters

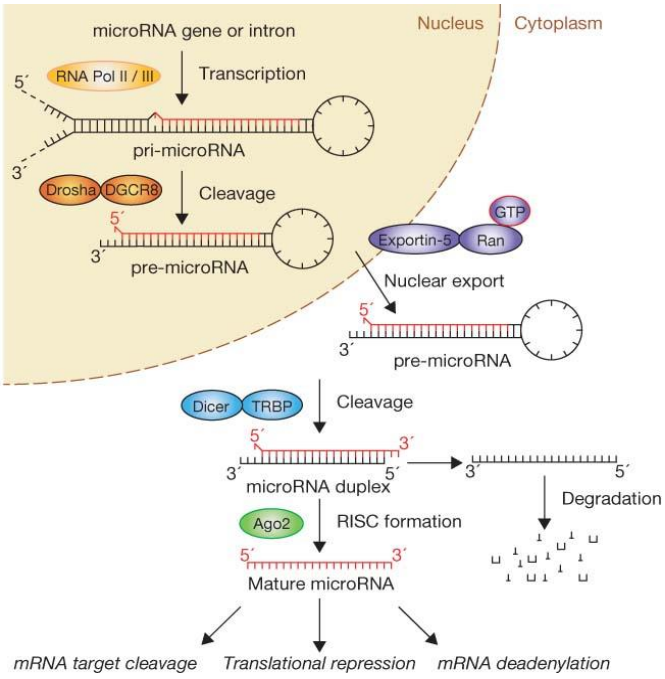


Diagram 3: The 'linear' canonical pathway of microRNA processing [56]

encoding several miRNAs in a single transcript. microRNAs are transcribed by RNA polymerase II to yield long precursors of more than 1000 bases. The transcription outcomes are referred to as primary miRNAs (pri-miRNAs). The pri-miRNAs are then processed by the Drosha/DiGeorge complex, which has double stranded RNA ribonuclease function. This process yields precursor microRNAs (pre-miRNA) [57] that consist of 70-100 nucleotide double-strand hairpin nucleic acids. The pre-miRNA are then actively exported to the cytoplasm by exportin-5 and then processed into mature double-stranded miRNA molecules by a second RNase III-like enzyme, Dicer [58, 59]. The mature miRNA is then incorporated into the RNA-induced silencing complex (RISC) where it is unwounded into a single-stranded mature form to bind to the 3'-untranslated regions (3'UTR) of target mRNA. The strand that is bound by RISC is guided to the target mRNA by sequence complementarity. Thereby, the "seed sequence" consisting of 6-8 nucleotides at the 5' end of the miRNA is essential for mRNA recognition and binding. microRNAs regulate mRNA levels by destabilizing the mRNA or by indirectly interfering with the translation machinery to reduce protein levels [60]. The fate of the mRNA appears to be determined by the degree of seed sequence complementarity. Typically, miRNA:mRNA interactions lead to suppressed translation of the target mRNA [61, 62] but when complementarity is very high, it can lead to mRNA degradation [63].

ii. microRNA mechanism of action

microRNAs regulate gene expression via two different pathways: canonical and unorthodox. The canonical pathway is more prevalent. In this pathway, microRNAs interact with targeted mRNAs primarily via their seed sequences, which are 6- to 8-nt-long fragments at the 5' end of the miRNA. Perfect seed complementarity increases the

stability of interaction between miRNA and mRNA targets but is not always necessary for posttranscriptional regulation. It has been shown that the seed pairing substantially enables and promotes target prediction reliability. Besides seed matching, microRNA:mRNA interactions can be further stabilized by complementary nucleotides outside the seed regions [64]. Seed matching is not always sufficient for repression of targeted mRNA, suggesting the involvement of other mostly unknown criteria. The unorthodox pathway functions independently of seed matching. One representative example is the interaction of human miR-24 with cell cycle genes. miR-24 targets cell-cycle genes through sites that are spread over almost the whole miRNA. These interactions lack obvious seed pairing and contain multiple mismatches, bulges, and wobbles [65]. Another example of the unorthodox pathway is the *let-7*: *lin-41* interaction in *C. elegans* [66], which displays base pairing at both 5' and 3' ends of the *let-7* binding site.

iii. microRNA deregulation in cancer and glioblastoma

Because microRNAs regulate the majority of human gene expression, they also regulate all possible biological processes. Consequently, their deregulation could lead to a wide array of pathological deregulations. Many miRNAs are deregulated in cancers, and aberrant expression of miRNAs can arise through a number of different mechanisms, such as genomic abnormalities, epigenetic factors, transcriptional regulation, and regulation of microRNA processing [60]. Genetic abnormalities consist of deletion, amplification, or translocation of miRNAs. These genetic alterations result in miRNA overexpression that could cause downregulation of tumor suppressor genes or miRNA underexpression that leads to oncogene up-regulation. Importantly, more than 50% of all

annotated human miRNA genes are located at fragile sites or areas of the genome that are associated with cancer and which are prone to breakage and rearrangement in cancer cells [67]. miR-15a and miR-16-1 in chronic lymphocytic leukemia (CLL) patients are representative examples of genetic alterations of microRNAs. These two microRNAs are underexpressed in CLL where they function as tumor suppressive microRNAs. They are subject to chromosomal deletions or mutations at the 13p13.4 locus in CLL patients [68]. More than 68% of CLL patients have genes that are deleted and downregulated at the 13p13.4 locus. Other research demonstrated that amplification in GBM of chromosome 12q, where miR-26a-2 is located, causes overexpression of miR-26a. miR-26a-2 directly targets the PTEN tumor suppressor in GBM [69].

A notable example for epigenetic regulation is hypermethylation of CpG islands in promoter regions. Such hypermethylation has been described for some microRNA genes. Some miRNAs in breast cancer are upregulated upon the exposure of cells to the demethylating agent 5-aza-2′-deoxycytidine [70], upon mutation of DNA methyltransferases (DNMTs) in colorectal cancer [71], and upon histone deacetylase inhibitor treatment [72]. Recent research also showed reduced expression of miR-137 in glial tumors and GBM stem cells (GSCs) as well as a tumor suppressive role in self-renewal and differentiation of stem cells by targeting RTVP-1. Coincidentally, miR-137 CpG island hypermethylation was observed in GBM tumors [73].

Many miRNAs are also deregulated via deregulation of transcription factors that regulate their expression. Examples include AR-miR-125b in prostate cancer [74], MYCN-miR-17 in neuroblastoma [75], STAT3-miR-12 in myeloma [76], and the Myc-miR-17-92 cluster in glioblastoma [77-80].

microRNA deregulation can also occur at the processing level. The expression levels of Dicer or Drosha are altered in a number of cancers including lung adenocarcinoma and cervical squamous cell carcinoma [81, 82]. Upregulation of Drosha is found in more than 60% of cervical squamous cell carcinoma (SCC) specimens and it is due to copy number gain at chromosome 5p, where the Drosha gene is located. Notably, some miRNAs are reduced upon Drosha overexpression, indicating that individual miRNAs respond differentially to deregulation of the miRNA processing machinery. In GBM some of microRNAs are upregulated and function as oncomiRs, such as miR-10b, miR-21 targeting PTEN and miR-221/222 targeting p27. Other microRNAs are downregulated, such as miR-34a, which functions as a tumor suppressor targeting c-MET, Notch and CDK6 in GBM.

III. Epidermal Growth Factor Receptor (EGFR) and Epidermal Growth Factor Receptor Family Receptor Feedback Inhibitor 1 (ERRFI1) in GBM

Numerous histopathological, genetic and large-scale sequencing studies identified the epidermal growth factor receptor (EGFR) and its downstream signaling networks as commonly deregulated components in GBM [18, 83]. EGFR is the prototypical member of the ErbB/EGFR family, which consist of four members in mammals (EGFR/ ErbB2/ ErbB3/ ErbB4) [83]. Depending on the particular ligand among 13 different ligands and the receptor to which it binds, the ErbB/EGFR family activates various signaling transduction cascades to induce cell division, migration, adhesion, differentiation, and apoptosis [84]. EGFR turnover is critical for the regulation of EGFR expression and activity. MIG6, which I discovered as a direct target of miR-148a, is a negative regulator of EGFR expression and activation [85].

i. EGFR in glioblastoma and cancer

EGFR is the most commonly deregulated and most studied receptor tyrosine kinase in GBM. Specifically, focal amplification of the EGFR gene occurs in 42% of GBM. The oncogenic role of EGFR in GBM has been functionally validated in both cell culture-based systems and animal models [83]. The cancer genome atlas identified EGFR as the fourth most highly mutated gene in GBM [18]. EGFR mutation leads to receptor activation. Also, autocrine loop formation can lead to the activation of EGFR in GBM [86]. EGFR amplification occurs in about 40% of primary GBMs but is not observed in lower-grade astrocytomas [87]. EGFR protein overexpression is not always accompanied by EGFR gene amplification, indicating that a fraction of GBM tumors show increased receptor abundance in the absence of gene amplification [18]. EGFR is also frequently mutated in GBM [88]. The most famous and best-studied EGFR mutation is the vIII mutation. EGFR vIII deletion shows constitutively activated EGFR through loss of exons 2 to 7 of the EGFR gene [25]. Approximately 60% of GBMs overexpress EGFR but mutations and amplification occur in only 40% of these cases. In this thesis, I uncover a new mechanism of EGFR, overexpression that involves miR-148a overexpression in GBM.

ii. EGFR regulation

After ligand binding, EGFR undergoes conformational alterations leading to receptor dimerization and stabilization. This conformational change allows the activation of the intrinsic kinase domain and self-phosphorylation that lead to docking of adaptor proteins and activation of intracellular signaling cascades involved in growth control and development. The activated EGFR is immediately targeted by inhibitory mechanisms that do not require *de novo* protein synthesis. There are two representative inhibitory

mechanisms: dephosphorylation of kinase domain by protein tyrosine phosphatases (PTPs) and endocytosis of the active form of EGFR. PTPs are a major switch of many signaling pathways as they regulate the activity of protein tyrosine kinases (PTKs) [89]. Several PTPs have been identified as negative regulators of EGFR, including RPTP-sigma and PTPN12 tyrosine phosphatase, the latter of which acts as a tumor suppressor in triple-negative breast cancer [90, 91]. Acting as docking sites for adaptor proteins and enzymes that dock with EGFR can induce either stimulatory effects, such as the mitogen-activated protein kinase (MAPK), or inhibitory effects, such as the Cbl ubiquitin ligase [92, 93]. Cbl is an E3 ubiquitin ligase with an unconventional Src-homology-2 (SH2) and a RING finger domain and is required primarily for receptor sorting after internalization from the plasma membrane [94]. The SH2 domain allows direct binding of Cbl to Tyr phosphorylated EGFR, whereas the RING domain participates in the transfer of ubiquitin from an E2 ubiquitin ligand to the receptor cytoplasmic domain [95]. This leads to the degradation of ligand-receptor complexes in the lysosome. EGFR is also regulated by inducible feedback inhibitors (IFIs), which bind directly to the receptor and inhibit its activation. IFIs include leucine-rich and immunoglobulin-like domain protein 1 (LRIG1) [96], suppressor of cytokine signaling 4 and 5 (SOCS4 and SOCS5) [97], and receptor-associated late transducer (RALT, also known as MIG6; mitogen-inducible gene 6) [98-100]. In my thesis research, I show for the first time that miR-148a regulates EGFR trafficking and activation by targeting MIG6.

iii. MIG6

MIG6 (MIG6; Mitogen-inducible gene 6, also known as ERRFI1; ERBB receptor feedback inhibitor 1, RALT; Receptor-associated Late Transducer, and gene-33) is a cytosolic protein [101]. MIG6 was originally identified as a mitogen-inducible gene and has been implicated in the feedback regulation of a variety of signaling processes,

including those of EGFR [98, 102]. Deletion of the mouse gene encoding MIG6 leads to hyperactivation and sustained signaling of EGFR through the mitogen-activated protein kinase (MAPK) pathway and gives rise to overproliferation and impaired differentiation of epidermal keratinocytes [103]. Another study found that MIG-6 gene disruption in mice leads to the development of epithelial hyperplasia, adenoma, and adenocarcinoma in the lung, gallbladder, and bile duct. These studies suggest that MIG-6 is a tumor-suppressor and is therefore a candidate gene for the frequent 1p36 genetic alterations found in cancer [104]. MIG6 is composed of a receptor endocytosis domain (RED), and an ErbB-binding region (EBR). The centrally located EBR allows specific binding to members of the ErbB receptor family. MIG6 inhibits downstream EGFR signaling, including activation of ERKs and AKT, as well as biological responses regulated by the EGFR, such as cell proliferation and cell locomotion, as determined by both gain- and loss-of function studies in a wide range of cultured cells [99, 105-107].

iv. Expression of MIG6 in GBM

MIG6 expression downregulation and recurrent genetic deletions of its locus on chromosome 1p36 are common events in multiple tumor types [85]. 1p36 deletions are found in 13.2% of GBM, but downregulation of MIG6 expression is observed in 50% of GBM [85]. Therefore, MIG6 deletions cannot account for its frequent downregulation. In my thesis, I propose miR-148a upregulation as a new mechanism of MIG6 downregulation in GBM.

v. Mechanisms of MIG6 action

MIG6 is recruited to the EGFR kinase domain through its EBR domain when EGF binds to the EGFR extracellular domain. MIG6 contains a RED (endocytic domain) and an EBR (EGFR binding region). Ectopic overexpression of the EBR domain of MIG6 is

sufficient for inhibition of EGFR autophosphorylation and downstream signaling [99, 108]. EBR binds to an extended surface of the EGFR catalytic domain and causes a conformational change that prevents downstream signal generation [101, 109]. The RED domain of MIG6 also participates in EGFR inhibition. It was shown that MIG6 binds to components of the endocytic machinery through RED. For example, it binds to AP-2, the major adaptor complex responsible for sorting cargo into clathrin-coated pits [110], and intersectins, which are SH3-domain-containing accessory proteins involved in cargo sorting and clathrin-coated pit maturation [111]. These molecular interactions allow coupling of EGFR-MIG6 complexes to clathrin-mediated endocytosis [112]. Lastly, beside promoting clathrin-coated endocytosis of the EGFR, MIG6 was also shown to mediate EGFR trafficking and sorting to late endosomes via Syntaxin8, which is a component of the endosomal SNARE complexes that are involved in cargo trafficking from early to late endosome [113, 114]. The above three mechanisms act sequentially and synchronously to induce EGFR downregulation.

IV. Apoptosis and BIM in Glioblastoma

Resistance to apoptosis and cell death is one of the hallmarks of cancer and GBM. It also contributes to resistance of GBM to therapy and leads to escape of tumor cells from surveillance by the immune system [115]. During apoptosis, caspases are activated to cleavage cellular substrates and lead to biochemical and morphological changes that are characteristics of apoptosis [116, 117]. There are two major pathways by which caspase activation occurs: *via* the extrinsic pathways and the intrinsic pathway.

i. Extrinsic pathway

The extrinsic apoptosis pathway (also referred to as alternative pathways or the death receptor apoptosis pathway) is initiated by extracellular death ligands which activate a family of cell surface death receptors [118, 119]. One cytokine ligand, TNF, is generated by activated macrophages and is a critical mediator of apoptosis in the extrinsic pathway. Binding of TNF ligand to the TNF-receptor in the cell membrane initiates interactions with the intermediate membrane proteins, TNF receptor-associated death domain (TRADD) and Fas-associated death domain protein (FADD). These intermediate proteins recruit the inactive forms of certain members of the caspase protease family to initiate caspase activation and induce apoptosis [120]. A transmembrane protein member of the TNF family, Fas ligand (FasL), binds to the Fas receptor (also known as Apo-1 and CD95) to generate the death-inducing signaling complex (DISC) that is composed of FADD, caspase-8 and caspase-10 [121]. Inactive procaspase-8 and procaspase-10 are then cleaved by DISC and become active initiator caspases [121, 122]. Activated Caspase-8 and 10 initiate cleavage and activation of the effector protein, caspases-3, 6, and 7. Activated caspase-8 can also crosstalk with the intrinsic pathway by cleaving BID at the mitochondrial membrane to facilitate the release of cytochrome c [123]

ii. Intrinsic pathway

The intrinsic apoptosis pathway (also referred to as the mitochondrial pathway) involves non-receptor–mediated intracellular signals that induce activities in the mitochondria that initiate apoptosis. The BCL-2 family proteins are critical mediators of apoptosis through the intrinsic pathway. Stimuli such as growth factor deprivation, oxidants, Ca^{2+} overload, oncogene activation, DNA-damaging agents, and microtubule-attacking drugs can initiate the intrinsic pathway of apoptosis [124]. The BCL-2 family is divided into two different subgroups: the anti-apoptotic proteins

(BCL2, BCL-X, MCL1, A1/BFL1, BOO/DIVA, and NR-13), and the pro-apoptotic proteins (BAX, BAK, BOK/MTD, BCL-X, BID, BIK, BLK, NIP3, NIX, NOXA, PUMA, BMF, and BIM). The pro-apoptotic subgroup can be further subdivided into two subgroups: the multi-domain pro-apoptotic proteins (BAX, BAK, and BOK) and the Bcl-2 homology domain 3 (BH3)-only pro-apoptotic proteins (BID, BAD, and BIM) [125, 126]. BIM is a Bcl-2 like pro-apoptotic protein that is essential in mediating targeted therapy-induced apoptosis and that connects growth factor signaling pathways such as EGFR to the mitochondria in many cancers [127, 128]. In normal conditions, BIM is complexed with interacting dynein light chain LC8 that sequesters BIM to block its proapoptotic function. After certain apoptotic stimuli, the complex interaction between LC8 and BIM is broken to release BIM to induce apoptosis [129]. The released BIM directly binds to Bcl-2, which is an anti-apoptotic Bcl-2-BCL-X_L member. Anti-apoptotic BCL2 inhibits the conformational oligomerization of Bax and Bak to activate Bax [130]. The activation of Bax initiates cytochrome c release from mitochondria [131]. A critical step in this process is mitochondrial outer membrane permeabiliation (MOMP), resulting in the release of apoptosis mediator proteins that are normally found in the space between the inner and outer mitochondrial membranes [118]. In the cytoplasm, cytochrome c binds to Apaf-1, a normally monomeric apoptosis adaptor protein, and results in the conformational change of Apaf-1 and subsequently to Apaf-1 oligomerizaton. This complex is called apoptosome and activates caspases [124]. Once activated, caspase-9 cleaves the executioner caspases-3,-6 and -7 to induce apoptosis in the stimulated cell.

iii. BIM in cancer and GBM

The role of BIM in regulating apoptosis has been shown in many different cell types such as lymphocytes, osteoclasts, osteoblasts, mast cells, epithelial cells, endothelial cells, and neurons [132, 133]. BIM is one of the BH3-only proteins that is strictly regulated through both transcriptional and post-transcriptional mechanisms [134]. Upregulated BIM expression triggers cytochrome c release from mitochondria, which consequently induces a chain reaction that entails the formation of the apoptosome and the activation of its effector, caspase-9 leading to induction of apoptosis. Alternative splicing generates at least three BIM isoforms, BIM_S, BIM_L, and BIM_{EL}; all three isoforms are potent inducers of apoptosis with different apoptotic potencies and can only be expressed stably in cultured cell lines if Bcl-2 or a functional homolog is coexpressed at high levels [135]. The BIM isoforms differ from each other in cytotoxicity, with BIM_S being the most potent. This is partly explained by the sequestration of BIM_L, and BIM_{EL} in the cytoskeleton-associated motor complex bound to dynein light chain, LC8, from which they are released upon various apoptotic stimuli [129]. BIM_S protein has been only recently detected in 293 human embryonic kidney cells [136], while BIM_L, and BIM_{EL} have been found in a variety of tissues and cell types [137]. BIM_S is the most effective killer and is encoded by exon 2, exon 5 (which includes the BH3 domain), and exon 6 (which includes the hydrophobic tail, required for insertion into the outer mitochondrial membrane). BIM_L further includes exon 4, which encodes a binding site for dynein light chain 1 (DLC1). BIM_{EL} is composed of 196 amino acids and includes exons 2, 4, 5 and 6, but additionally includes exon 3 containing an ERK1/2 docking domain (FSF) and ERK1/2 phosphorylation sites, such as Ser 69 [138, 139]. ERK1/2-dependent phosphorylation of BIM_{EL} targets it for proteasomal degradation and may prevent binding to Bax. Apoptotic stimuli, such as chemotherapy or cytokine withdrawal cause the upregulation of BIM mRNA through activation of FOXO3a which is a

forkhead-like transcription factor [140]. The chemotherapeutic drug, paclitaxel increases the BIM_{EL} expression level via increasing FOXO3a expression without affecting the levels of other Bcl-2 family members [140]. A critical role of BIM is also shown in gastric cancer. Approximately one half of human gastric cancer cells do not express the tumor suppressor, RUNX3 [141]. RUNX3, a suppressor of gastric cancer also increases BIM transcription following treatment with transforming growth factor β (TGF-β) [142]. TGF-β induced apoptosis is also regulated by BIM post-transcriptional pathways. TGF-β transcriptionally induces the mitogen-activated protein kinase (MAPK) phosphatase (MKP) 2 through SMAD3. MKP2 abrogates ERK activity and ERK-induced BIM ubiquitination and proteasomal degradation, further enhancing apoptosis [143, 144]. Conversely, the breakdown of BIM homeostasis can also result in tumor cell survival, metastasis, and chemo-resistance [145-147]. For example, baby mouse kidney epithelial (BMK) cells transformed by E1A and dominant negative p53 (p53DD) form tumors in nude mice. Importantly, BIM deficiency in these cells, brought about by lack of p53-mediated BIM expression, supports the formation and growth of the tumors in nude mice suggesting that BIM is a key regulator of epithelial tumors [148].

Cells usually undergo apoptosis after loss of attachment with their extracellular matrix or adjoining cells. Detachment-induced apoptosis is referred as anoikis which is important barrier against metastases. Recent research shows that BIM plays a role in anoikis stimuli-activating mitochondrial pathways in various tumors, such as breast cancer, lung cancer, osteosarcoma, fibrosarcoma and melanoma [149, 150]. The most remarkable evidence for a relationship between cell detachment signal and BIM is found in breast cancer [147]. It was shown that BIM is as a key regulator of anoikis that downstream of the EGFR-ERK pathway. Suppression of EGFR caused

by cell detachment results in inhibition of ERK signaling and upregulation of BIM expression. However, EGFR overexpression can maintain ERK activation after cell detachment and block BIM expression and anoikis [147]. BIM also plays a role in mediating the effects of EGFR inhibitors. Gefitinib is an EGFR-targeting tyrosine kinase inhibitor (TKI), which has been successfully used in treatment of NSCLCs [151]. Gefitinib-treated NSCLC cells show accumulation of BIM through blockade of the MEK/ERK pathways [151]. BIM RNA levels in EGFR-mutant lung cancer specimens predicted response and duration of clinical benefit of EGFR inhibitors. Therefore, BIM RNA levels may be used to determine which patients will benefit from kinase inhibition [152]. BIM is also downregulated in 29% of GBMs based on TCGA data analysis [127, 128]. However, the mechanism of BIM downregualtion in GBM is not known. In this thesis, I show that BIM downregulation in GBM is at least partially due to microRNA-148a upregulation.

V. Rationale and hypothesis

Despite the most advanced therapy consisting of combinations of surgery, radiation and chemotherapy, GBM remains one of the deadliest human cancers [153]. These tumors are highly invasive, proliferative and resistant to apoptosis [154]. It has been shown that malignancy and resistance to therapy are in large part driven by the concomitant activation of several oncogenic pathways [155]. We hypothesized that because microRNAs can simultaneously regulate multiple molecules and pathways, studying them would provide new knowledge on the concomitant deregulation of gene expression, and targeting them could lead to more efficient GBM therapies. To identify important microRNAs in GBM, we reasoned that such microRNAs would correlate with patient survival. We therefore analyzed TCGA data for microRNAs that correlate with patient

survival and found micoRNA-148a as one of few miRNAs that negatively correlated with patient survival. We also noticed through target prediction analysis that this miRNA is predicted to target several tumor suppressors that are known to play a role in GBM malignancy. We therefore hypothesized that miR-148a is an oncogenic and prognostic miRNA that acts by regulating tumor suppressors in GBM and initiated a comprehensive analysis of miR-148a's role in GBM. Diagram 4 shows the schematic of the hypothesis. To test the hypothesis, we conducted mechanistic, functional, translational, and expression studies on miR-148a in GBM cells, stem cells, xenografts and patient tumor specimens. We show that miR-148a is a prognostic oncomiR that acts by inhibiting MIG6 and BIM and activating EGFR in GBM.

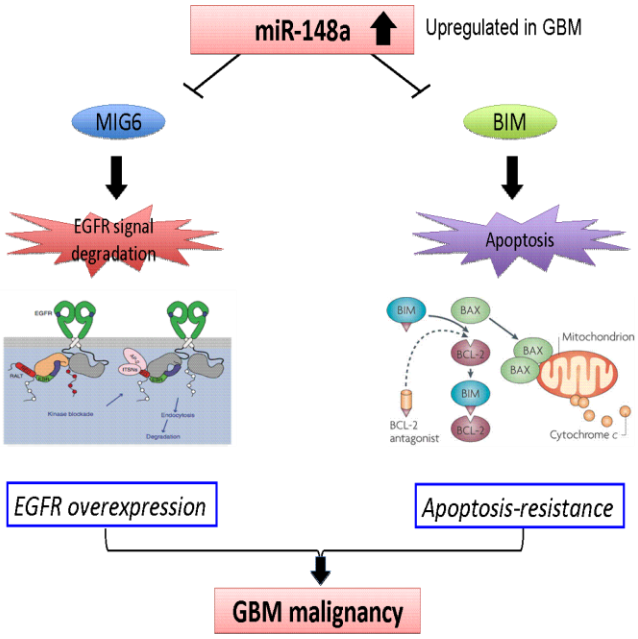


Diagram 4: Schematic diagram of Thesis Hypothesis. Schematic drawing of miR-148a effect on MIG6, BIM to induce GBM malignancy

Chapter 2: MATERIALS AND METHODS

I. Cell culture and tumor specimens

GBM cell lines U87, U373, A172, T98G, SNB-19 and U251 were from ATCC. GBM stem cells (GSCs) 1228, 0802 and 0308 (a kind gift from Dr. Jeongwu Lee, Cleveland Clinic) were cultured in MEM supplemented with 0.15% sodium bicarbonate, 1 mmol/L sodium pyruvate, and 0.1 mol/L nonessential amino acids. U373 cells were grown in DMEM with 1 g/L glucose supplemented with HEPES buffer. A172 cells were grown in DMEM with 4.5 g/L glucose. T98G cells were grown in MEM with 1 mmol/L sodium pyruvate, and 0.1 mol/L nonessential amino acids. SNB-19 cells were grown in DMEM F12. GSCs were grown in Neurobasal Media with N2 and B27 supplements (0.5 X each) and human recombinant bFGF and EGF (50ng/ml each). All cells were grown in 10% fetal bovine serum (except GSCs) at 37°C in 5% CO_2-95% O_2. GBM surgical specimens (n=18) and normal brain (n=7) were obtained from patients undergoing surgery at the University of Virginia Hospital according to procedures that were reviewed and approved by the Review Board of the University of Virginia.

II. TCGA data analysis

The collection of the data from The Cancer Genome Atlas (TCGA) was compliant with all applicable laws, regulations and policies for the protection of human subjects, and necessary ethical approvals were obtained {Cancer Genome Atlas Research Network : 2008 gr}. Analysis of all data was done in the R project [156]. For analysis of differential expression and determination of the effects of miR-148a on patient survival, Agilent 8x15k microRNA expression for 491 glioblastoma and 10 normal unmatched brain samples was downloaded along with clinical information from the TCGA database

(Level 2 (normalized) data, November 2012). Cox regression analysis of all samples with miRNA and survival data (n=482) was performed to determine whether miR-148a levels were a risk indicator for survival. The expression of miR-148a was also compared in normal brain (n=10) to GBM (n=491) using the R-based Limma package [157].

III. Quantitative RT-PCR

miScript Primer Assay Hs-miR-148a was used for measuring miR-148a levels. Total RNA was extracted from GBM cell lines and GSCs using the miScript RNA extraction kit (QIAGEN, Valencia, CA), according to the manufacturer's instructions. MicroRNA primer assay kits were purchased from QIAGEN (Valencia, CA), and samples were prepared according to the recommended protocol. Each RNA sample was reverse-transcribed using the miScript Reverse Transcriptase kit (QIAGEN, Valencia, CA), and quantitative real-time PCR analysis was performed using the 7500 Real-time PCR System (Applied Biosystems, Carlsbad, CA). The qRT-PCR was also used to assess the mRNA levels of MIG6 and BIM. The primer sequences used were: MIG6-forward: 5′-GACAATTTGAGCAACTTGACTTGG-3′, MIG6-reverse: 5′-GGTTACTTAGTTGTTGCAGGTAAG-3; BIM-forward: 5′-TGGCAAAGCAACCTTCTGATG-3′ and BIM-reverse: 5′-GCAGGCTGCAATTGTCTACCT-3′. Human U6B and GAPDH primers (QIAGEN, Valencia, CA) were used as endogenous controls.

IV. Cell transfections

GBM cells and GSCs were transfected with 20 nM pre-miR-148a, anti-miRNA-148a or control-miR (Ambion, Carlsbad, CA), using Oligofectamine or Lipofectamine RNAimax (Invitrogen, Grand Island, NY) according to the manufacturer's instructions. Plasmid transfections were performed with Fugene 6 (Roche, Indianapolis, IN).

V. Generation of anti-miR-148a stable expressing GBM cells

U87 cells were infected with the pEZX-AM04 expression plasmid containing a hygromycin resistance gene as well as the antisense sequence for miR-148a and the mCherry gene under the U6 promoter (pEZX-AM04; GeneCopoeia) using the Lentivector-based anti-miRNA technology. An empty vector was used as the control. Anti-miR-148a expression and control constructs were packaged with pPACKH1 Lentivector packaging Plasmid mix (System Biosciences, Mountain View, CA) in 293-TN packaging cell line. Viruses were concentrated using PEG-*it* Virus Precitipatation Solution (System Biosciences.) After 48-hour of culturing, red fluorescent protein (mCherry) was detected by fluorescence microscopy. An infection efficiency of ~100% was verified by fluorescent microscopy.

VI. Cell growth and apoptosis assays

To determine the effects of miR-148a on cell growth, GBM cells and GSCs were transfected with pre-miR-148a, anti-miR-148a, or control. Three days post-transfection, the cells were collected every day for five days and counted with a hemocytometer. To determine the effects of miR-148a on cell death, cells were transfected as described

above and Annexin V-PE and 7AAD flow cytometry was used to determine the apoptotic dead cell fractions, respectively as previously described [158].

VII. Cell migration and invasion assays

The effects of miR-148a expression on cell migration and invasion were assessed using the wound healing and trans-well assays as previously described [159]. Briefly, the cells were transfected with pre-miR-148a, anti-miR-148a or control. For wound healing, seeded cells were removed from the middle of the plates by scratching and allowed to migrate back into the scratch. For invasion, the cells were placed in the upper chamber of wells separated by a collagen IV-coated membrane. After incubation for 8-18hrs, the cells on the upper membrane surface were mechanically removed. Cells that had invaded to the lower side of the membrane were fixed and stained with 0.1% crystal violet. Invaded cells were counted under a microscope in five randomly chosen fields and photographs were taken.

VIII. Neurosphere formation assay

GSCs were grown in low EGF and FGF medium (20 ng/ml each) and transfected with either anti- or pre-miR-148a or scrambled controls for 72 h. The cells were dissociated into single cells with dissociation buffer (EDTA 1mM, BSA 0.5% in PBS), and 1000 single cells were transferred to 24 well plates and incubated for 7 days. The number of secondary neurospheres containing more than 30 cells was counted.

IX. *In vivo* tumor formation

Tumor xenografts were generated by implantation of the 1228 GSC cell line transfected with anti-miR-148a or U87 cells engineered to stably express anti-miR-148a encoding plasmids. 1228 (1×10^5 cells; n=6) and U87 cells (3×10^5 cells; n=10) were stereotactically implanted into the striata of immunodeficient mice. Four weeks after tumor implantation, the animals were subjected to a cerebral magnetic resonance imaging (MRI). To measure tumor size, 30 μl of gadopentetate dimeglumine (Magnevist, Bayer Healthcare, NJ) was intraperitoneally injected 15 minutes prior to scanning, and tumor volume was quantified as previously described [160].

X. Immunoblotting

Immunoblotting was performed as previously described using antibodies specific for MIG6 (Santa Cruz Biotechnologies, Santa Cruz, CA), BIM, EGFR (Cell Signaling, Danvers, MA) and p-EGFR Tyr 845 (Santa Cruz Biotechnologies, Santa Cruz, CA) [161]. All blots were stripped and re-probed with β-actin or GAPDH (Santa Cruz, Dallas, Texas) antibodies as loading controls. Blots in which differences were not clearly visible were quantified by densitometry on film as previously described [162].

XI. Generation of MIG6 and BIM 3'UTR constructs

The MIG6 3′-UTR reporter plasmid was constructed via insertion of the MIG6 3'-UTR (2561 bp) downstream of the Renilla luciferase stop codon in the pMIR vector (Promega, Madison, WI) generating the pMIR-MIG63'UTR plasmid. MIG6 primer pairs used were MIG6-forward: 5'-GTACCTGCTAGCACCTTGGGGTCATGGAGCAC-3 and MIG6-

reverse: 5'-GTCCCAGCTAGCATCCTTTGTCCAATACTGTACAC-3'. For BIM a commercially available 3'-UTR reporter plasmid, pEZX-BIM3UTR-1, was used (Gene copoeia, Madison, WI). QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used to generate mutations in the 3′ UTR of MIG6 and BIM by PCR using the pMIR-MIG6 3'UTR and pEZX-BIM 3'UTR constructs as templates. The following primers containing the mutation TGCACTGA (1370-1377) →CCGGGCCG in the 3′ UTR of MIG6 gene and TGCACTG (1029-1035) →GCGCGCC 3'UTR of BIM were designed and used for the site-directed mutagenesis: MIG6-forward primer 5′-CATGCTTAAGAAAAACCGGGCCGTTTCTGCATTATGTG -3′, and reverse primer 5′-CACATAATGCAGAAACGGCCCGGTTTTTCTTAAGCATG -3′ and BIM-forward primer 5′-CCCATGGTCACAGAGCGCGCCTCAGCATCAGGTCCCAGAGG -3′, and reverse primer 5′-CCTCTGGGACCTGATGCTGAGGCGCGCTCCTGTGACCATGGG -3′ (the mutation sites are underlined).

XII. 3'UTR reporter assays

Luciferase reporter vectors were used to validate the specificity of the predicted miRNA-target mRNA interactions. GBM cells were transfected with pre-miR-148a or pre-miR control for 6 hrs. For MIG6, the cells were then transfected with either the reporter vector with 3′UTR-MIG6 or with mutant MIG6 3'UTR, in addition to a control CMV promoter-driven β-galactosidase reporter plasmid for 48hrs using FUGENE 6. For BIM, the cells were transfected with either 3'UTR BIM or BIM mutant 3'UTR for 48 hrs using FUGENE 6. Luciferase assays were performed using the Luciferase System Kit (Promega, Madison, WI) for MIG6 or the Dual Luciferase Assay (Promega, Madison, WI) for BIM, and luminescence was measured on a Promega GloMax 20/20 luminometer.

Firefly luciferase activity was double normalized by dividing each well first by β-galactosidase activity and then by an average luciferase/β-galactosidase value in a parallel set done with a constitutive luciferase plasmid.

XIII. Rescue experiments

To determine if MIG6 and BIM mediate the effects of miR-148a on cell growth and apoptosis, rescue experiments were conducted in which the effects of anti-miR-148a were measured in the setting of inhibited MIG6 or BIM. Cells were either transfected with anti-miR-148a for 6hrs (1228), or anti-miR-148a stable expressing U87 cells were used. The cells were then transfected with siRNA against MIG6 (Thermo Fisher Scientific, Waltham, MA) or BIM (Cell Signaling, Danvers, MA) and incubated for a further 72 hours. The effects of miR-148a on cell growth and death were then measured as described above. MIG6, EGFR and BIM expression changes were verified by immunoblotting.

XIV. EGFR tracking assays

Cells were plated and transfected with either pre-miR-148a or pre-miR control for 24hrs followed by transfection with Rab7-mCherry for 24 hrs (kindly provided by Marc G. Coppolino, University of Guelph). Cells were serum starved overnight, followed by stimulation with 50 ng/mL EGF for 30 minutes. Samples were then washed, fixed with 4% (w/v) paraformaldehyde (w/v)/PBS, permeabilized with 0.2% (v/v) Triton X-100 in PBS and blocked with 5% (w/v) milk in PBS before staining with primary (EGFR, Abcam, Cambridge, MA; Mig6, Santa Cruz, Dallas, Texas) and secondary antibodies. Samples

were imaged using a 63X (NA 1.4) lens on a Zeiss LSM 700 with 405, 488, 543, 633 nm lasers using ZEN software (Carl Zeiss, Oberkochen, Germany). Captured images were analyzed for colocalization using ImageJ software. Briefly, images were initially thresholded, and the Colocalization Finder tool was used to determine the area and intensity of colocalizing pixels of EGFR.

XV. Statistics

All experiments were performed at least 3 times. When appropriate, two group comparisons were analyzed with a t-test, and p values were calculated. For rescue experiments, the anti-miR-148a-induced change in the setting of inhibited target protein was compared with the anti-miR-148a-induced change in the control setting. For TCGA data, Cox regression analysis was performed to determine the correlation between miR-148a expression and patient survival. More detailed TCGA data statistical analyses are described in the corresponding sections. For all analyses, $P < 0.05$ was considered significant and symbolized by an asterisk in the graphs.

Chapter 3: microRNA 148a is a prognostic oncomiR in Glioblastoma and Cancer

I. Introduction

GBM is the most common primary brain tumor and one of the most lethal human cancers (Diagram 1). Genetic diversity and multi-pathway deregulation contributes to GBM treatment resistance [163]. Thus the inhibition of single oncogenic molecules with specific inhibitors seems insufficient to achieve better therapies. Combination therapy with targeted molecular drugs, such as cocktails of inhibitors, are additive in their toxicity, which may limit the doses that patients can tolerate [45]. We hypothesize that more effective cancer treatment could be achieved by using small noncoding RNA that can be targeted to influence multiple molecules simultaneously and to promote tumor suppressor gene expression in cancer. Herein we investigate miR-148a as one such non-coding RNA.

Factors responsible for GBM malignancy and poor prognosis include rapid cell growth, resistance against apoptosis, and distant invasion of the surrounding brain [10, 33]. We therefore studied the effects of miR-148a on cell proliferation, apoptosis and invasion using several complementary approaches. Also, small subpopulations of cancer cells, glioma stem cells (GSC), show a greater potential for tumor initiation, pluripotency, self-renewal and resistance to therapy [36, 164, 165]. We therefore included GSCs in all studies and additionally studied the effects of miR-148a on GSC self renewal and tumorigenesis using a neurosphere formation assay and in vivo xenografts.

microRNAs regulate gene expression by binding to the 3' untranslated region (3'UTR) of target mRNA and inducing mRNA degradation and/or inhibition of protein synthesis [60, 166]. Deregulation of miRNA expression has been associated with cancer formation through

alterations in either oncogenic or tumor suppressor gene targets [60, 167]. A number of miRNAs are deregulated in GBM and play important roles in tumor formation and growth [168-176]. In this study, we therefore investigated the involvement of several predicted miR-148a targets in mediating the effects of miR-148a. We experimentally verified two targets: MIG6 and BIM with immunoblotting and 3'UTR assays. We used rescue experiments to show that they mediate the effects of miR-148a. Because, MIG6 regulates EGFR activation and expression, we also studied the effects of miR-148a on these parameters using various approaches including EGFR trafficking with confocal microscopy.

The starting point of our research was a preliminary observation of a correlation between miR-148a expression and clinical outcome of GBM patients (Figure 1). We screened all known microRNAs for correlation with GBM patient survival using the CaBIG Cancer Genome Atlas (TCGA) data analysis website. The Cancer Genome Atlas is a comprehensive and coordinated effort to improve our understanding of the molecular basis of cancer through application of genome analysis technologies such as large-scale genome sequencing. We found only few microRNAs that correlated with patient survival. Among these, microRNA-148a exhibited one of the best inverse correlations with GBM patient survival (Figure 1). A thorough literature analysis revealed no publications on miR-148a in GBM. Previous published research had described miR-148a as a tumor suppressor in hepatocellular carcinoma, pancreatic cancer, gastric cancer and colorectal cancer [179-182]. However, a role for miR-148a in GBM and its characterization as oncogene have not been described before. We therefore embarked on a comprehensive study of the role of miR-148a in GBM Our study demonstrated for the first time that miR-148a is an oncogenic and prognostic microRNA in GBM.

Diagram 4 shows a schematic diagram of the thesis hypothesis. The hypothesis stipulates that upregulated microRNA-148a inhibits the EGFR negative regulator MIG6, and the proapoptotic molecule BIM. The inhibition of these two important tumor suppressors would then lead to GBM malignancy. According to our findings, microRNA-148a is a critical risk factor indicator and has significant oncogenic function in GBM. Through a xenograft in vivo model, this study shows a role of upregulated microRNA-148a in tumor initiation. Lastly, we directly show microRNA-148a inhibits EGFR degradation through MIG6 regulation. Altogether, the findings show that microRNA-148a is a critical regulator and promising therapeutic target in GBM (Diagram 4).

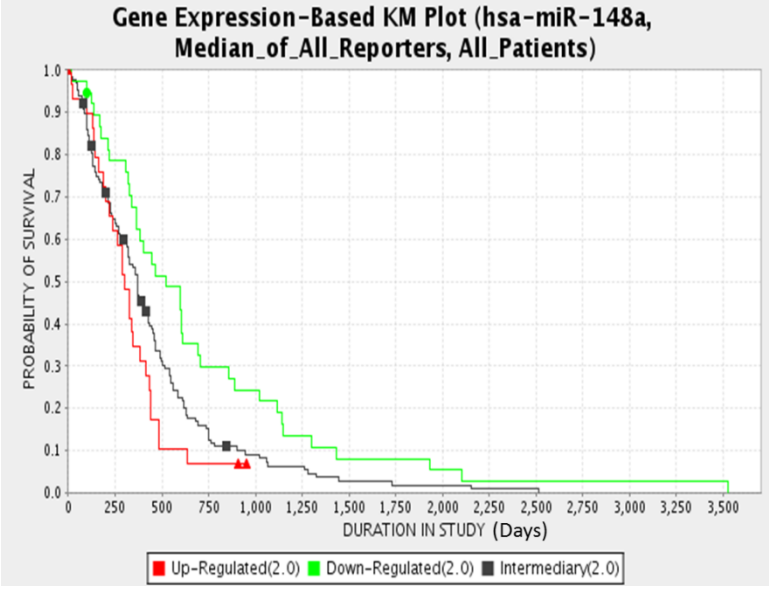


Figure 1: Analysis of TCGA GBM. The Cancer Genome Atlas (TCGA) data and Cancer Biomedical Informatics Grid (caBIG) show that miR-148a is associated with poor prognosis of glioblastoma patients.

RESULTS

i. MicroRNA-148a inversely correlates with patient survival

The compared 491 tumor samples with 10 normal tissue samples and showed a significant (59%) increase of miR-148a levels in the tumors as compared to normal brain ($p=3\times10^{-4}$) (Figure 2). Cox regression analysis of 482 GBM samples in the TCGA dataset revealed that elevated miR-148a expression was a highly significant negative risk factor (p=9.9×10-6). The hazard ratio was 1.19 with confidence intervals 1.10-1.29. The Kaplan-Meier curve of the TCGA patient cohort is shown in Figure 3. The lower quartile (with the lowest miR-148a expression) had longer overall survival than those with higher miR-148a expression. The median survivals of the different groups in the Kaplan Meier curve are <25% expression = 515, 25-50%= 463, 50-75%= 377, 75-100% = 382 (days). Log-rank analysis of these samples revealed that miR-148a was highly significant as a negative risk factor (p=9.18×10-5). These data show that miR-148a is elevated in GBM where it acts as a prognostic indicator of poor survival.

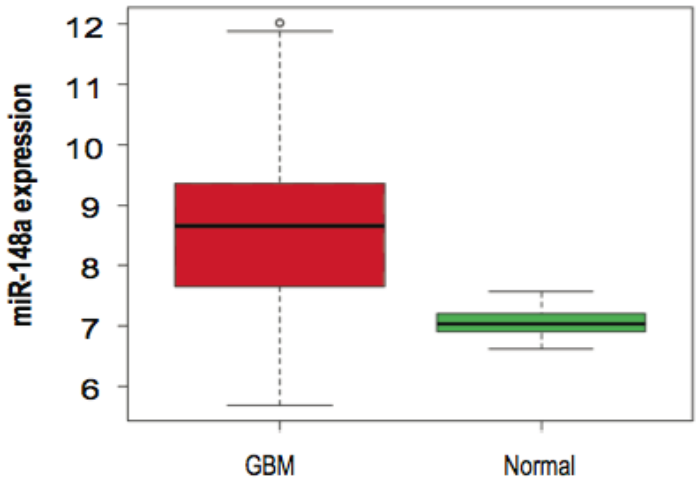


Figure 2: miR-148a is upregulated in GBM tissues and is a risk indicator. Analysis of TCGA microRNA expression data showing significantly higher expression of miR-148a in GBM tumors (n=491) than in normal brain (n=10).

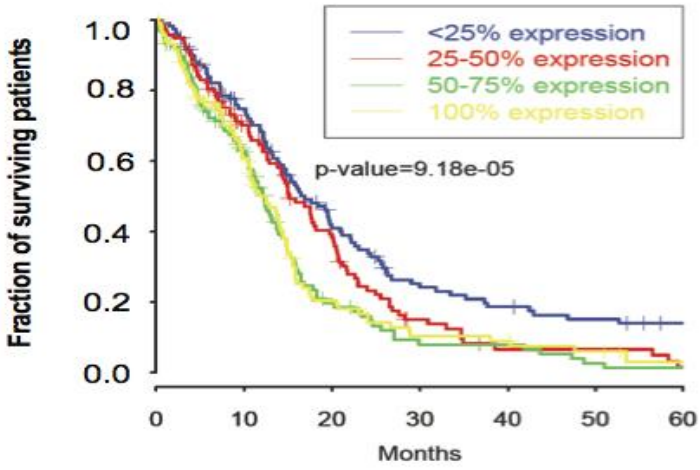


Figure 3: miR-148a inversely correlates with patient survival. Correlation analysis of expression data and patient survival data (n=482) from TCGA demonstrate that miR-148a levels are a risk indicator for survival.

ii. MiR-148a expression is upregulated in GBM cells, GSCs and human tumors

We next sought to determine of the expression data from TCGA, could be reproduced in an independent set of human GBMs from our tumor bank as well as in GBM cells and GSCs. The miR-148a expression level was determined with quantitative polymerase chain reaction (qPCR) in five established GBM lines and three GSCs. We measured miR-148a levels in GBM cells (U87, U373, T98G, A172, and SNB19), GSCs (0308, 0822, and 1228) and human tumor specimens (n=18) as well as normal human astrocytes and normal brain (n=7). MiR-148a was on average three fold higher overexpressed in GBM cells and GSCs than in astrocytes ($p<0.05$; n=3/line; Figure 4) and on average 2.7 fold increased in tumors relative to normal brain ($p<0.05$; n=3/line; Figure 5). These data expand the expression data form the TCGA analysis and preside further evidence that miR-148a is overexpressed in GBM cells, stem cells and tumors as compared to normal cells and brain. Overexpression of miR-14a and its inverse correlation with survival suggest an oncogenic role for miR-148a in GBM.

To determine if miR-148a has oncogenic functions, we assessed its effects on GBM cell and GSC growth, survival, migration, invasion and GSC self renewal. We used complementary overexpression and inhibition approaches as well as transient and stable expression of the anti-miR-148a. We also assessed the effects of anti-miR-148a inhibition on the in vivo growth of GBM xenografts established from GBM cells and GSCs. The results of these functional experiments are described below.

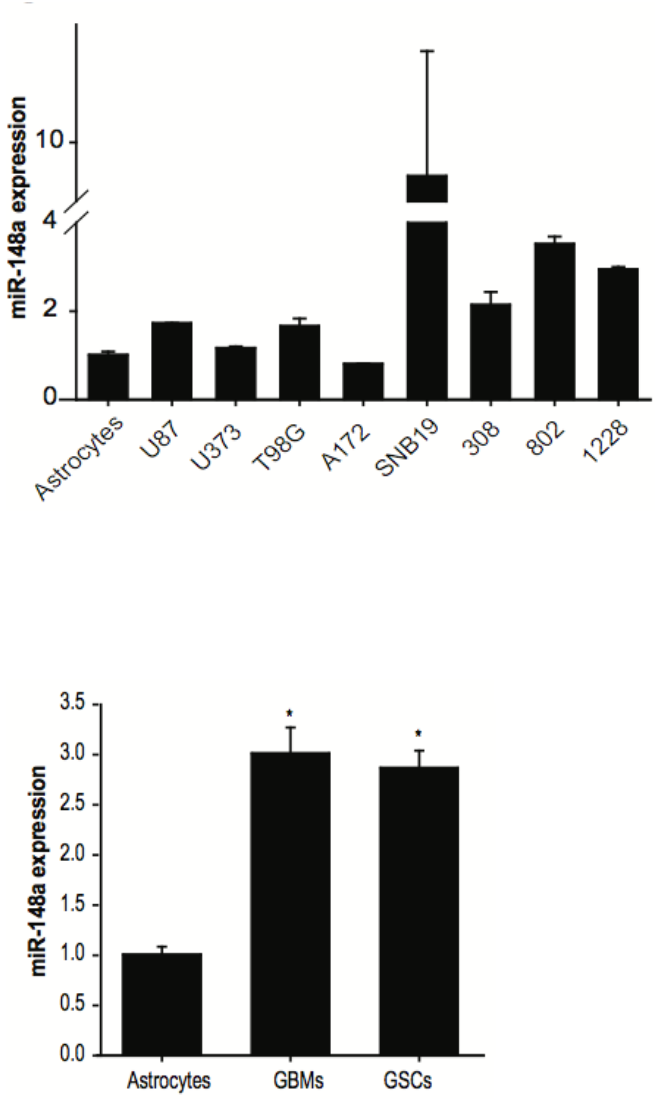


Figure 4: miR-148a is upregulated in GBM cells and GSCs. Quantification of miR-148a in glioblastoma (GBM) cell lines (U87, U373, T98G, A172, SNB19) and stem cell lines (GSCs) (0308, 0802, 1228) showing relative expression compared to normal human astrocytes. Single cell lines are shown in the upper panel and averages in the bottom panel. *, $p<0.05$

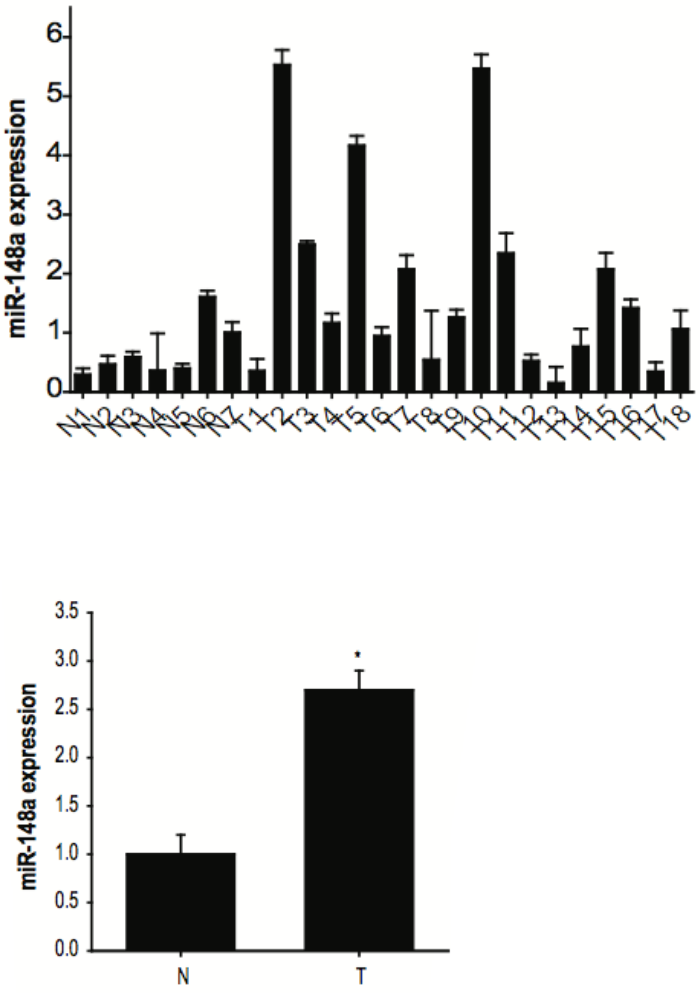


Figure 5: miR-148a is upregulated in human tumors. Quantification of miR-148a in human GBM tumors (T) (n=18) showing relative levels compared to normal human brain (N) (n=7). Single tissues are shown in the upper panel and averages in the bottom panel. *, $p < 0.05$

iii. **MiR-148a promotes glioblastoma cell and GSC growth**

One of the characteristics of GBM that contributes to malignancy and resistance to therapy is rapid cell growth. We therefore assessed the effects of miR-148a on GBM cell and GSC growth. We determined the effects of miR-148a overexpression or inhibition in GBM cells (A172, SNB19, U87, and U373) and GSCs (0308, 0822, and 1228) by cell counting. miR-148a was overexpressed by transfection with pre-miR-148a and inhibited by transfection with anti-miR-148a or scrambled controls for 48 hrs prior to cell counting for five days. miR-148a inhibition with antisense miRNA significantly decreased the growth rate of GBM cells and GSCs ($P < 0.05$) (Figure 6 and 7). miR-148a overexpression significantly increased the growth rate of GBM cells and GSCs ($P < 0.05$) (Figure 10 and 11). MiR-148a levels after overexpression were verified by qRT-PCR (Figure 8 and 9). The above data show that miR-148a promotes the growth of GBM cells and GSCs.

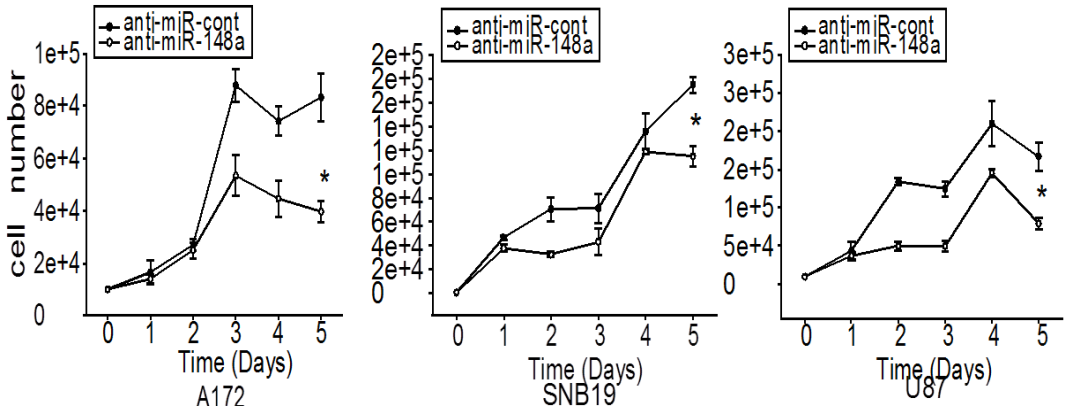


Figure 6: miR-148a inhibition reduced GBM cell growth. A172, SNB19, and U87, GBM cell lines were transfected with anti-miR-148a or anti-miR-cont. The cells were assessed 48 hrs later for cell growth by cell counting for 5days. The data show that miR-148a inhibition reduces GBM cell growth. *, $p < 0.05$, n=3.

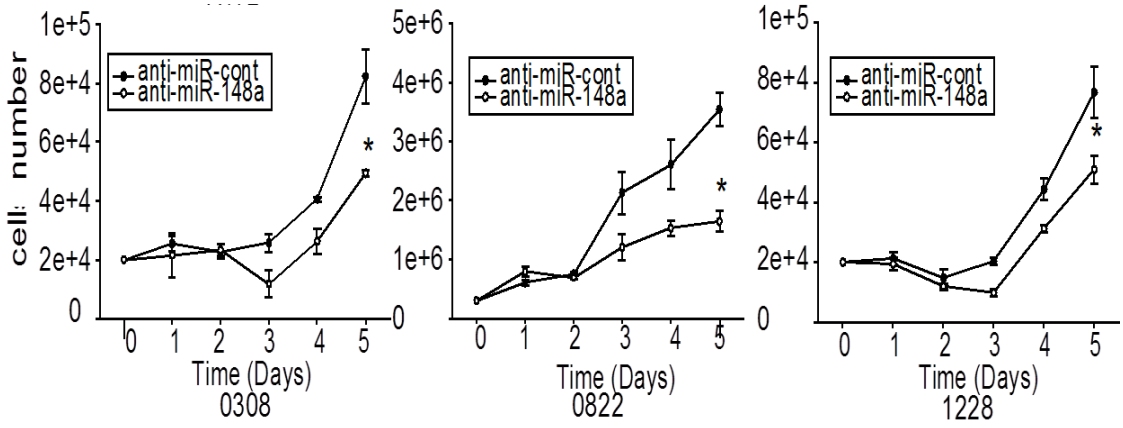


Figure 7: miR-148a inhibition reduced GSC cell growth. 0308, 0822, and 1228, GSCs were transfected with anti-miR-148a or anti-miR-cont 48 hrs later the cells were assessed for cell growth by cell counting for 5 days. The data show that miR-148a inhibition reduces GCS cell growth. *, $p < 0.05$, n=3.

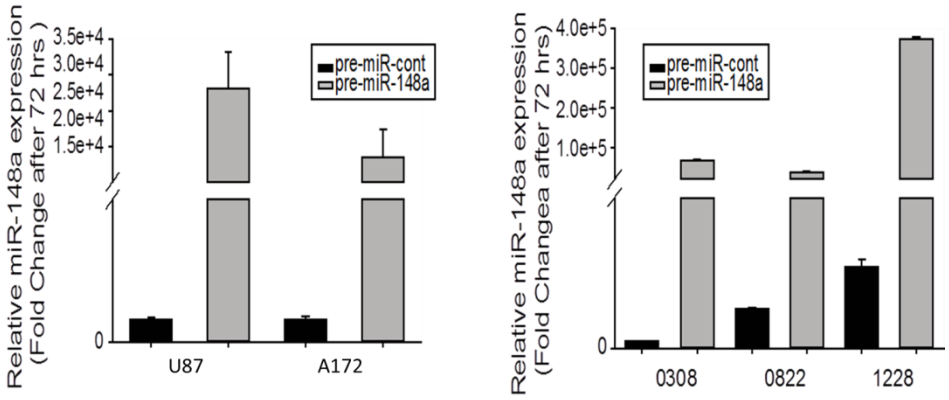


Figure 8: miR-148a levels 3 days after transfection with pre-miR-148a. Quantitative RT-PCR of miR-148a in GBM cells (U87 and A172) and GSCs (0308, 0822 and 1228) showing the levels of miR-148a expression 72 hrs (3 days) later pre-miR-148a transfection as compared to control (pre-miR-cont)- transfected cells. *, $p < 0.05$, n=3.

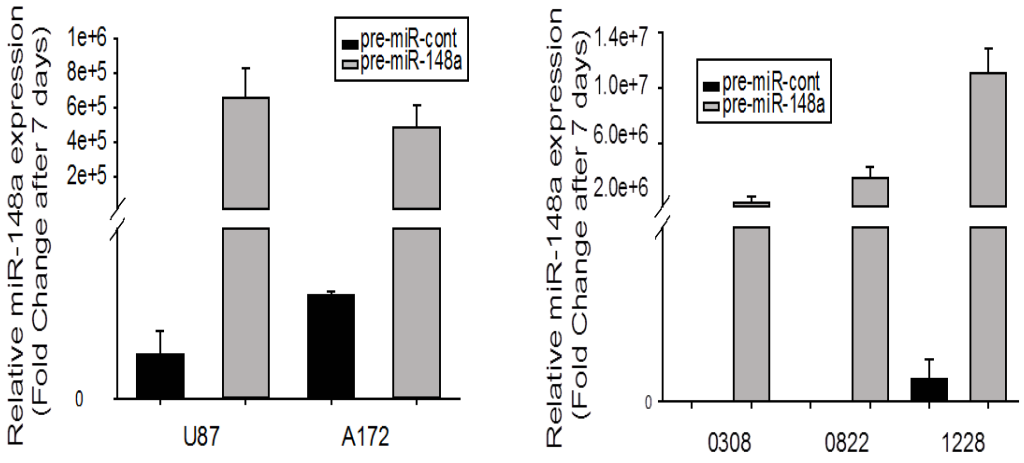


Figure 9: miR-148a levels 7 days after transfection with pre-miR-148a. Quantitative RT-PCR of miR-148a in GBM cells (U87 and A172) and GSCs (0308, 0822 and 1228) showing the levels of miR-148a expression 7 days later pre-miR-148a transfection as compared to control ***,** $p < 0.05$, n=3

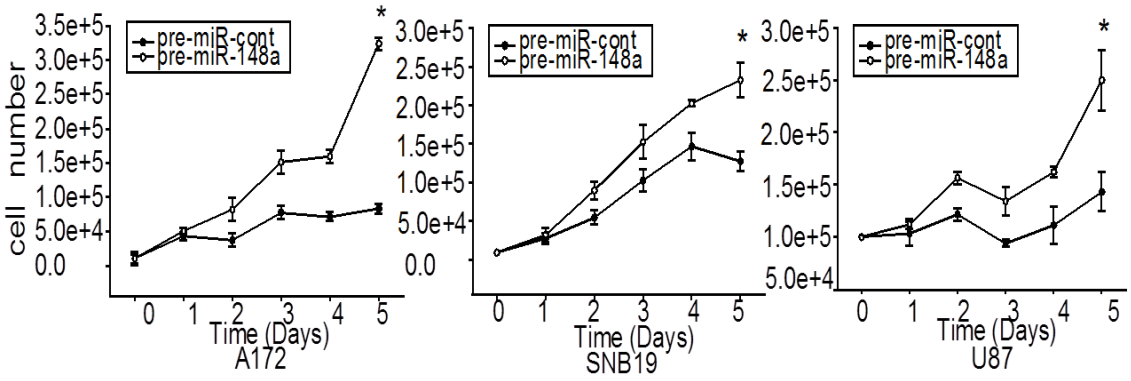


Figure 10: miR-148a promotes GBM cell growth. A172, SNB19, and U87 GBM cell lines were transfected with pre-miR-148a or controls 48 hrs later the cells were subsequently assessed for cell growth by cell counting for 5 days. The data show that miR-148a overexpression promotes A172, SBN19, and U87 cell growth. *, $p < 0.05$, n=3.

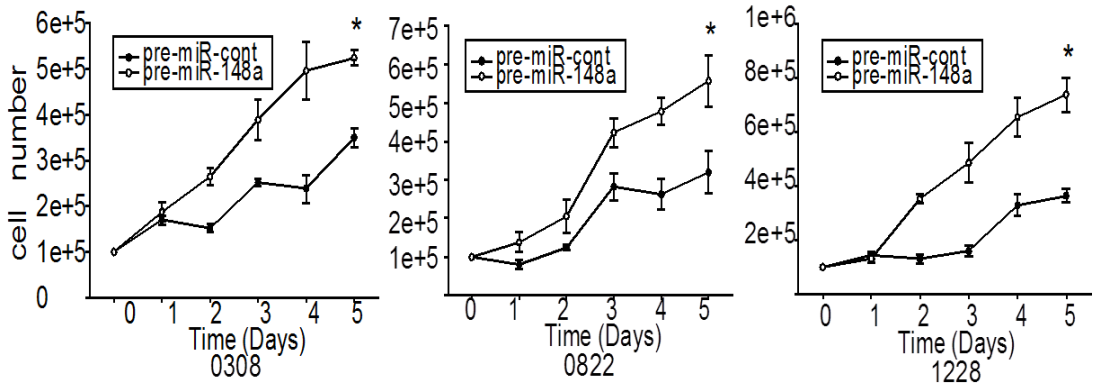


Figure 11: miR-148a promotes GSCs cell growth. 0308, 0822, 1228, GSC were transfected with pre-miR-148a or controls, and 48 hrs later the cells were subsequently assessed for cell growth by cell counting for 5 days. The data show that miR-148a overexpression promotes 0308, 0822, and 1228 cell growth. *, $p < 0.05$, n=3.

iv. **MiR-148a promotes glioblastoma cell and GSC survival**

GBM is notoriously resistant to apoptosis and cell death, particularly in response to chemotherapy and radiation therapy. We therefore overexpressed or inhibited miR-148a in GBM cells (A172, SNB19, U87, and U373) and GSCs (0308, 0822, and 1228) and determined the effects on cell apoptosis and death. The cells were transfected with either pre-miR-148a or anti-miR-148a or scrambled controls for 48 hrs prior to the measurement of apoptosis and cell death using Annexin V-7 AAD flow cytometry. Inhibition of miR-148a led to a significant induction of apoptosis and cell death in all GBM cells and GSCs ($P < 0.05$) (Figure 12). Overexpression of miR-148a led to a significant inhibition of apoptosis and cell death in GBM cells and GSCs ($P < 0.05$) (Fig. 13). MiR-148a levels after overexpression were verified by qRT-PCR (Figure 9 and 10). The above results show that miR-148a reduces cell death in GBM.

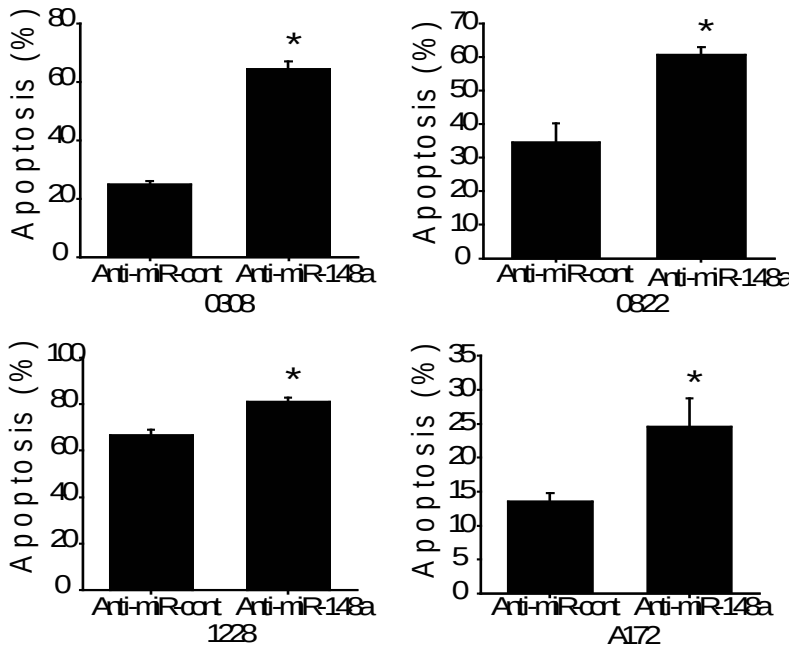


Figure 12: miR-148a inhibition reduces cell survival in GSC and GBM cells. GSCs (0308, 0822, and 1228) and the GBM cell line (A172) were transfected with either anti-miR-148a or control-miRs and 48hrs later were assessed for cell death and apoptosis by AnnexinV-PE/7-AAD flow cytometry. The data show that miR-148a inhibition reduces survival. *, $p < 0.05$, n=3.

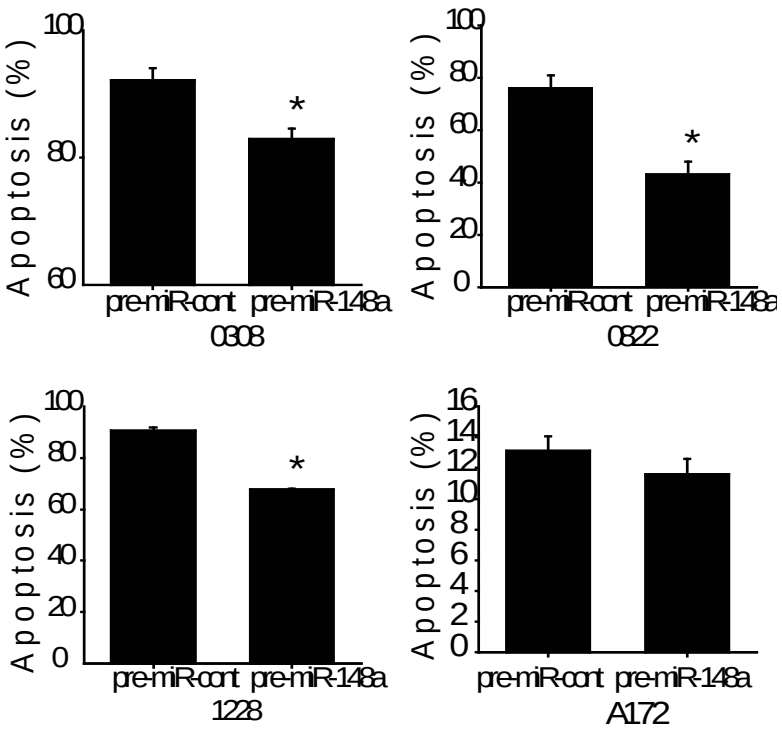


Figure 13: miR-148a promotes GSC and GBM survival. GSCs (0308, 0822, and 1228) and GBM cell line (A172) were transfected with either pre-miR-148a, or controls and 48 hrs later were assessed for cell death and apoptosis by AnnexinV-PE/7-AAD flow cytometry. The data show that miR-148a overexpression decreases cell survival. *, $p < 0.05$, n=3.

v. MiR-148a promotes glioblastoma cell migration

Glioblastoma cells are known to migrate and invade the surrounding brain, making a surgical removal of the entire tumor practically impossible. These m and invaded cells regenerate the tumor leading to tumor recurrence, which is almost always lethal. To determine of miR-148a affected cell migration or invasion, we treated GBM cells but not GSCs because they grow as neurospheres that do not attach to tissue culture plates. Anti–miR-148a or pre–miR-148a was transfected into glioblastoma to either inhibit or overxpress miR-148a. The cells were then assessed for migration using a wound healing assay. Inhibition of miR-148a expression decreased the migration of glioblastoma cells (Figure 14). Overexpression of miR-148a increased the migration of glioblastoma cells (Figure 15). These data show that miR-148a promotes glioblastoma cell migration.

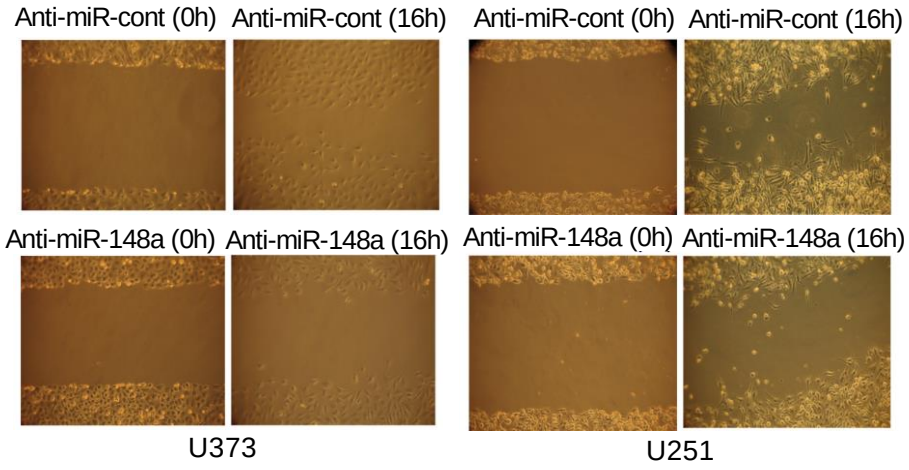


Figure 14: Inhibition of miR-148a decreases GBM cell migration. GBM (U373, U251) cell lines were transfected with either anti-miR-148a or controls and assessed for migration with the wound-healing assay. The data show that miR-148a inhibition inhibits GBM cell migration.

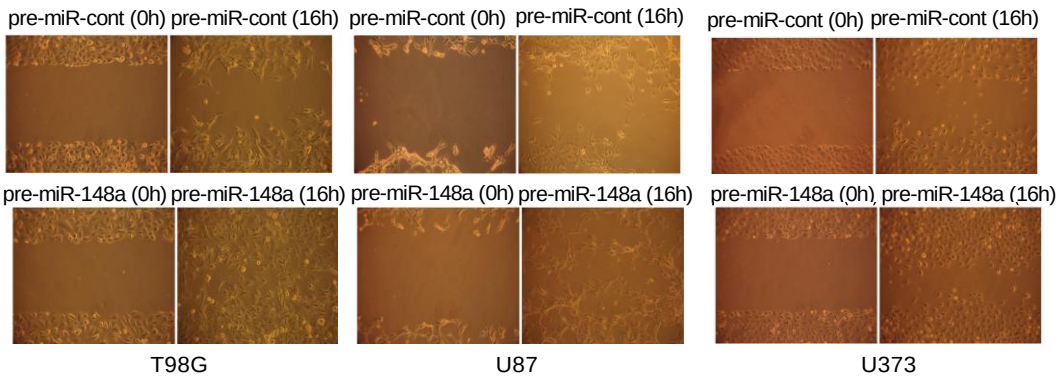


Figure 15: MiR-148a promotes GBM cell migration. GBM (T98G, U87, U373) cell lines were transfected with either pre-miR-148a or controls and assessed for migration with the wound-healing assay. The data show that miR-148a overexpression increases GBM cell migration.

vi. MiR-148a promotes glioblastoma cell invasion

GBM cell invasion of the surrounding brain is one of the hallmarks of this tumor that is responsible for tumor recurrence and resistance to therapy. We therefore assessed the effects of miR-148a on glioblastoma cell invasion. We determined the effects of miR-148a overexpression and inhibition on cell invasion using a transwell invasion assay. Similar to migration, GSCs were not used for these experiments because they grow as neurospheres that do not attach to tissue culture plates. Anti–miR-148a or pre–miR-148a or scrambled controls were transfected into glioblastoma cells and 16 hrs later followed by transwell invasion though a collagen IV coated membrane. Collagen IV was chosen because it is a common component of the brain's extracellular matrix. Invaded cells were stained, photographed and counted. Inhibition of miR-148a significantly decreased GBM cell invasion ($P < 0.05$) (Figure 16). Overexpression of miR-148a significantly increased GBM cell invasion ($P < 0.05$) (Figure 17). These data show that miR-148a can exert oncogenic effects in GBM by promoting tumor cell invasion.

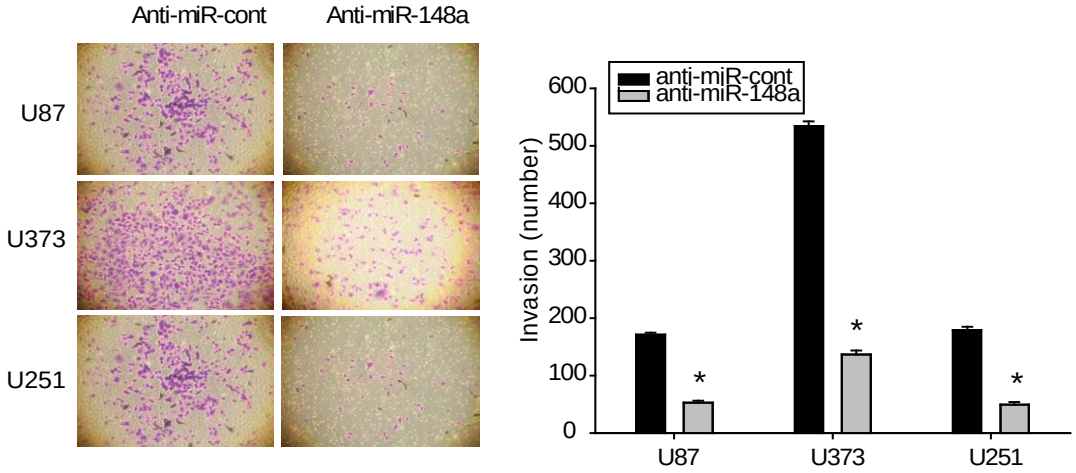


Figure 16: MiR-148a inhibition decreases GBM cell invasion. GBM (U87, U373, and U251) cell lines were transfected with either anti-miR-148a or controls and 16 hrs later assessed for invasion with the transwell invasion assay; Left panels show representative invasion assays, right panels show the quantification of invasion. The data show that miR-148a inhibition inhibits GBM cell invasion. *, $p < 0.05$, n=3.

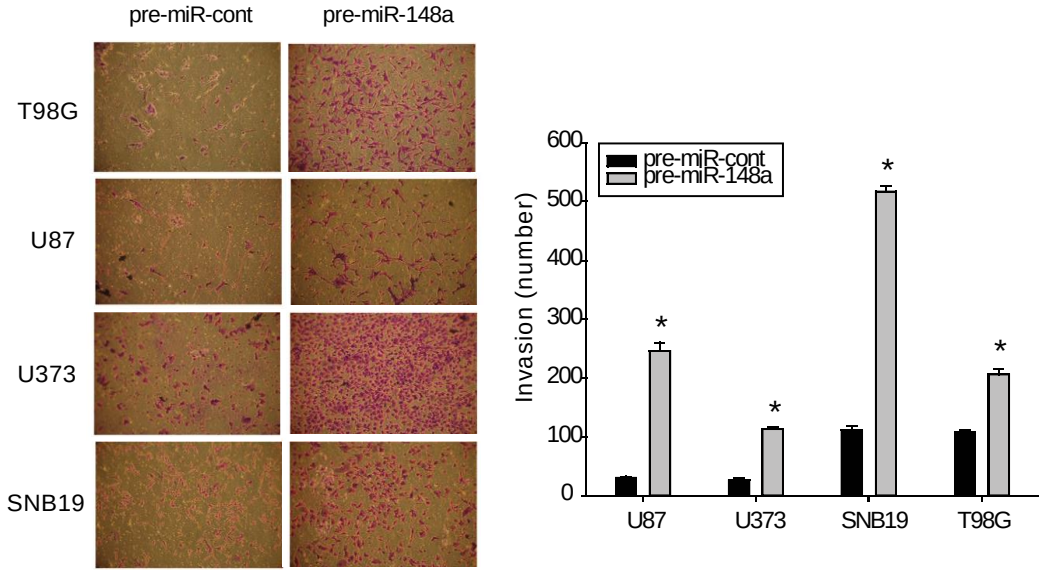


Figure 17: MiR-148a promotes GBM invasion. GBM (U87, U373, SNB19, and T98G) cell lines were transfected with either pre-miR-148a or controls and 16 hrs later assessed for invasion with the transwell invasion assay; Left panels show representative invasion assays, right panels show the quantification of invasion. The data show that miR-148a overexpression increases GBM cell invasion in GBM. *, $p < 0.05$, n=3.

vii. MiR-148a induces GSC neurosphere formation

According to the cancer stem cell theory, GSCs are responsible for tumor initiation, recurrence and resistance to therapy. One of the main characteristics of cancer stem cells is the ability to self renew. To determine if miR-148a acts as an oncogene by regulations glioblastoma stem cell functions, we tested the effects of miR-148a on stem cell self renewal. We analyzed the effects of miR-148a on GSC self-renewal using a neurosphere formation assay. This assay is based on the premise that cancer stem cells grow in neurospheres. We transfected GSCs with either anti–miR-148a, pre–miR-148a or controls and measured neurosphere formation for a period of one week. We counted the number of neurospheres that were larger than a specific arbitrary size corresponding to ~ 30 cells. MiR-148a inhibition significantly reduced neurosphere size and number ($P < 0.05$) (Figure 18). miR-148a overexpression significantly increased neurosphere size and number ($P < 0.05$) (Figure 19). These data show that miR-148a promotes the self-renewal ability of GSCs and suggest that this microRNA is an important regulator of stem cell functions in GBM.

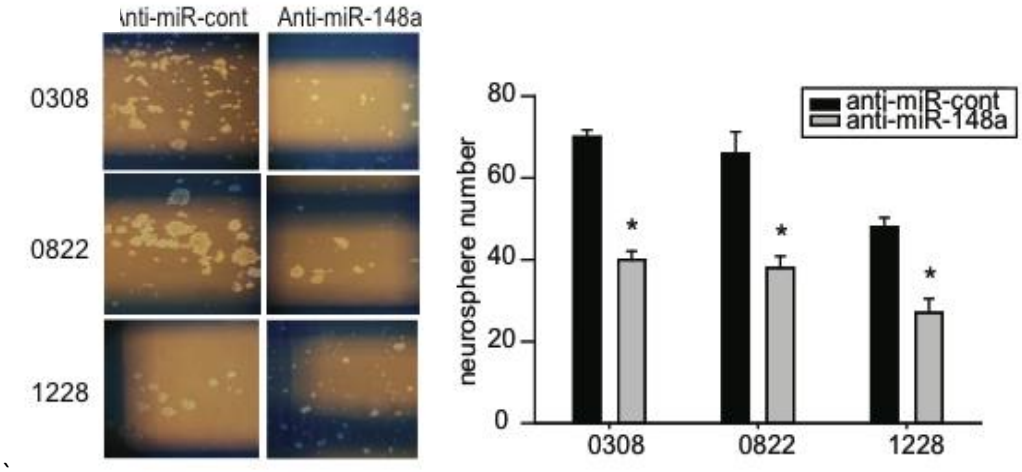


Figure 18: miR-148a inhibition reduced GSC neurosphere formation. GSCs (0308, 0822, and 1228) were transfected with either anti-miR-148a or miR controls and measured for self-renewal with the neurosphere formation assay. The data show that inhibition of miR-148a significantly reduces neurosphere formation Left panels show representative assays; right panels show quantification of neurosphere formation. *, $p < 0.05$, n=3.

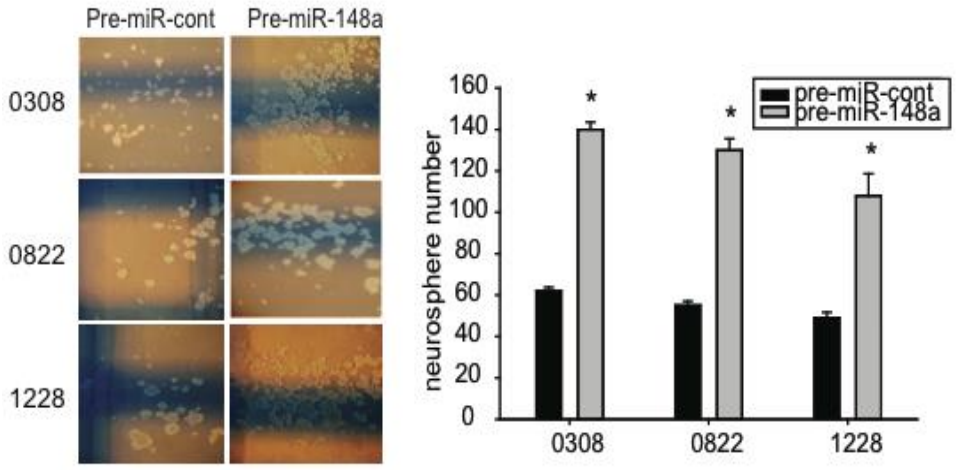


Figure 19: **MiR-148a overexpression promotes GSC neurosphere formation.** GSCs (0308, 0822, and 1228) were transfected with either pre-miR-148a or controls and assessed for self-renewal with the neurosphere formation assay. The data show that overexpression of miR-148a significantly increased neurosphere formation. Left panels show representative assays, right panels show quantification of neurosphere formation. *, $p < 0.05$, n=3.

viii. Transiently expressed anti-miR-148a reduces the *in vivo* growth of GSC-derived xenografts

Since miR-148a promotes GBM cell and GSC gowth, survival, migration, invasion and self-renewal, it appears to act as an oncogene. To further investigate these findings, we next tested the effects of miR-148a on in vivo xenograft formation and growth. GSCs (1228) were transfected with anti-miR-148a or anti-miR-control and stereotactically implanted into the striata of immunodeficient mice. Tumor sizes were measured with MRI four weeks after implantation. The data show that anti-miR-148a reduces in vivo tumor formation by GSCs ($P<0.05$) (Figure 20). These findings suggest that miR-148a is an oncogene that is involved in GBM tumor initiation and development.

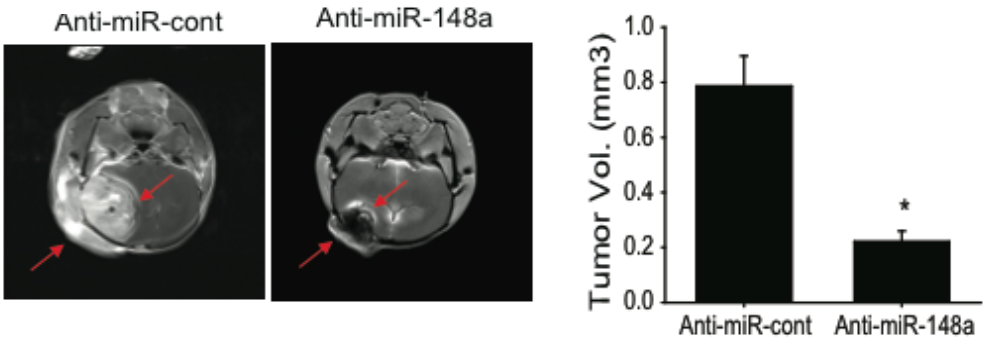


Figure 20: antisense miR-148a inhibits the growth of GSC-derived orthotropic xenografts. GSCs (1228) were transfected with anti-miR-148a or control and orthotopically implanted in immunodeficient mice (n=6). After 4 weeks, MRI measured tumor volumes. The data from Figure 19 show that miR-148a inhibition leads to inhibition of GSC-derived xenograft growth. Arrows point to tumors. *, $p < 0.05$, n=6

viii. Stably expressed miR-148a inhibitor reduces the in vivo growth of GBM-derived xenografts

We also assessed the effects of stable anti-miR-148a expression on the growth of a GBM xenograft derived from the U87 cell line. These experiments were performed to complement the GSC xenograft experiments described above and show the effects of longer term miR-148 inhibition on in vivo tumor growth. U87 cells were infected with lentiviruses encoding the pEZX-AM04 containing the antisense sequence for miR-148a, mCherry fluorescent protein and a hygromycin resistance gene. An empty vector was used as control. After growth in appropriate media, transfection efficiency was verified by fluorescent microscopy of the red colored mCherry protein, which is driven by the same promoter as anti-miR148a. The data show ~ 100% transfection efficiency (Figure 21). The sequence of miR-148a antisense in pEZX-AM04 vector is shown in Figure 22. U87 cells stably expressing anti-miR-148a were orthotopically injected into NOD/SCID immunodeficient mice brains (n=10) and tumor size was measured by magnetic resonance imaging (MRI) after 3 weeks. The result shows significantly reduced tumor volume in anti-miR-148a expressing xenografts as compared to controls ($p<0.05$) (Figure 23). These data show that miR-148a promotes GBM tumor formation and growth and further confirm the miR-148a oncogenic effects.

Altogether, the above data from chapter 2 show that miR-148a is upregulated in GBM where it predicts poor prognosis and where it exerts powerful oncogenic effects. Therefore, miR-148a functions as a new oncogene in GBM. Chapter 3 will uncover the targets and oncogenic mechanisms of action of miR-148a.

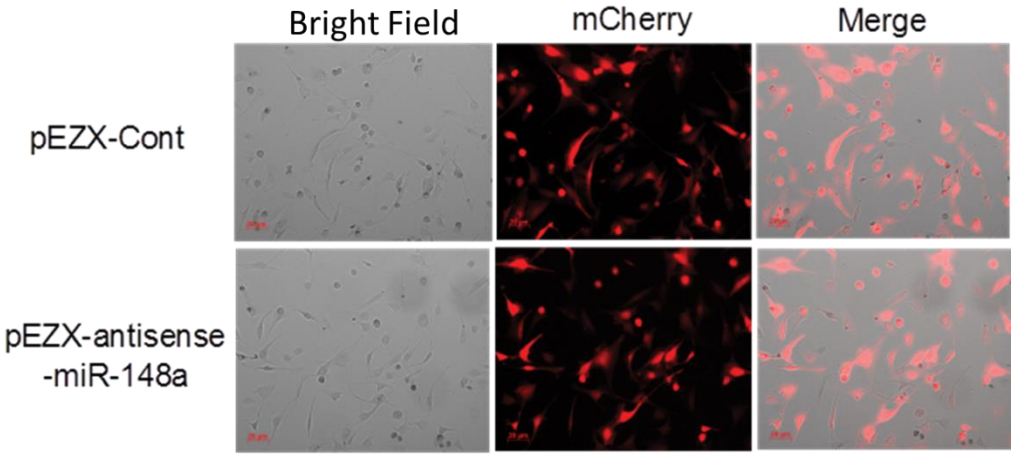


Figure 21: Transfection efficiency of U87 cells infected with lentiviruses encoding anti-miR-148, mCherry, and hygromycin resistance gene. Representative mCherry fluorescent positive U87 cells.

A

Anti-miR-148a 5' ACAAAGUUCUGUAGUGCACUGA 3'

B

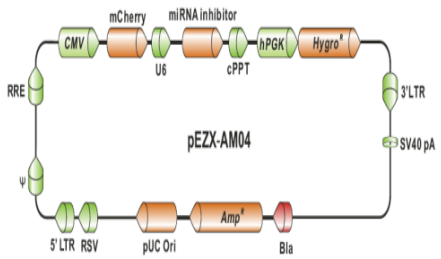


Figure 22: A) Sequence of miR-148a antisense, B) Map of pEZX-AM04.

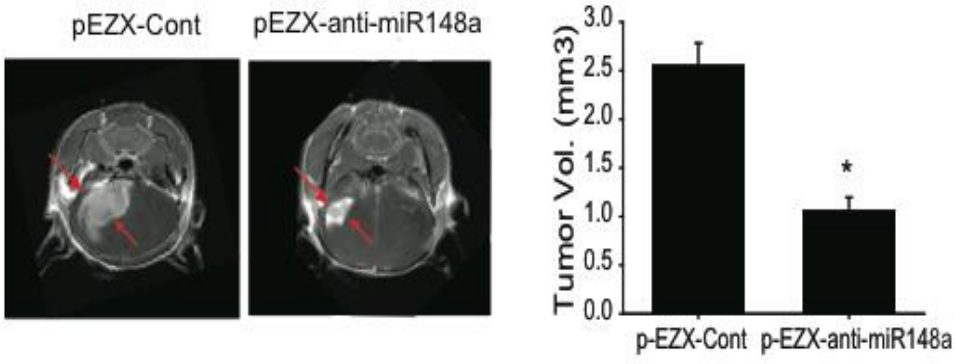


Figure 23: antisense miR-148a inhibits the growth of GBM cell-derived orthotropic xenografts. Anti-miR-148a expressing U87 stable cells were orthotopically implanted in immunodeficient mice (n=10). After 3 weeks, tumor volumes were measured by MRI. The data show that miR-148a inhibition leads to inhibition of GBM cell derived xenograft growth. Arrows point to tumors. *, $p < 0.05$

II. DISCUSSION

The above extensive loss and gain of expression and functional studies provide strong evidence that miR-148 is a prognostic and oncogenic microRNA in glioblastoma. Previous reports have shown that miR-148a is a suppressive microRNA in development [183, 184], and in several cancers such as hepatocellular carcinoma, colorectal cancer, non-small cell lung cancer cells, gastric cancer, pancreatic cancer, and breast cancer [179, 181, 185-190]. It was shown that miR-148a represses DNA methyltransferase 1 (DNMT1) leading to ATP-citrate lysate (ACL)-miR-148a-dependent regulation of DNMT 1 during adipogenesis in gastric cancer [184, 187]. miR-148a also was shown to play a role in myogenic differentiation. It was shown that overexpression of miR-148a significantly promoted myogenic differentiation of both C2C12 myoblast and primary muscle cells. In addition to role of miR-148a in development, miR-148a has been shown as a tumor suppressive microRNA that was significantly decreased in hepatocellular carcinoma (HCC) tissues. In vitro and in vivo studies showed that restoration of miR-148a expression significantly repressed the migration and pulmonary metastasis of hepatoma cells [179]. In colorectal cancer, overexpression of miR-148a inhibited colon cancer cell proliferation and lower levels of miR-148a expression were associated with shorter disease-free survival rates and poorer overall survival rates [185]. Two different studies showed DNA methyltransferases (DNMTs) as targets of miR-148a adipocyte differentiation and gene regulation in gastric cancer [184, 187]. However, miR-148a has not been studied in GBM before.

Our study shows for the first time that miR-148a is a prognostic and oncogenic microRNA (oncomiR) in GBM. The study initiated from a preliminary analysis of a limited set of TCGA data that we performed and that showed that miR-148a expression inversely correlated with patient survival. Using the Cancer Biomedical Informatics Grid (caBIG)

analysis (a website that has since been shut down) we observed that miR-148a is associated with poor prognosis of glioblastoma patients. We therefore hypothesized that miR-148a, which has been described as a tumor suppressive microRNA in other cancers, is an oncogenic microRNA in glioblastoma. We speculated that if miR-148a acts as an oncogene, its expression in GBM would likely be elevated. We therefore first examined the expression of miR-148a in GBM cells, GSCs and tumor specimens from our tumor bank. We found that miR-148a was highly expressed in GBM cells and human tumor specimens as compared with normal human astrocytes and normal brain. We the confirmed these findings by analyzing miR-148a expression in ~ 500 tumors from TCGA data and showing that miR-148a was significantly elevated in GBM as compared to normal brain, which was compliant with all applicable laws, analysis of all data was done in the R project. Moreover, Cox regression analysis of all the above samples for miRNA and survival data was performed and showed that miR-148a was a risk indicator for GBM patient survival. We therefore convincingly showed that miR-148a is upregulated in GBM and that its expression predicts poor patient survival. Interestingly, other miRNAs have also been shown to have opposite effects in different cancers. For example, miR-34s (miR-34a and miR-34b/-34c clusters) recently acquired notoriety because they are induced by p53. Consistent with p53's role as a tumor suppressor, miR-34s are downregulated in several tumors such as non–small cell lung cancers [191] and pancreatic cancers [192]. Reduced miR-34 expression is not always correlated with p53 loss; instead, miR-34a is located in 1p35, a locus that is frequently deleted in a number of cancers [193]. However, it is not the case for all type of cancers. MiR-34 have been found to be upregulated in several cancers, including renal cell carcinoma [194], colon cancer [195], and hepatocellular carcinoma [196]. microRNAs can have complex context-dependent that vary in different cancer types. The ratio between target genes and microRNAs expression is one possible reason to explain the opposite effects of microRNAs in different cancer types. Also, the microRNA target genes themselves can have context-dependent effects. For

example, PTEN is a well known tumor suppressor in many cancers including GBM [197]. However, our lab has shown that PTEN can have oncogenic properties by enhancing the stability of gain-of-function p53 mutants and that PTEN exerts its unconventional oncogenic effects in glioblastoma through a novel PTEN/mutant p53/c-Myc/Bcl-XL molecular and functional axis. [198, 199]. Therefore, a microRNA that targets PTEN could exert opposite effects in wildtype and mutant p53 cancer cells. These and other unknown reasons could explain the opposite effects of miR-148a in GBM and other cancers.

We then studied the functional role of miR-148a in GBM. We used complementary miRNA inhibition with anti-miR and overexpression with pre-miR to test the effects of miR-148a on GBM cell and GSC proliferation, survival, migration, invasion, stem cell self renewal and in vivo tumor growth. The attenuation of miR-148a with antisense produced striking anti-tumor effects. Conversely, overexpression of miR-148a with precursor-mimicking endogenous miR-148a, promoted GBM malignant endpoints. MiR-148a increased GBM cell and GSC growth, survival, migration, and invasion as well as GSC neurosphere formation. Inhibition of miR-148a led to the inhibition of in vivo GBM cell and GSC-derived xenograft growth. These data suggest that miR-148a is a new potential therapeutic target in GBM. Interstingly, transient antisense transfection elicited greater inhibitory effect on tumor formation compared to stable antisense transfection using lentiviruses. Transient antisense transfection might have exerted a strong initial inhibition of tumor formation due to a higher antisense expression than the one achieved via lentivirus transfection. In fact, it is well known that lentiviral infections usually lead to lesser transgene expression levels than those achieved with lipid-based transfections. Having shown that miR-148a is an upregulated prognostic oncomiR in GBM, we next went on to investigate the targets and mechanisms of action of miR-148a.

Chapter 4: MIG6, EGFR and BIM as targets of miR-148a

I. Introduction

Deregulated cell signaling in cancer leads to unchecked growth, uncontrolled migration, invasion and metastasis, evasion of apoptosis and other cancer phenotypes [43]. Owing to relatively recent technological advances that have allowed us to increase our understanding of the mechanisms by which these alterations occurs the development of therapeutic agents that target specific proteins responsible for oncogenic signaling is likely to be a more promising therapeutic strategy than the canonical chemotherapy and radiation that are used to non-specifically kill rapidly dividing cells. Despite the promise and advance of such approaches, a number of difficulties remain to be overcome, the most important of which is therapy resistance. Due to its frequent deregulation and important role, the epidermal growth factor receptor (EGFR) is one such promising molecular target in GBM.

The epidermal growth factor receptor (EGFR) is a member of a family of four ErbB receptor tyrosine kinases; EGFR (HER1)/ErbB1), HER2/ErbB2, HER3/ErbB3, and HER4/ErbB4. EGFR plays a critical role in regulating tumor cell growth, invasion, angiogenesis, metastasis, protein translation, and cell metabolism [25]. Binding of specific partner ligands and growth factors such as epidermal growth factor (EGF) and transforming growth factor alpha (TGF-α) to the extracellular domain of ErbB receptors stabilize them and induce conformational changes leading to receptor dimerization, which is an essential requirement for transactivation of the tyrosine kinase. Various adaptor and effector molecules like Shc (Src homology 2 containing transforming protein); Grb2 and Grb7 (growth factor receptor -bound protein); kinases such as JAKs (Janus Kinases), Src (c-Src tyrosine kinase), and PI3K

(protein tyrosine phosphatases), E3 ligase Cbl; and transcription factors like STATs (signal transducer and activator of transcription) are activated to trigger critical signaling cascades-Ras/Raf (rat sarcomma/rapidly accelerated fibrosarcoma), MAPK (mitogen activated protein kinase), PKC (protein kinase C), PI3K/PKB (protein kinase B), and JAK/STAT. These signaling cascades result in the activation of Erk (extracellular signal-regulated kinase), JNK (c-Jun N-terminal kinase), p38-MAPK, Akt and transcription factors like c-Myc, among others. The biological effects of these phosphorylations vary from target to target, including stabilization, degradation and increased transcriptional activity. ERK can also directly phosphorylate and inactivate pro-apoptotic proteins such as BAD [200] and BIM-EL [201], resulting in pro-survival factors being favored in the cell. In general, activation of EGFR signaling is pro-survival and pro-proliferation in almost all cell contexts [18, 202]. Therefore, it is not surprising that dysregulation of EGFR signaling and oncogene addiction to this receptor is found in many cancers [203].

EGFR has received much attention as a potential target for GBM treatment because of its high frequency of mutation, genomic and expression alterations causing increased protein expression and activated signaling [203, 204]. Almost 40% of GBMs have overactivated EGFR [18]. Moreover, 30% of GBM have the most common mutation of EGFR, EGFRvIII (in frame deletion spanning exons 2-7, imparting ligand-independent constitutive activation and high oncogenecity) [27]. In primary GBM, EGFR alterations are associated with loss of the tumor suppressors p16Ink4a, p19Arf, and PTEN, while secondary GBMs are commonly accompanied by mutations or deletions of p53, and aberrant activation of PDGFR (platelet-derived growth factor receptor) pathways and IDH1 (isocitrate dehydrogenase 1) mutations [205]. Remarkably, EGFR is also involved in sustenance of glioblastoma stem cells (GSCs) making it an even more attractive therapeutic target [206, 207]. It has been shown that the

proportion of cells in the stem cell niche is decided by the balance between EGFR signaling and a canonical stem-cell signaling like Notch [206]. It has also been shown that EGFR signaling promotes hypoxia-induced GSC growth [207].

Several EGFR inhibitors such as monoclonal antibodies and tyrosine kinase inhibitors have been developed for GBM therapy [208]. The first-generation reversible small molecules tyrosine kinase inhibitors (TKIs) of EGFR, like erolotinib and gefitinib or the monoclonal antibodies against EGFR such as cetuximab and panitumumab, are validated therapeutic inhibitors for EGFR and EGFR downstream signaling pathways. Erlotinib and Gefinibib compete with ATP binding in the catalytic TK domain of EGFR. The most effective outcome using these two drugs is shown in non-small cell lung cancer (NSCLC) where the common EGFR mutations are located around the ATP-binding site [209]. In vitro studies showed that these small molecule inhibitors primarily inhibit phosphorylation of the receptor and downstream signaling, and influence angiogenesis, cell cycle regulation and apoptosis [210]. However, the clinical response rates to these two small molecule TK inhibitors in GBM have been disappointing. Combination therapies with TKIs showed lack of efficacy and revealed high toxicity [211]. Anti-EGFR monoclonal antibodies that target the extracellular domain of EGFR, like cetuximab and panitumumab, have been developed and tested in several cancers. These monoclonal antibodies disrupt ligand binding to EGFR, abrogate downstream signaling and promote internalization and degradation of the receptor leading to cell cycle arrest and cell death [212, 213]. Clinical trials using these antibodies have shown some benefit in head and neck, lung and other cancers [212]. However, preclinical data show mixed results in GBM [213], and clinical trials with cetuximab in combination with radiotherapy and temozolomide are in progress [214].

Despite the increasing interest and significant research to identify the potency of TKI and monoclonal antibodies, EGFR inhibition has not been as successful in vivo in glioblastoma as in other cancers. These are due to the compensatory mechanisms of alternative signaling pathways and cellular heterogeneity of glioblastoma as well as the insufficient penetration of drugs across the blood-brain barrier [215]. It is necessary to find another strategy that targets multiple genes simultaneously. Herein, we propose microRNAs that target either oncogenic or tumor suppressive genes without resistance.

EGFR signaling and protein half-life are tightly regulated [216]. Mitogen-inducible gene 6 (MIG6) regulates EGFR signaling and turnover by binding EGFR and directly inhibiting tyrosine kinase activity, increasing clathrin-dependent EGFR endocytosis and trafficking into the lysosome, and promoting EGFR degradation [85, 98, 101]. Ablation of MIG6 induces tumor formation, supporting a tumor suppressor function of MIG6 [85, 217]. The MIG6 gene is located on chromosome 1p36, which is subject to focal deletions in GBM. A Cancer Genome Atlas (TCGA) data analysis showed that 15 out of 430 GBM samples contain homozygous deletions in 1p36 [217] but that MIG6 expression is downregulated in ~50% of primary tumor samples and GBM cell lines [85]. Therefore MIG6 deletions only account for a small fraction of the GBM tumors with reduced MIG6 expression. In my thesis research, I show for the first time that miR-148a regulates EGFR trafficking and activation by targeting MIG6.

Resistance to apoptosis is one of the biggest obstacles in GBM therapy [125, 218]. Apoptosis via the intrinsic pathway is regulated by the balance between pro-apoptotic (Bax, Bak, BIM and Bad) and anti-apoptotic (Bcl-2 and Bcl-xL) members of the Bcl-2 family [219]. BIM

(*BCL2L11*) is a member of the BD-3-only proteins [135]. Under the proliferation conditions, Bim is bound to dynein light chain (LC8) of the microtubular motor complex and is sequestered away from other Bcl-2 family members preventing its proapoptotic function [129]. Following cell death signaling or proapoptotic stimuli, BIM is translocalized to the mitochondria where it initiates the mitochondrial cell death pathway by directly interacting with Bcl-2 and releasing Bax protein at the mitochondrial outer membrane. Bax induces pore formation at the mitochondrial membrane leading to cytochrome c release. BIM is regulated by ERK1/2 signaling. The phosphorylation of BIM by activated ERK induces BIM degradation by the ubiquitin-proteasome system [220]. BIM has been shown as an important mediator of targeted therapy-induced apoptosis in solid tumors [127]. Moreover, BIM is downregulated in 29% of GBM cases based on TCGA analysis [127, 128]. However, the causes of BIM downregulation in GBM are not known. In my thesis, I show that miR-148a directly targets and inhibits BIM. This provides an explanation for BIM downregulation in GBM that is not due to gene deletion. It also partly explains the anti-apoptotic effects of miR-148a that we uncovered and makes this microRNA a more attractive therapeutic target, as its inhibition would lead to both EGFR ihhibition and induction of apoptosis through BIM activation.

II. Results

In chapter 2, I investigated the expression and function of miR-148a and showed that it was a prognostic oncogene in GBM. This chapter uncovers the mechanism of miR-148a action.

i. MiR-148a inhibits MIG6 and BIM expression and indirectly enhances EGFR expression and activation

MicroRNAs exert their actions by binding to the 3'UTR of targeted mRNA and leading to mRNA degradation or/and inhibition of translation. Several publicly available websites use algorithms to predict potential targets of microRNAs. To uncover mRNA targets of miR-148a in GBM, we therefore first used bioinformatics databases (Targetscan, Pictar, RNAhybrid) to identify potential tumor suppressor targets. We focused on tumor suppressors based on our demonstration that miR-148a is oncogenic and therefore would be expected to act by inhibiting the expression of tumor suppressors. The following tumor suppressor genes contained predicted binding sites for miR-148a: ERRFI1 (MIG6, NM_018948), BCL2L11 (BIM, NM_001204106), PTEN (NM_000314), SOCS3 (NM_003955), DNMT1 (NM_001130823) and JMY (NM_152405). To experimentally verify these potential targets, GBM cells were transfected with miR-148a and 48 hrs later were assessed for protein and mRNA target of these proteins levels by immunoblotting and qRT-PCR. Only two of the candidates were confirmed: MIG6 (ERRFI1) and BIM (BCL2L11) (Figure 24-30). As MIG6 is a critical negative regulator of EGFR trafficking, degradation and activation, we also determined the effects of miR-148a on EGFR expression and activation. MiR-148a inhibition led to increased expression of MIG6 and BIM extra-long (most abundant form of BIM) in GBM cells and GSCs (Figure 24, 26). MiR-148a inhibition led to a 20-50% and 100-250% increase in MIG6

protein expression in GBM cells and GSCs, respectively. Conversely, miR-148a overexpression significantly reduced MIG6 and BIM protein levels (Figure 25, 27). Pre-miR-148a transfected cells showed an average 50% reduction of MIG6 protein in GBM (A172, U87, and SNB19) cells. Moreover, the effects of miR-148a on EGFR expression and activation were opposite to those on MIG6, as miR-148a overexpression led to increased EGFR and phospho-EGFR (Figure 25). We confirmed the above results in U87 cells stably expressing anti-miR-148 (Figure 28). MiR-148a also inhibited MIG6 (Figure 29) and BIM (Figure 30) mRNA levels as shown by qRT-PCR, suggesting that the microRNA effects are via translation inhibition as well as via mRNA degradation. These data demonstrate that miR-148a regulates MIG6 and BIM expression and EGFR expression and activation in GBM.

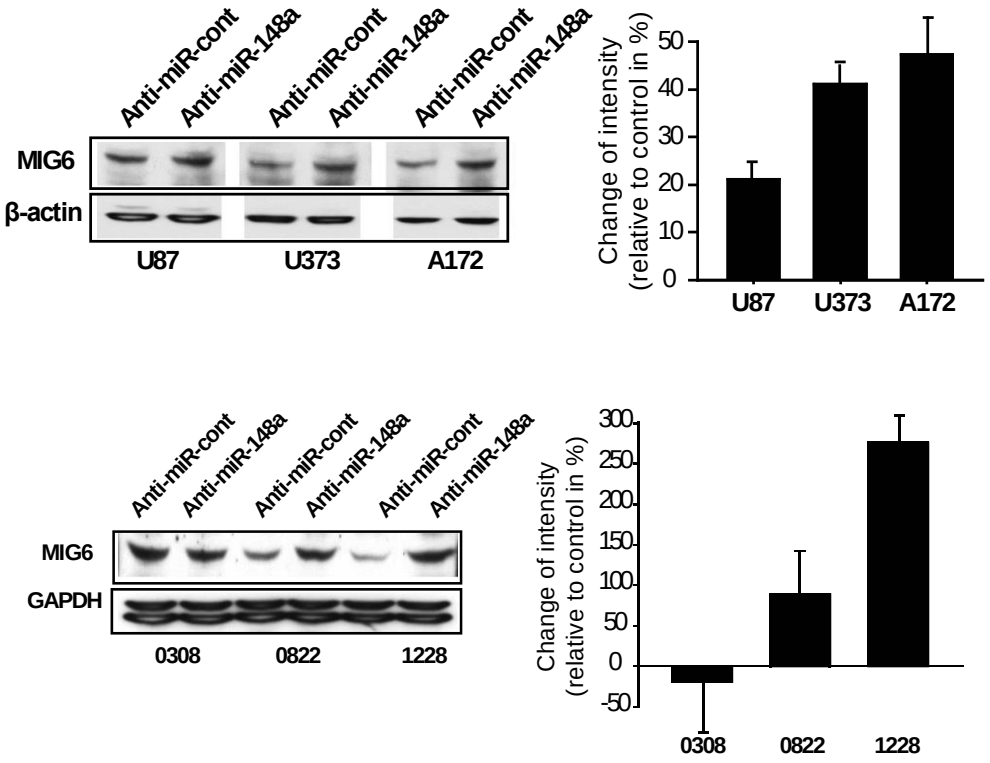


Figure 24: miR-148a inhibition increases MIG6 protein expression in GBM. Human GBM (U87, U373, and A172) cell lines and GSCs (0308, 0822, and 1228) were transfected with anti-miR-148a, or controls. 48 hrs later, the cells were assessed for MIG6 expression by immunoblotting. The data show that miR-148a inhibition promotes MIG6 protein expression. Immunoblots are from representative experiments, and bar graphs show quantification of the immunoblots in the right panel.

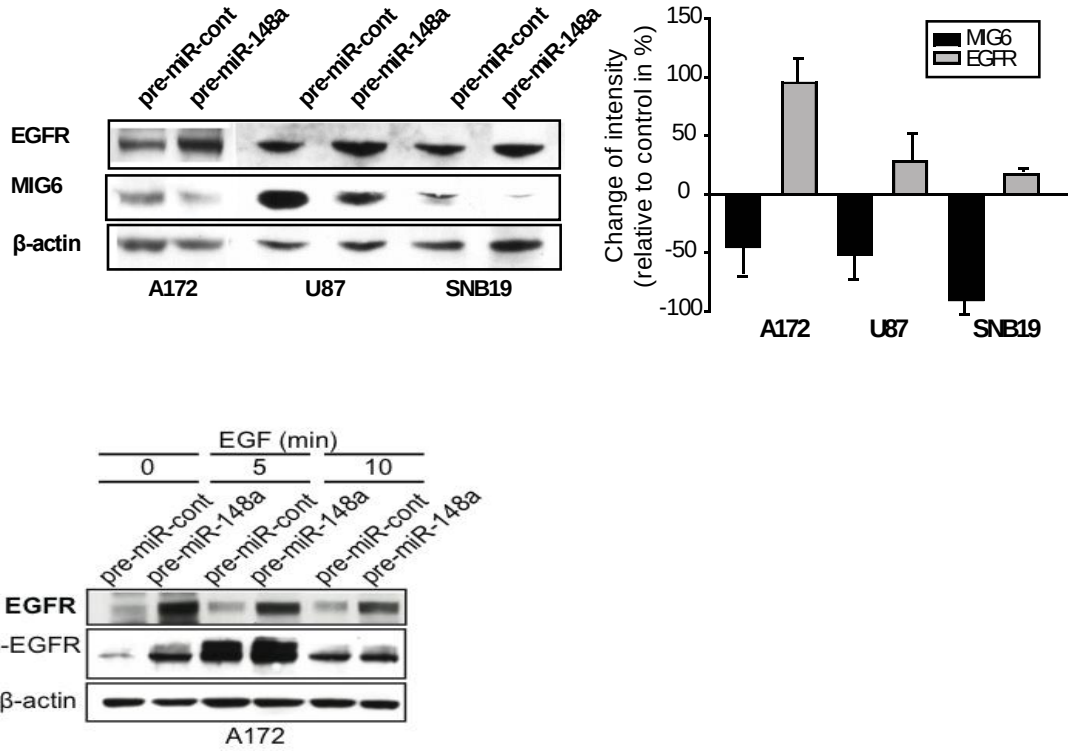


Figure 25: miR-148a inhibits MIG6 expression and enhances EGFR expression and activation. Human GBM (A172, U87, and SNB19) cell lines were transfected with pre-miR-148a or controls. 48 hrs later, the cells were assessed for MIG6 and EGFR expression/activation (Tyr 845) by immunoblotting. The data show that miR-148a overexpression inhibits MIG6 and enhances EGFR expression and activation. Immunoblots are from representative experiments, and bar graphs show the quantification of the immunoblots relative to loading control.

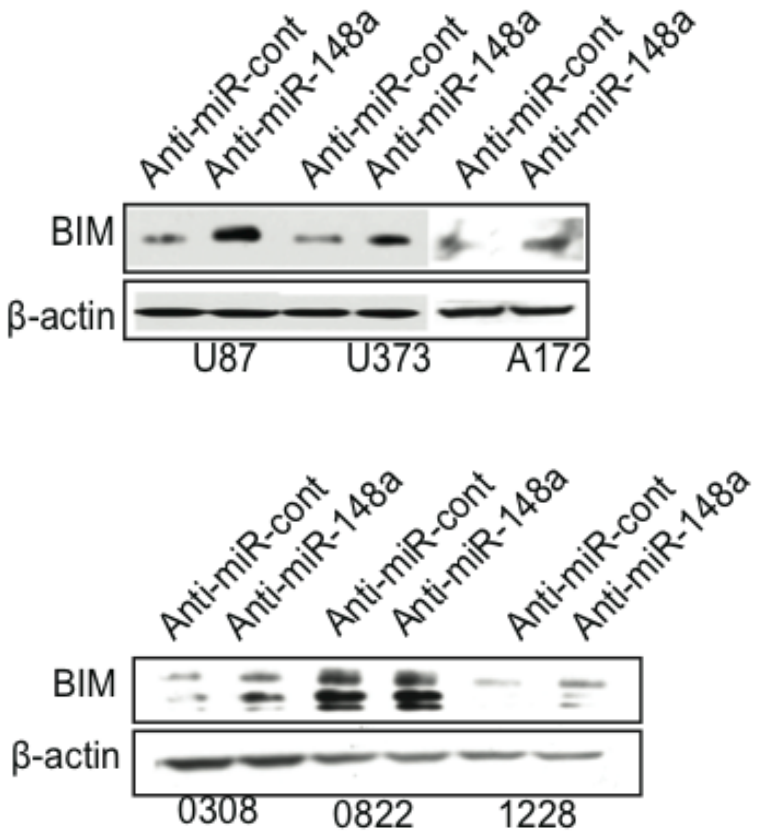


Figure 26: miR-148a inhibition promotes BIM expression in GBM. Human GBM (U87, U272, A172) cell lines and GSCs (0308, 0822, 1228) were transfected with anti-miR-148a or controls. 48 hrs later, the cells were assessed for BIM expression by immunoblotting. The data show that miR-148a inhibition increases BIM in GBM cells and GSCs.

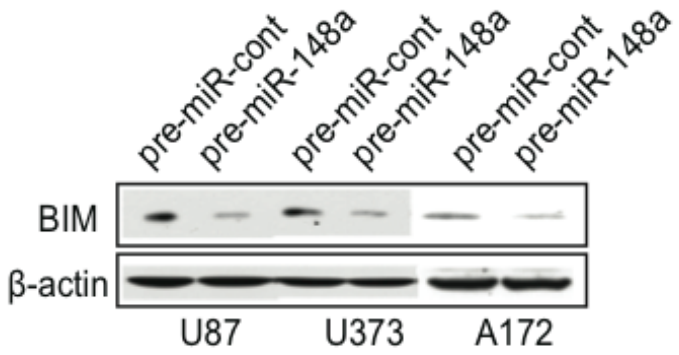


Figure 27: miR-148a inhibits BIM in GBM. Human GBM (U87, U272, A172) cell lines were transfected with pre-miR-148a or controls. 48 hrs later, the cells were assessed for BIM expression by immunoblotting. The data show that miR-148a overexpression inhibits BIM protein expression.

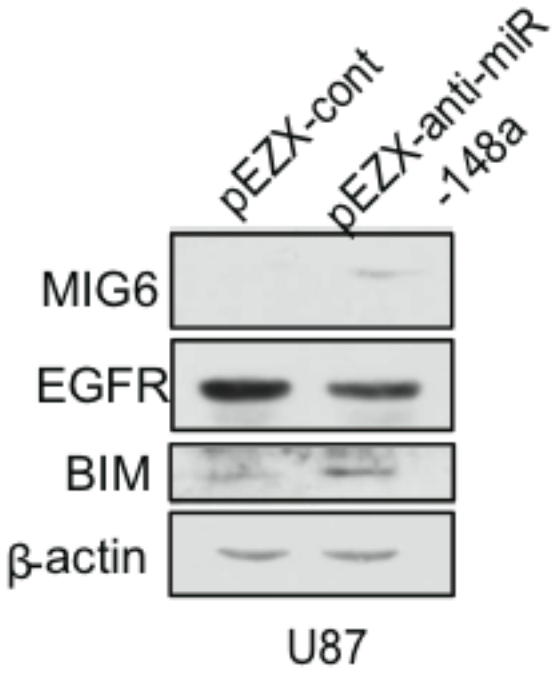


Figure 28: Stable expression of miR-148a inhibitor inhibits MIG6 and BIM and enhances EGFR expression. Immunoblots show regulation of MIG6, EGFR and BIM proteins in stable anti-miR-148a expressing U87 cells.

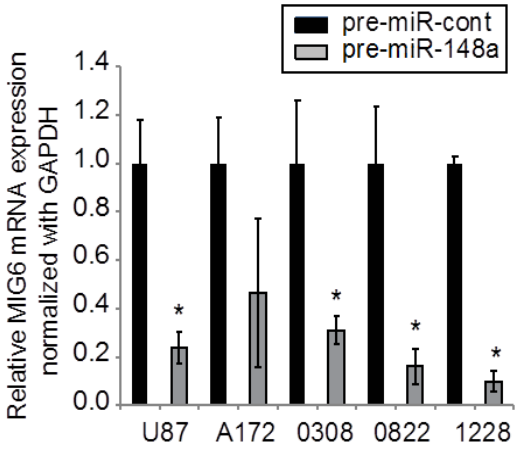


Figure 29: miR-148a inhibits MIG6 mRNA expression. MIG6 mRNA levels were measured by quantitative RT-PCR in GBM cells (U87 and A172) and GSCs (0308, 0822 and 1228) after pre-miR-148a transfection. The data show that miR-148a reduces BIM mRNA levels in all cells, suggesting that miR-148a induces MIG6 mRNA degradation. *, $p < 0.05$

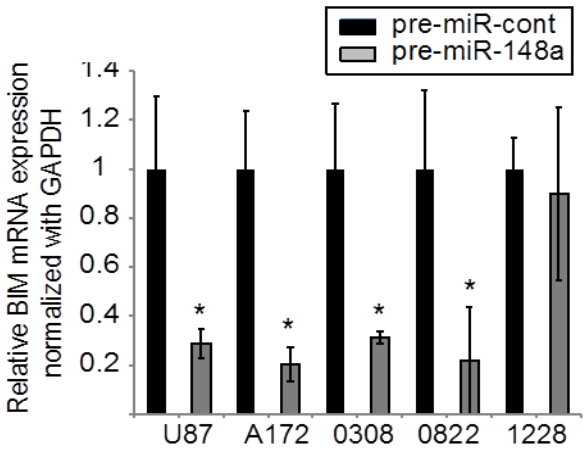


Figure 30: miR-148a inhibits BIM mRNA expression. BIM mRNA levels were measured by quantitative RT-PCR in GBM cells (U87 and A172) and GSCs (0308, 0822 and 1228) after miR-148a transfection. The data show that miR-148a reduces BIM mRNA levels in almost all cells suggesting that miR-148a induces BIM mRNA degradation. *, $p < 0.05$

ii. MiR-148a directly inhibits MIG6 and BIM expression by binding to their 3'UTRs

miR-148a could regulate MIG6 and BIM either directly by binding to their mRNA 3'UTR or indirectly by regulating genes that regulate them. To distinguish between these two possibilities and determine if MIG6 and BIM 3'-UTRs are direct targets of miR-148a, luciferase reporter assays were performed. To construct the reporter vectors, we inserted the MIG6 3'UTR or BIM 3'UTR downstream of a luciferase gene into a reporter vector and tested in a cotransfection assay with pre-miR-148a. Mutated 3'UTR sequences were used as controls. Seed matches and mutated binding sites of miR-148a in the 3'UTRs of MIG6 and BIM are shown in Figures 31 and 33. MIG6 or BIM 3'-UTR reporter constructs or 3'UTR mutant controls were transfected into GBM cells prior to transfection with miR-148a, and luciferase activity was measured. Overexpression of miR-148a significantly reduced luciferase activity by more than 35% for both MIG6 (Figure 32) and BIM (Figure 34). The above data show that miR-148a directly inhibits MIG6 and BIM by binding their 3'UTRs.

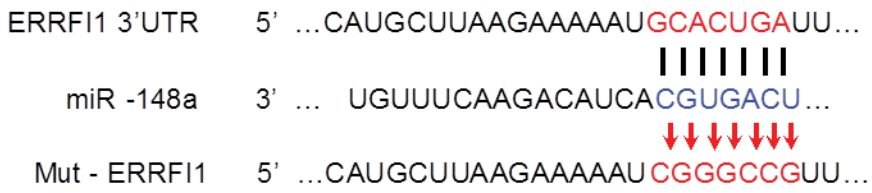


Figure 31: Seed matches and mutated binding sites of miR-148a in the 3'UTR of MIG6. MiR-148a seed sequence alignment with the MIG6 3'UTR. Black bars indicate the seed matches. Red arrows indicate mutation sites for the generation of the control 3'UTR reporter vectors.

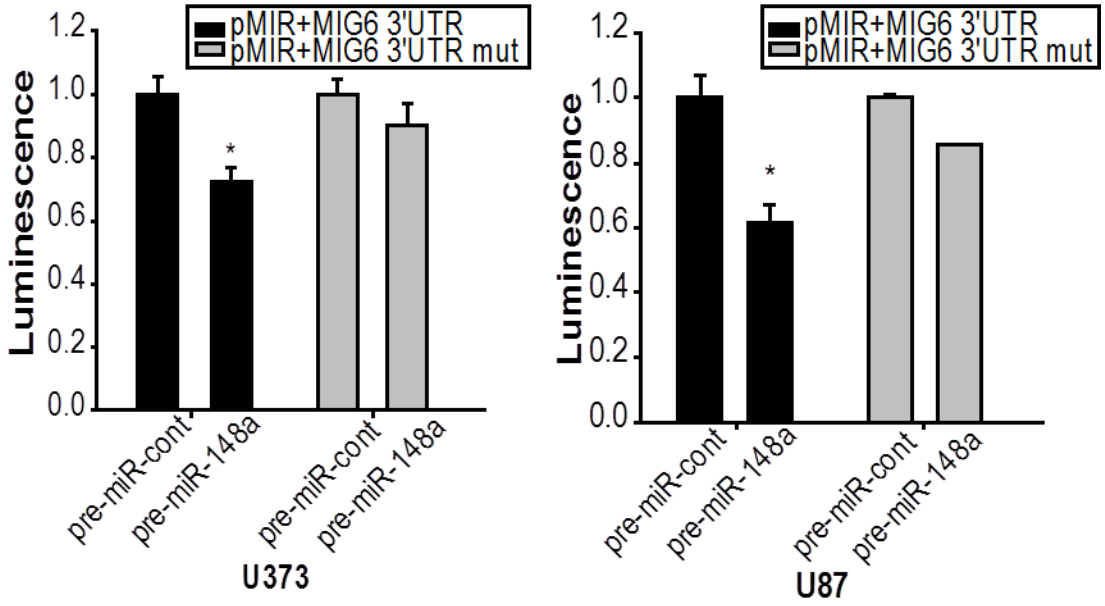


Figure 32: miR-148a directly targets and inhibits MIG6 by binding to its 3'UTR. 3'UTR luciferase assays for MIG6 showing the inhibition of luciferase activity by miR-148a in GBM (U373, U87) cells relative to mutant (mut) controls. *, $p < 0.05$

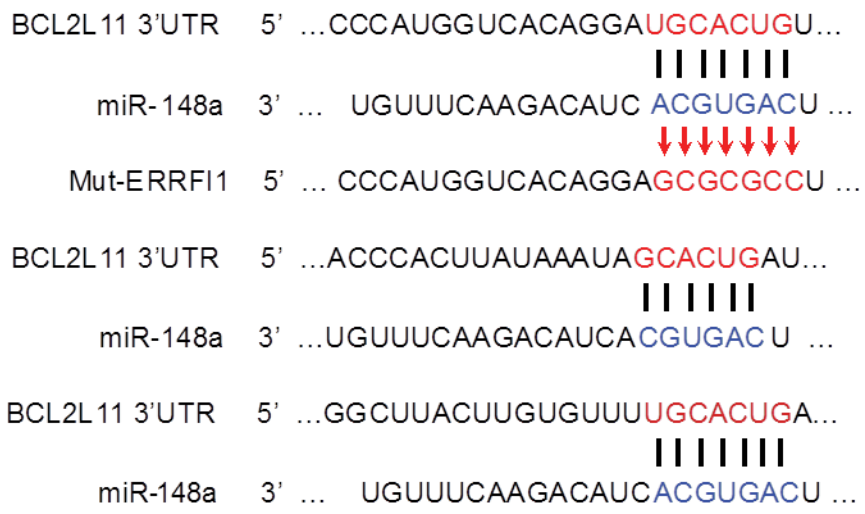


Figure 33: Seed matches and mutated binding sites of miR-148a in the 3'UTR of BIM. MiR-148a seed sequence alignment 8a with the BIM 3'UTRs. Black bars indicate the seed matches. Red arrows indicate mutation sites for the generation of the control 3'UTR reporter vectors.

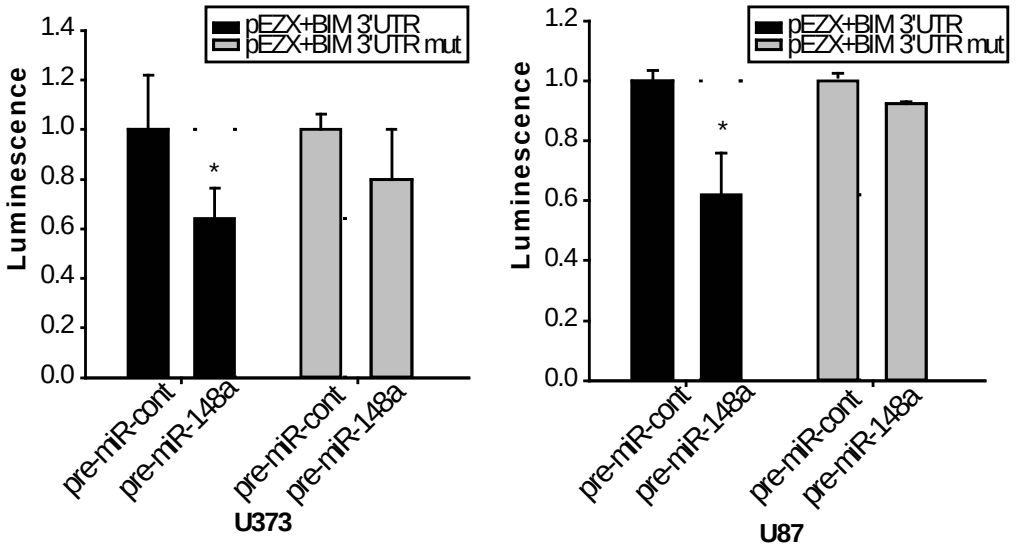


Figure 34: miR-148a directly targets and inhibits BIM by binding to its 3'UTR. 3'UTR luciferase assays for BIM showing the inhibition of luciferase activity by miR-148a in GBM (U373, U87) cells relative to mutant (mut) controls. *, $p < 0.05$

iii. MIG6 and BIM mediate the effects of miR-148a on GBM cell growth and survival

To determine if the oncogenic effects of miR-148a are mediated by MIG6 and BIM, MIG6 or BIM upregulation by anti-miR-148a was prevented using siRNAs prior to assessment of cell growth (MIG6) or apoptosis (BIM). GBM cells were transfected with MIG6, BIM or control siRNAs prior to transfection with anti-miR-148a followed by assessment of cell growth or apoptosis by cell counting and Annexin V-7AAD flow cytometry, respectively. Inhibition of miR-148a significantly inhibited GBM and GSC cell growth. MIG6 knockdown partially prevented the effects of miR-148a inhibition on cell growth (Figure 35). Similar to earlier results, inhibition of miR-148a increased GBM and GSC cell-line apoptosis; and BIM knockdown prevented the increased apoptosis induced by anti-miR148a expression (Figure 37). MIG6 and BIM knockdown with siRNA was confirmed by immunoblotting (Figure 35, 37). A similar rescue to the above was obtained in U87 cells stably expressing anti-miR-148a (Figure 36, 38). The above data show that the oncogenic effects of miR-148a are partially mediated by MIG6 and BIM.

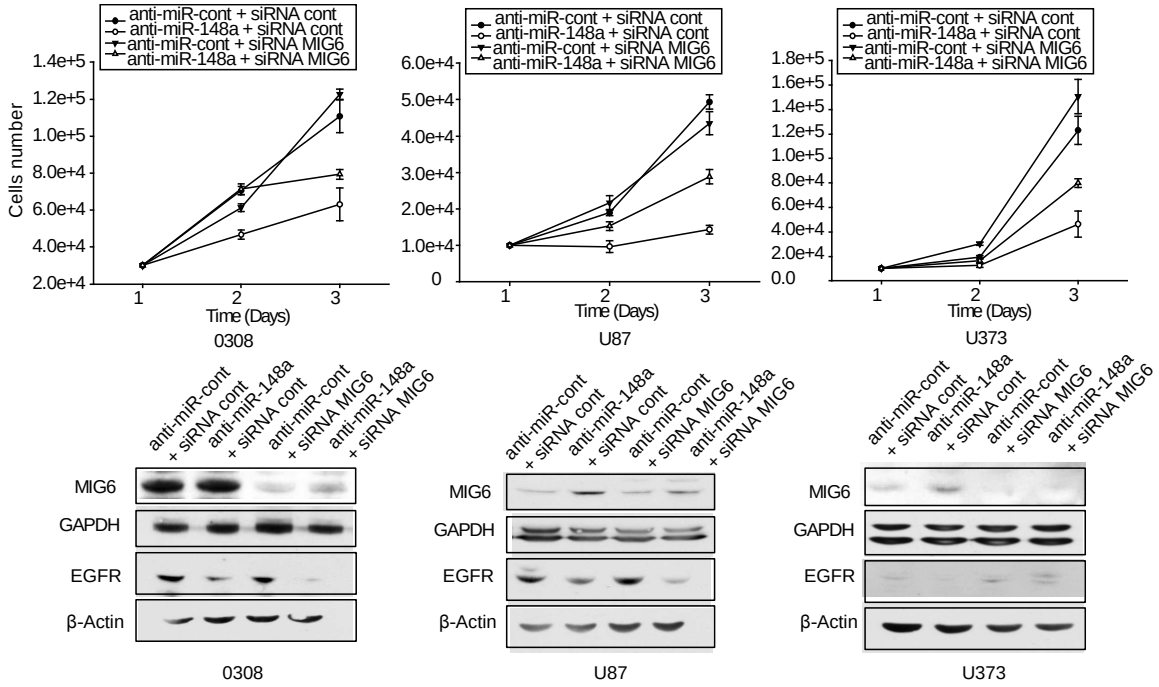


Figure 35: MIG6 mediates the effects of miR-148a on GBM cell growth. GBM (U87, U373) cells and GSCs (0308) were transfected with anti-miR-148a prior to transfection with either MIG6 siRNA or siRNA control. The upper panels depict growth assay showing that MIG6 inhibition partially rescues the proliferative effects of miR-148a inhibition. Immunoblots in the lower panels showing the rescue of anti-miR-148a-induced upregulation of MIG6 and downregulatiopn of EGFR by the corresponding siRNA.

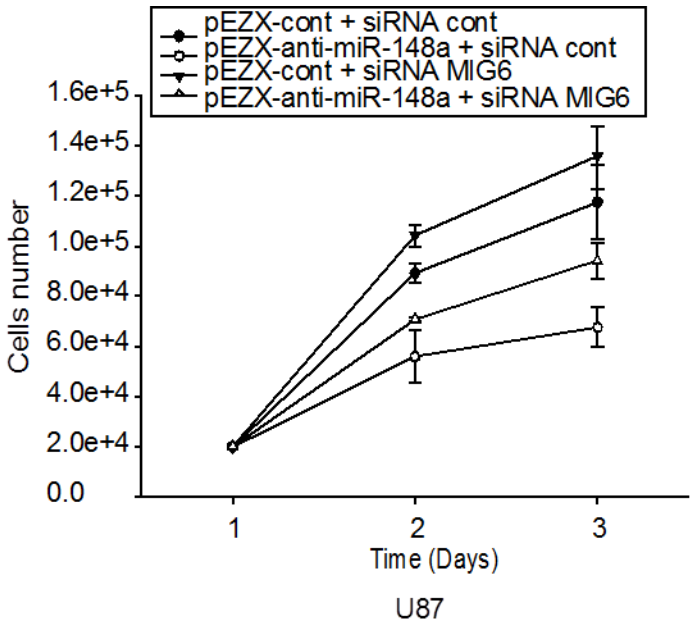


Figure 36: MIG6 partially mediates the effects of miR-148a on cell growth. Anti-miR-148a stably expressing U87 cells were transfected with siRNA against MIG6 (40 nM) for 72hrs prior to the measurement of cell growth by cell counting. The data show that MIG6 inhibition partially rescues the effects of anti-miR-148a on cell proliferation.

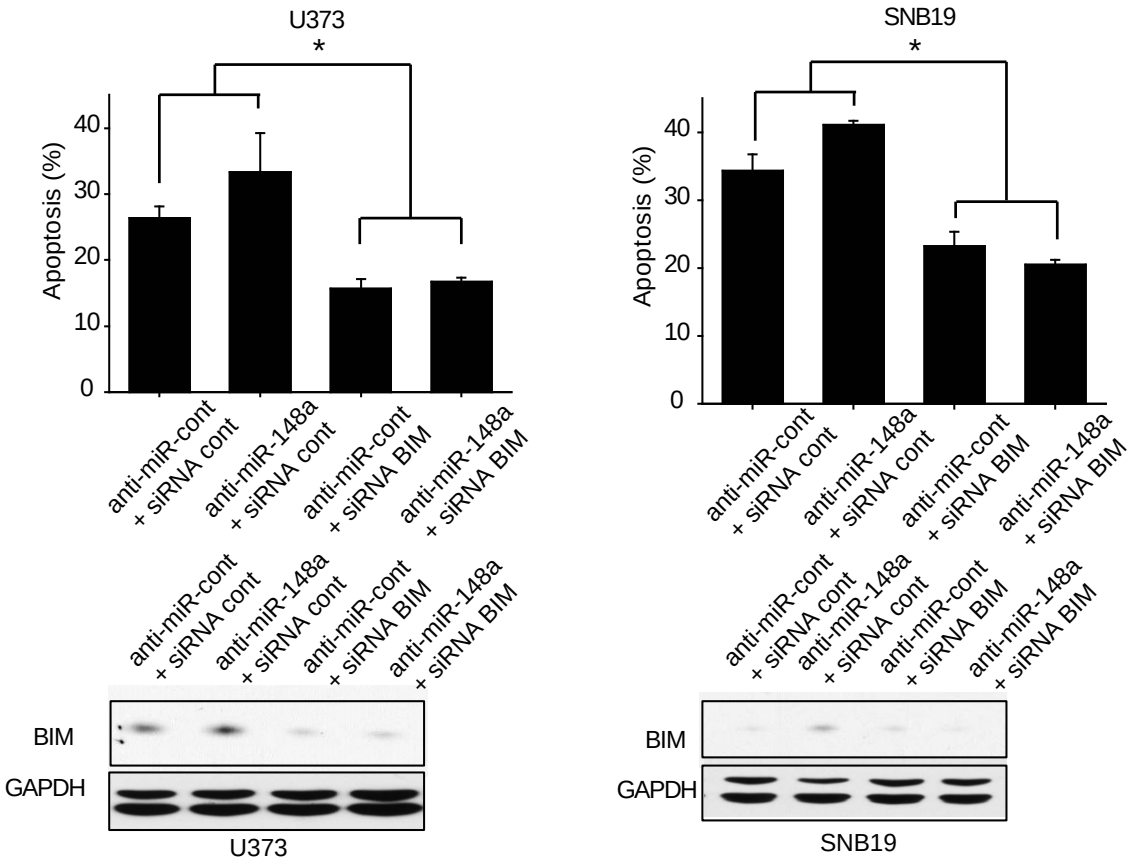


Figure 37: BIM mediates the effects of miR-148a on GBM cell survival. GBM (U373, SNB19) cells were transfected with anti-miR-148a prior to transfection with either MIG6 siRNA BIM siRNA or control. Apoptosis/cell death assays show that BIM inhibition partially rescues the apoptotic effects of miR-148a inhibition (upper panels). Immunoblots showing the rescue of anti-miR-148a-induced upregulation of BIM by the corresponding siRNA (lower panel). The relative change in apoptosis induced by microRNA-148a inhibition was compared to the relative change of apoptosis induced by microRNA-148 inhibition in BIM siRNA-treated cells and P values were calculated. *, $p < 0.05$

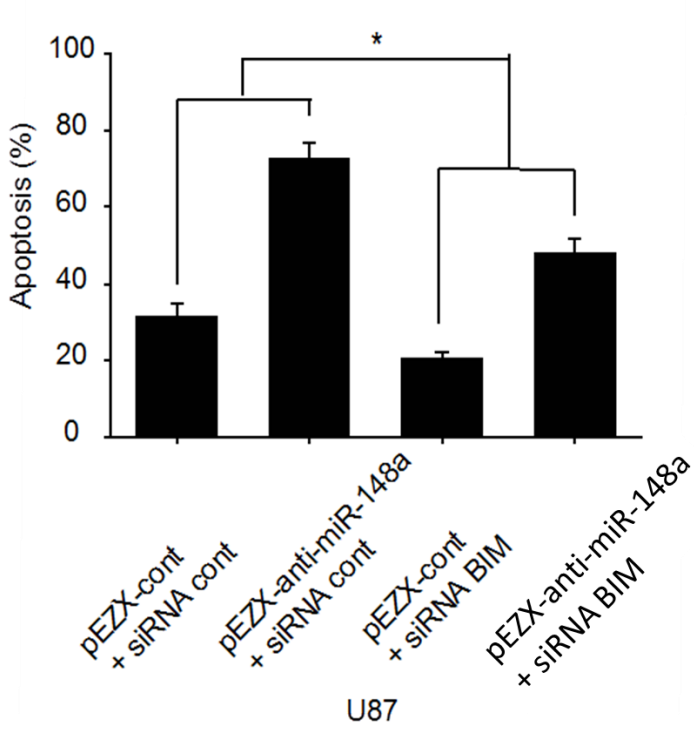


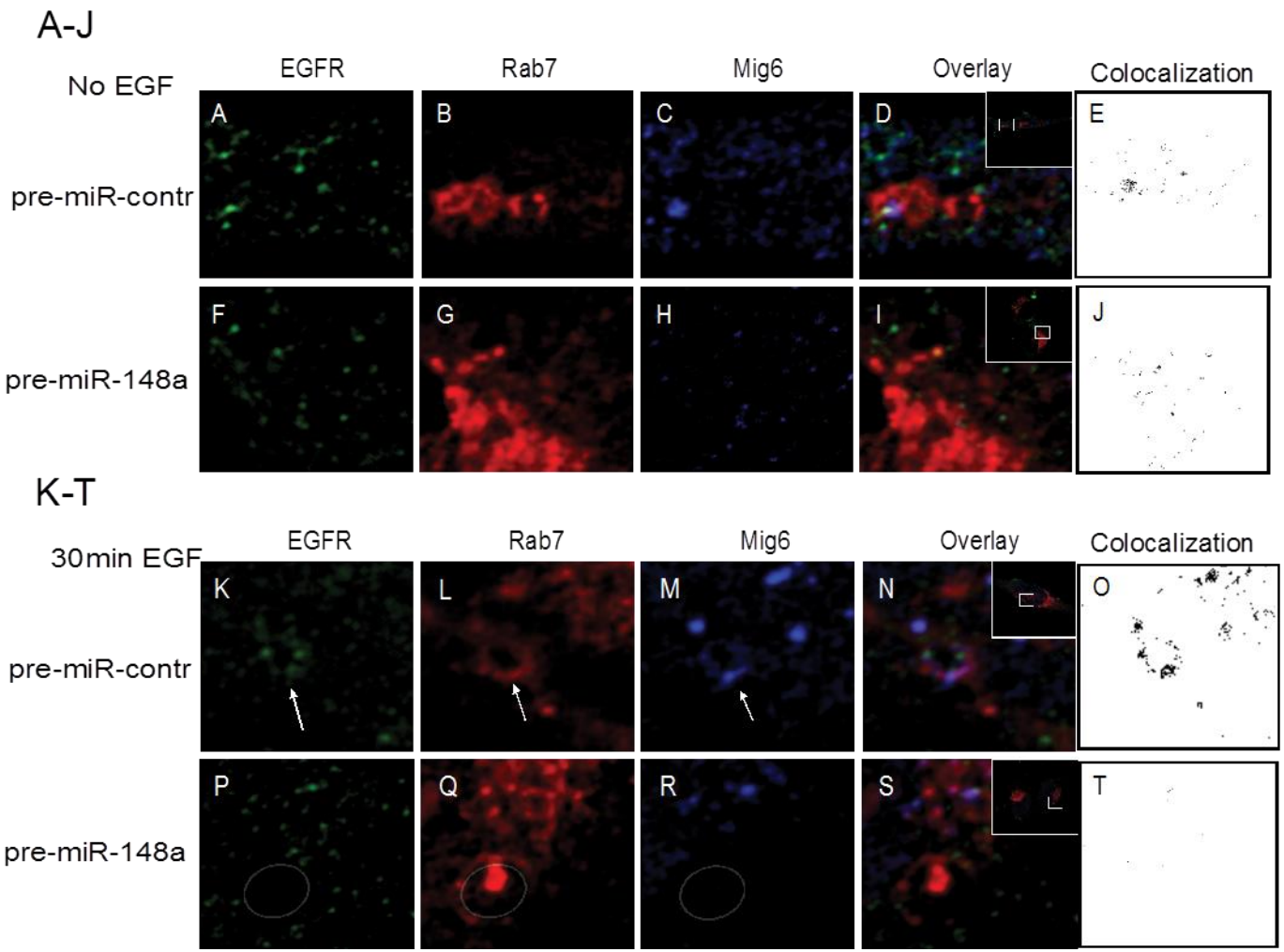
Figure 38: BIM partially mediates the effects of miR-148a on GBM cell death. Anti-miR-148a stable expressing U87 cells were transfected with siRNA against BIM (40 nM) for 5 days prior to the measurement of apoptosis and cell death by Annexin V/7 AAD flow cytometry. The data show that BIM inhibition partially rescues the effects of anti-miR-148a on cell death and apoptosis. The relative change in apoptosis induced by microRNA-148a inhibition was compared to the relative change of apoptosis induced by microRNA-148 inhibition in BIM siRNA-treated cells and P values were calculated. *, $p < 0.05$

iv. MiR-148a inhibits EGFR trafficking and degradation

Previous research has shown that MIG6 regulates EGFR trafficking into the late endosome/lysosomes to promote EGFR degradation [85]. We used confocal microscopy to determine whether miR-148a affects EGFR trafficking into the Rab7-positive late endosome/lysosomal compartment in GBM cells. Rab7 has been shown to localize to late endosomes and to be important in the maintenance of the late endosomal compartment. Rab7 also controls the fusion of late endosome with lysosomes where EGFR degradation occurs [221]. First, GBM cells were transfected with miR-148a or control before transfection with fluorescently labeled Rab7. Confocal microscopy analysis revealed reduced levels of MIG6 protein in miR-148a over-expressing cells as compared to control (data not shown). In control cells, MIG6 and EGFR colocalized in relatively large Rab7-labeled structures, likely multivesicular bodies (MVB)/late endosomes (Figure 39A-E, K-O). This colocalization occurred at all-time points, but was particularly evident 30 min after EGF stimulation in control cells (arrows, Figure 39K-O). Importantly, in miR-148a transfected cells colocalization between EGFR, MIG6, and Rab7 was rarely seen and never found in the large Rab7-labelled structures (MVBs) (grey circle, Figure 39P-T). Co-localization is also shown in black and white for a clearer alternative image (Figure 39E, J, O, and T). Quantification of the percentage of EGFR that co-localized with Rab7 and Mig6 showed a significant reduction in colocalization in miR-148a over-expressing cells compared with control cells (Figure 39U). These data demonstrate that miR-148a reduces EGFR trafficking and degradation in GBM cells.

Altogether, the data from this chapter uncover the mechanism of action of miR-148a in GBM. They show that the oncogenic effects of miR-148a are mediated by the direct downregulation of MIG6 and BIM and indirect upregulation and activation of

EGFR, an important driver of GBM malignancy.


A-J
No EGF
EGFR
Rab7
Mig6
Overlay
Colocalization
A
B
C
D
E
pre-miR-contr
F
G
H
I
J
pre-miR-148a
K-T
30min EGF
EGFR
Rab7
Mig6
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Colocalization
K
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O
pre-miR-contr
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pre-miR-148a

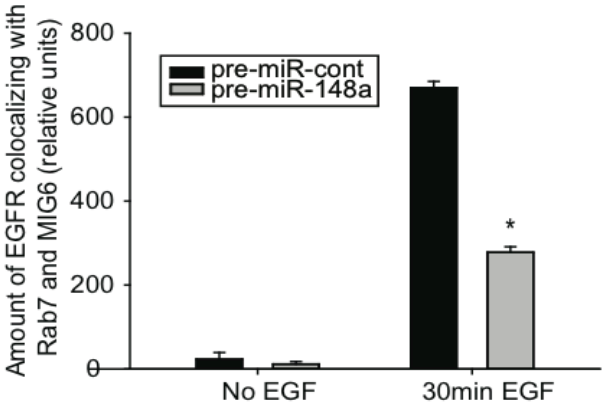

Figure 39: miR-148a inhibits EGFR trafficking and degradation. GBM (U87) cell lines were transfected with control (A-E and K-O) or pre-miR-148a (F-J and P-T) for 24 h and then transfected with Rab7-mCherry (red; B, G, L and Q) for 24 h. The cells were serum-starved for the last hour before being treated with EGF (50 ng/ml) for the indicated times (0 min; A-J, 30 min; K-T). Cells were fixed and stained with anti-EGFR (green; A, F, K and P) and anti-MIG6 (blue; C, H, M and R). Arrows point to the EGFR and MIG6-containing Rab7 compartment in control-transfected and EGF-treated cells (K, L and M). Note the increased amount of EGFR colocalizing with Rab7 and Mig6 in control cells (N and O) as compared with miR-148a overexpressing cells (S and T). In miR-148a-expressing cells, light gray circles point to Rab7 compartment structures, but colocalization between EGFR, MIG6 and Rab7 is rarely seen and not in large Rab7-labeled structures (MVBs) (P, Q and R). Colocalization of MIG6, EGFR and Rab7 are shown in black and white in E, J, O and T). Colocalization of EGFR with MIG6 and Rab7-labeled structures was quantified on the confocal images. The results are the means ± SEM of > 30 cells scored from 2 separate experiments. *, $p < 0.05$

III. DISCUSSION

MicroRNAs are deregulated in cancer where they can act as oncogenes or tumor suppressors by targeting and inhibiting tumor suppressors or oncogenes, respectively. We show for the first time that miR-148a acts as an oncomiR in GBM by directly targeting and inhibiting MIG6 and BIM. We demonstrate that MIG6 inhibition by miR-148a induces the inhibition of EGFR trafficking and degradation leading to EGFR overexpression and activation. As EGFR is an important oncogene in GBM, several oncogenic effects of miR-148a are likely mediated by EGFR activation. Furthermore, miR-148a inhibits BIM, an important pro-apoptotic molecule, to reduce apoptosis and promote cell survival.

In previous research, large scale cancer genome sequencing projects and many molecular genetics and histopathological studies have shown EGFR and its downstream signaling networks as one of the most deregulated components of human GBM and other cancers [42, 222]. The EGFR gene is either amplified and/or mutated in a total of about 40% of GBM patient samples [222]. However, published research as well as TCGA have shown that the EGFR gene is overexpressed at the mRNA and protein levels in up to 97% of human GBM. Therefore, amplification only partly accounts for EGFR overexpression, suggesting the existence of an alternative way to induce EGFR overexpression. There are multiple possible mechanisms that can result in activation of EGFR pathways without genetic alteration during initiation and development of GBM. One important mechanism involves the inhibition of EGFR trafficking and degradation that is regulated by MIG6, a downregulated tumor suppressor in GBM. Besides MIG6 regulation of EGFR trafficking, another well known mechanism of EGFR trafficking regulation is through the Cbl cytosolic protein [225]. Here, we suggest miR-148a upregulation in GBM as an important contributor to EGFR overexpression and activation. In fact, we show that miR-148a directly

targets and inhibits MIG6, leading to inhibition of EGFR trafficking and endosomal degradation and subsequent EGFR overexpression and activation. In support of our hypothesis, recent research showed that MIG6 promotes EGFR turnover through its regulation of the vesicle trafficking pathways through complex formation with STX8, a SNARE family protein mediating the fusion of late endosomes [85]. We first showed that miR-148a reduces MIG6 protein and induces EGFR expression activation. Our data from the 3'UTR reporter experiments demonstrated that microRNA-148a targets and inhibits MIG6 mRNA directly. We then used immunofluorescence analysis to show that miR-148a-inhibited MIG6 regulated EGFR trafficking to the late endosome. Our findings also have potential therapeutic implications. A phase III clinical trial for GBM treatment using monoclonal antibodies (mAbs) against EGFR is currently underway [226]. It is noticeable that one potential mechanism of action of EGFR mAbs is its ability to target EGFR for degradation in the lysosome. MIG6 loss as seen in GBM might counteract this degradation and lead to therapy resistance. Therefore, MIG6 upregulation via miR-148a inhibition might sensitize GBM to anti-EGFR mAbs, making miR-148a a promising therapeutic target in GBM.

We also identified the important pro-apoptotic molecule BIM as a target of miR-148a. BIM expression is downregulated in 29% of GBM cases based on TCGA analysis. Interestingly, a recent study demonstrated that elevated BIM expression levels in cancers strongly increased the anti-tumor activity of EGFR and other RTK inhibitors [152]. Independent studies also have shown possible mechanism to overcome resistance to TKI with alteration of BIM expression [227]. It was shown that the induction of the proapoptotic protein Bim is essential for apoptosis triggered by EGFR TKI treatment [127, 228]. Moreover, a polymorphism in BIM that generates a dysfunctional form of the protein led to intrinsic EGFR TKI resistance in EGFR mutant NSCLC cell lines [229]. Further evidence for the implication of BIM in overcoming resistance to TKIs is

that BIM is induced by lung cancer cell lines that are sensitive to erlotinib but not by those that are resistant [128]. Based on the above, it was hypothesized that resistance to EGFR inhibition can be modulated by alterations in the intrinsic apoptotic pathway controlled by the BCL-2 family of proteins. These findings strongly suggest that combined upregulation of BIM and inhibition of EGFR is likely to achieve synergistic anti-tumor effects. Our study shows that such combined targeting of BIM and EGFR can be achieved by inhibition of miR-148a, providing a rationale for the therapeutic targeting of this microRNA.

The role of microRNA-148a on GBM was not explored before. Preliminary analysis of a subset of TCGA data suggested an oncogenic role for microRNA-148a in GBM, which is the opposite of what was previously shown in other cancers. Previous research described miR-148a as a tumor suppressor in hepatocellular carcinoma, pancreatic cancer, gastric cancer and colorectal cancer. It was shown that microRNA-148a suppresses tumor cell invasion and metastasis by downregulating ROCK1 in gastric cancer [181], promotes apoptosis by targeting Bcl-2 in colorectal cancer [182], and suppresses the epithelial-mesenchymal transition and metastasis of hepatoma cells by targeting Met/Snail signaling [179]. However, we were not able to verify the above as targets of miR-148a in GBM. Our comprehensive study describes miR-148a as a prognostic oncogenic microRNA in GBM and uncovers its mechanisms of action.

We analyzed ~ 500 patient data from TCGA and found that miR-148a expression predicts patient survival. High miR-148a expression correlated with poor prognosis while low expression correlated with better prognosis. This correlation suggested an important oncogenic role for miR-148a in GBM. Additionally, the patient derived GBM tissues also expressed higher endogenous miR-148a as compared to the normal human brain. The high endogenous microRNA-148a

expression was confirmed with qPCR assay in our own independent set of tumors and in GBM cells and GBM stem cells. We then studied the functional effects of miR-148a by determining the effects of its inhibition and overexpression on cell proliferation, survival, migration, invasion and self-renewal ability of tumor stem cells. The data from these complementary approaches were consistent and convincingly showed that miR-148a exerted oncogenic effects by promoting tumor cell and stem cell proliferation, survival, migration, invasion and stem cell self renewal. miR-148a inhibition had the opposite effects on the above endpoints. In vivo experiments further confirmed the oncogenic effects of miR-148a. Importantly, inhibition of miR-148 inhibited stem cell self renewal and the initiation of tumors in vivo. In light of the hypothesized importance of tumor stem cells in tumorigenesis and therapy resistance, this further suggests that miR-148a is a promising target for GBM therapy. Further, we uncovered the targets and oncogenic mechanisms of action of miR-148a. We showed that miR-148a directly targets and inhibits MIG6 and BIM and indirectly upregulates the expression and activation of EGFR via inhibition of its trafficking and degradation. Thereby, we uncovered a new mechanism of EGFR overactivation in GBM. Our study also provides new mechanistic explanations for the non-genetic downregulation of the tumor suppressors MIG6 and BIM in GBM.

In summary, the present study shows that miR-148a is elevated in GBM, where it predicts poor patient survival. It demonstrates that miR-148a has powerful oncogenic and cancer stem cell regulatory effects that are mediated by BIM, MIG6 and EGFR. The study therefore represents a first characterization of miR-148a as a prognostic oncogene and promising therapeutic target in GBM.

Charter 5: Overall Discussion and Future Directions

I. Discussion

The research described in this dissertation has prognostic, mechanistic and potential therapeutic implications. We studied microRNAs because they are frequently deregulated in many cancers and because single microRNAs can regulate several genes and therefore exert considerable tumor regulatory effects. To find potentially important microRNAs, we speculated that correlation with patient survival would strongly suggest that microRNA is an important regulator of malignancy. We therefore started the work by searching for microRNAs that correlate with patient survival in a subset of TCGA data. The purpose was to identify differentially expressed prognostic microRNAs that act as either oncogenes or tumor suppressors and to then investigate their targets and mechanisms of action and evaluate their usefulness as therapeutic agents or targets. The preliminary search showed that microRNA-148a had one of the best inverse correlations with patient survival, suggesting that it might be oncogenic in GBM (Figure 1). Searching the literature, we noticed that it was described as a tumor suppressor in some cancers and that nothing was known about it in GBM. I therefore chose miR-148a as the focus of my thesis research and conducted a comprehensive study of its role in GBM.

First, I investigated the expression of miR-148a in GBM cells, stem cells and human tumors. I found that miR-148a was upregulated in GBM cells as compared to normal astrocytes and in GBM tumors as compared to normal brain. Then, I analyzed the TCGA data comprehensively for miR-148a correlation with patient survival and overall expression. I analyzed ~ 500 patient data from TCGA and found that miR-148a expression predicts patient survival. High miR-148a expression correlated with poor prognosis while low expression correlated with better prognosis.

Several microRNAs have been previously shown to be deregulated in GBM and the expression of very few correlated with patient survival. For example, the oncogenic miR-21 levels are elevated in human GBM as compared to normal glial cells and/or brain [230, 231]. Also, miR-21 levels in glioma correlated to tumor grade and low miR-21 levels in human tumors are associated with slightly better survival according to the cancer genome atlas (TCGA) [178, 232]. In addition, a recent study identified miR-182 as a prognostic marker for glioma progression and patient survival [233]. This research showed upregulated miR-182 in glioma cell lines and primary glioma specimens as compared to normal brain. miR-182 expression levels in the tumors statistically significantly correlated with tumor grade and clinical features. The 5-year survival rates of patients with low miR-182 levels were significantly better than the survival rate of patients with high miR-182 levels. The study therefore suggests that miR-182 could serve as a marker of glioma progression and predictor of patient survival [233]. However, the expression of miR-148a and its potential correlation with survival in GBM had not been investigated before. My data demonstrate for the first time the upregulation of miR-148a and its inverse correlation with patient survival, and suggest that miR-148a is an important new oncogene in GBM.

MicroRNAs are expressed in specific spatiotemporal patterns, and dysregulation or mutation of miRNA genes causes or contributes to several human diseases including cancer, mainly due to repression or loss of mRNA targets. Deregulation of miRNAs and aberrant expression of miRNAs can arise through a number of different mechanisms, such as genomic abnormalities, epigenetic factors, transcriptional regulation, and regulation of microRNA processing [60]. For instance, genetic deletion, amplification or translocation of miRNAs result in miRNA overexpression or underexpression [59]. The chromosomal deletions or mutations at the 13p13.4 locus in chronic lymphocytic leukemia (CLL) patients result in underexpressed miR-15a and miR-16-1 [67, 68]. Another example of microRNA deregulation mechanisms is shown in

glioblastoma stem cells (GSCs). Recent research showed that a hypermethylated CpG island of miR-137 reduced expression of miR-137 in glial tumors and GBM stem cells (GSCs) to induce malignancy, self-renewal and differentiation of stem cells [73]. The mode of upregulation of miR-148a in GBM is not known. There are several potential mechanisms that could theoretically explain the upregulation of miR-148a such as amplification of its chromosome locus or regulation via transcription factors. In fact, microRNA-148a is located in chromosome 7 that is one of the most frequently genetically amplified in GBM. Further studies are required to determine if the miR-148a gene is amplified in GBM. Also, transcriptional upregulation of miR-148a expression needs to be explored. This could be achieved by first screening for potential transcription factor consensus sequences in the miR-148a promoter, followed by molecular and functional studies to confirm the involvement of a specific transcription factor in inducing miR-148a expression in GBM. Irrespective of the above, our findings uncover miR-148a as one of a very few prognostic miRNAs in GBM, highlighting its potential importance in regulating GBM malignancy.

Having demonstrated the upregulation and prognostic significance of miR-148a in GBM, I then studied the functional effects of miR-148a by determining the effects of its inhibition and overexpression on cell proliferation, survival, migration, invasion and self-renewal ability of tumor stem cells. The data show that miR-148a exerted oncogenic effects by promoting tumor cell and stem cell proliferation, survival, migration, invasion and stem cell self renewal. miR-148a inhibition had the opposite effects on the above endpoints. In vivo experiments further confirmed the oncogenic effects of miR-148a. Importantly, inhibition of miR-148 inhibited stem cell self renewal and the initiation of tumors in vivo. Figure 8 and 9 show the efficiency of precursor microRNA transfection. It shows over 10^5 fold change in microRNA expression after precursor transfection. This might not reproduce physiological levels of microRNA-148a. However, to avoid this potential caveat and further verify the effects and mechanisms of action

of miR-148a in GBM we also used complementary inhibition of miR-148a via antisense transfection. Antisense oligonucleotides for microRNAs are steric blocking antisense reagents that inhibit microRNA (miRNA) function by hybridizing and repressing the activity of a mature miRNA. The efficiency of antisense transfection cannot be measured by pRT-PCR because microRNAs are not degraded by their antisense. For this reason, we indirectly measured anti-miR efficiency by analyzing changes to the miRNA target levels and also used the word inhibition instead of knock down of microRNA to refer to the downregulated activity of miR-148a. The data obtained from these latter experiments were consistent with the overexpression data.

Standard treatment for glioblastoma patients includes surgery followed by a total of 60 Gy focal fractionated radiotherapy with concomitant and adjuvant administration of the alkylating chemotherapy agent, temozolomide [234]. The addition of temozolomide significantly improves the median, 2- and 5-year survival compared to radiotherapy alone in patients with newly diagnosed glioblastoma. Nevertheless, glioblastoma patients have a poor prognosis with a median survival of only 14.6 months. Besides, it has long been observed that after surgical removal, tumors frequently recur within 1 cm of the resection cavity. This is mainly due to the fact that at the time of surgery, cells from the bulk tumor have already invaded normal brain tissue [235]. Furthermore, the Food and Drug Administration (FDA) approved anti-angiogenic drugs, such as bevacizumab, which targets VEGF-A, that have been shown to increase glioma invasiveness via MET regulation [236]. Evasion of apoptosis is also a hallmark of GBM, because defects in its regulators invariably accompany tumourigenesis and sustain malignant progression [44]. Growth factor pathways that stimulate cell proliferation are constitutively activated in malignant GBM through overexpression or genetic amplification of growth factor receptor genes such as EGFR, ERBB2, PDGFRA, MET [18]. Lastly, tumor initiating cells, GSCs are also an important reason for radiotherapy resistance. It has been demonstrated that diverse mechanisms of radio- and

chemo- therapeutic resistance of GSC populations exist, including increased expression of drug efflux transporters, a more robust DNA damage response, reduced sensitivity to apoptotic signals, and increased expression of growth factors [237]. In vitro and in vivo xenograft studies have demonstrated that CD133+ GSC are more resistant to ionizing radiation than matched CD133- tumor cells [39]. Remarkably, the increased phosphorylation and activation of Chk1 and Chk2 in CD133+ cells enable an enhanced capacity of these cells to sense and repair DNA damage [39]. All of the above demonstrates the importance of tumor cell proliferation, survival, migration and invasion as well as tumor stem cell pathology in promoting GBM tumor growth and mediating resistance to therapy. By showing that miR-148a strongly regulates all of the above factors; my findings demonstrate a powerful oncogenic role for miR-148a in GBM. Together with the expression and prognostic studies, the findings identify miR-148a as a new oncogene in GBM.

I then moved on to study the oncogenic mechanisms of action of miR-148a. I started by looking for potential targets of miR-148a using computer prediction analysis with PICTAR and TARGET SCAN. I found more than 20 candidates tumor suppressor genes as potential targets but was able to experimentally verify two of them, MIG6 and BIM. Because MIG6 is a known regulator of EGFR and because EGFR is an important oncogene in GBM, I also investigated the effects of miR-148a on EGFR. I showed that miR-148a directly targets and inhibits MIG6 and BIM and indirectly upregulates the expression and activation of EGFR via inhibition of receptor trafficking and degradation in the endosome. Using rescue experiments, I demonstrated that MIG6 and EGFR contribute to the oncogenic effects of miR-148a in GBM. Therefore, these data supported my hypothesis of miR-148a being a new prognostic marker and oncogene in GBM that acts via regulation of MIG6 and BIM.

I also uncovered a new mechanism of EGFR overactivation in GBM. EGFR is one of the most studied receptor tyrosine kinases that regulates cell proliferation, angiogenesis, migration, and invasion in GBM [24]. The EGFR locus on chromosome 7 is amplified and mutated is about 40% of human GBM [238]. However EGFR is overexpressed in a much higher fraction of human GBM [42, 223, 224]. Therefore, amplification only partly accounts for EGFR overexpression. A possible mechanism for EGFR overexpression in GBM is the inhibition of EGFR degradation via downregulation of its negative regulator, MIG6. MIG6 regulates both EGFR expression and activation. MIG6 allosterically inhibits EGFR tyrosine kinase activity and induces EGFR endocytosis, eventually leading to EGFR degradation [85, 101, 108, 239]. Although 50% of GBM display MIG6 downregulation, only 13.2 % of GBM tumors show MIG6 gene deletion on its chromosome 1p32 locus [85]. Similar to EGFR, this suggests an alternative mechanism for MIG6 downregulation in GBM regardless of gene deletion. My findings that miR-148a directly regulates MIG6 and indirect by regulates EGFR provide a plausible new mechanism for MIG6 downregulation and EGFR upregulation and overactivation that is not due to genetic alterations of the EGFR gene.

The other direct target of miR-148a that I discovered is BIM, a critical proapoptotic molecule. 28% of GBM show BIM downregulation, but the mechanism of underexpression has not been shown. Also, recent research shows that pretreatment RNA levels of BIM strongly predicted the capacity of an EGFR inhibitor to induce apoptosis in many cancer types [152]. My findings provide a new potential mechanism for BIM downregulation in GBM via upregulation of miR-148a. Because of the prognostic/therapeutic link between BIM and EGFR that others have shown before, the findings of my thesis suggest that targeting miR-148a for therapy might be doubly beneficial, as it will affect both molecules simultaneously.

The findings of my thesis also suggest that miR-148a is a potential new target for GBM therapy. In fact, I show that inhibition of miR-148a leads to the inhibition of GBM cell and stem cell derived tumorigenesis and tumor growth in vivo. MiRNAs hold great promise as therapeutic agents and targets in human disease and cancer. The rationale for developing miRNA therapeutics is based on the premise that aberrantly expressed miRNAs play key roles in human disease, and that correcting these deficiencies by either antagonizing or restoring miRNAs provides a therapeutic benefit. Synthetic miRNA mimics used to overexpress miRNAs include siRNA-like oligoribonucleotide duplexes or chemically modified oligoribonucleotides [58]. miRNAs can be inhibited by variously modified antisense oligonucleotides such as 2′-O-methyl antisense oligonucleotides [240]. miRNA therapeutic use has several potential advantages. First, single miRNAs can target multiple mRNAs that act as functional units. Manipulating such miRNAs would therefore achieve greater and more coordinated effects than targeting single molecules. This is evidenced by my findings of the simultaneous regulation of BIM and EGFR by miR-148a. Second, inhibition of oncogenic miRNAs can lead to upregulation of tumor suppressors as shown in my work demonstrating the upregulation of MIG6 and BIM after miR-148a inhibition. Such an effect cannot be achieved with siRNA or pharmacological agents. Third, miRNAs are naturally occurring small molecules that are more readily delivered to cells than larger genes. Fourth, miRNA inhibitory molecules (anti-miRs) are effective and easy to synthesize, rendering both miRNA replacement and miRNA inhibition feasible approaches. Lastly, there is evidence that miRNAs are secreted in microvesicles to reach and transfect adjacent and distant cells, thereby amplifying their own effects [241].

The major hurdle for microRNA based therapies for GBM and other CNS diseases is delivery of miRNAs or anti-miRNAs into the brain. The blood-brain barrier (BBB) prevents the transport of most systemically-delivered molecules into the brain. Research suggests the usefulness of local

agent delivery via convection-enhanced delivery (CED) [242, 243] or systemic delivery via focused ultrasound (FUS) with microbubbles (MB) [244, 245]. miRNAs and anti-miRNAs could be delivered using the above approaches as either naked nucleic acids or encoded in lentiviral vectors and attached to brain penetrating nanoparticles (BPN) [242]. CED uses movement of fluid in the interstitial space of the brain or tumors to distribute molecules, which do not readily penetrate the blood-brain barrier. In addition to providing effective delivery, it eliminates or reduces systemic exposure to the therapy, and also permits use of smaller doses [246]. A MRI-guided approach that utilizes focused ultrasound (FUS) with microbubbles (MB) to deliver miRNA/plasmid BPNs to tumors might be useful for systemic delivery. With this approach, MBs are first I.V. injected with the delivery payload of interest. Upon exposure to FUS, which is targeted to the tumor, MBs oscillate and open the surrounding endothelial barrier, permitting convective and/or diffusive payload delivery. FUS-MB-mediated drug delivery to solid tumors has been shown extensively in-vivo [244, 247], and FUS parameters can be tuned to provide reversible BBB sonoporation [248-250] to permit the delivery drugs such as Herceptin, which is a humanized anti-human epidermal growth factor receptor 2 (HER2/c-erbB2) monoclonal antibody and 1-3-bis(2-chloroethyl)-1-nitrosourea (carmustine) (BCNU), which is chemotherapeutic drug [251, 252] to the brain. FUS-MB-mediated tumor transfection has also been reported, yielding reduced carcinoma growth via the delivery of herpes simplex virus thymidine kinase, ganciclovir[253, 254], and shRNA [255, 256], supporting potential usage of FUS and MBs for BPN/miR/anti-miR delivery. The above approaches could therefore be developed to deliver anti-miR148a for GBM therapy.

In summary, my research work shows that miR-148a is elevated in GBM, where it predicts poor patient survival. It demonstrates that miR-148a has powerful oncogenic and cancer stem cell regulatory effects that are mediated by BIM, MIG6 and EGFR. The study therefore represents a

first characterization of miR-148a as a prognostic oncogene and promising therapeutic target in GBM.

II. Future directions

This work has demonstrated the deregulated expression of and oncogenic role of miR-148a in GBM. However, the mechanism of miR-148a overexpression in GBM has not been investigated. Possible mechanisms of deregulation of miR-148a include genetic alterations or transcriptional regulation. It has been shown that microRNAs are frequently located in fragile site of genomic loci. For example, microRNA-148a is on chromosome 7, which is frequently amplified in GBM. In the future, it would be informative to determine if miR-148a locus is amplified in GBM. To investigate transcriptional upregulation, the promoter region of miR-148a can be researched to find potential oncogenic transcriptional factor binding sites that might regulate miR-148a overexpression. Various molecular approaches including ChIP can be used to verify transcriptional regulation.

MicroRNAs are known to regulate multiple targets simultaneously. Until recently, the method for microRNAs target identification relied on computational prediction followed by experimental validation of single targets. This general methodology is restricted to a few selected targets chosen based on prior biological knowledge. To overcome this restriction approaches for genome-wide unbiased survey of all miRNA targets in the cell, such as phostoactivatable ribonucleoside-enhanced Crosslinking and Immunoprecipitation Experimetns (PAR-CLIP) and Crosslinking, ligation and sequencing of hybrids (CLASH), have been developed [257] [258]. The advent of large-scale sequencing technologies and new crosslinking methods for identifying protein/RNA interaction has enabled the systematic, genome-wide unbiased survey of all miRNA

targets in a given cell using PAR-CLIP and CLASH. These methods are based on crosslinking the miRNA and target mRNAs to the argonaute (AGO) proteins, key members of the RNA-induced silencing complex (RISC), by irradiation with UV light. AGO-ribonucleoprotein (RNP) complexes can then be recovered by immunoprecipitation, and the precise miRNA target sites of the co-purified target genes are identified by high-throughput sequencing [257, 258]. I identified two targets of miR-148a, but other targets that might mediate its powerful oncogenic effects could be discovered using the above screening approaches.

To target miR-148a for therapy as I propose in my thesis, methods for microRNA delivery to brain tumors have to be developed and tested. Our lab is working developing new approaches for local and systemic delivery of miRs and anti-miRs using convection enhanced delivery, nanoparticles and focused ultrasound microbubbles. Also, the toxicity of miR-148a inhibitor has not been investigated. The threshold at which normal tissues or cells could tolerate miR-148a inhibitor is not known. This will be an important step before moving miRNA replacement or inhibition therapy to the clinic.

It would also be interesting to investigate the effects of anti-miR-148a in combination with radiation, chemotherapy and EGFR inhibitors based on the rationale that was described in the discussion above. Additionally, it might be very useful to try to exploit the prognostic value of miR-148a in the clinic.

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