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MicroRNAs in Cancer: Glioblastoma and Glioblastoma Cancer Stem Cells

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Abstract

MicroRNAs represent an abundant class of endogenously expressed 18–25 nucleotide non-coding RNA molecules that function to silence gene expression through a process of post-transcriptional modification. They exhibit varied and widespread functions during normal development and tissue homeostasis, and accordingly their dysregulation plays major roles in many cancer types. Gliomas are cancers arising from the central nervous system. The most malignant and common glioma is glioblastoma multiforme (GBM), and even with aggressive treatment (surgical resection, chemotherapy, and radiation), average patient survival remains less than two years. In this review we will summarize the current findings regarding microRNAs in GBM and the biological and clinical implications of this data.

Keywords

cancer; cancer stem cells; glioblastoma; glioma; microRNA

Introduction

microRNA

MicroRNAs represent an abundant class of endogenously expressed short 18–25 nucleotide small non-coding RNA molecules that function to silence gene expression through a process of post-transcriptional modification (Bartel, 2004; Esquela-Kerscher and Slack, 2006; He et al., 2005; Lu et al., 2005; Pang et al., 2009; Sassen et al., 2008). Van der Krol et al. (1988) were the first to recognize plants as the initial eukaryotic organism found to possess anti-sense genes regulating target gene expression (van der Krol et al., 1988). Further, Rosalind Lee et al. (1993), while working with lin-14 in the model organism *caenorhabditis elegans*, found that lin-14 protein abundance was regulated by a short RNA product encoded by the

lin-4 gene (Lee et al., 1993). They demonstrated that a nucleotide precursor from the *lin-4* gene was modified to a 22-nucleotide RNA that contained sequences partially complementary to a number of 3' UTR sequences of the *lin-14* mRNA (Lee et al., 1993; Pang et al., 2009). Unbeknownst to the group, they had identified the first microRNA and demonstrated its interaction with and regulation of a specific transcript. Many biologists at the time did not give much credence to the finding and believed that Lee et al. (1993) had identified a biological oddity of the *C. elegans* developmental system (Lee et al., 1993). It was not until 1998, when Andrew Fire and Craig Mello successfully characterized the inhibitory mechanism and function of double stranded RNA (microRNAs) for which they subsequently shared the Nobel Prize, that it became evident microRNAs represented a new mechanism of genetic regulation (Fire et al., 1998). Further, in 2000 when Reinhart et al. demonstrated the presence of a second RNA, *let-7*, which was widely conserved and repressed the expression of *lin-14*, *lin-28*, *lin-41*, *lin-42*, and *daf-12* during developmental stage transitions in *C. elegans*, that a role for microRNAs in non-nematode species became apparent (Reinhart et al., 2000).

The mechanism by which microRNAs function to regulate gene expression has been elucidated by many laboratories over the last few years subsequent to their initial discovery (Bartel, 2004; He et al., 2005; Pang et al., 2009; Reinhart et al., 2000). MicroRNAs generated by the canonical biogenesis pathway (Figure 1) are transcribed as primary RNAs (pri-microRNAs) by RNA polymerase II prior to recognition by Drosha/RNase III nuclease complex or "spliceosome" in the nucleus and further modification to a pre-microRNA stem-loop structure (He et al., 2005; Iorio and Croce, 2009; Pang et al., 2009). In an alternate pathway microRNAs are transcribed directly as short hairpin RNAs. Regardless of pathway, the short hairpin RNAs are then exported to the cytosol and interact with Dicer and RNase III where further modification occurs and leads to formation of approximately 20–22 nucleotide double stranded microRNA (Esquela-Kerscher and Slack, 2006; Iorio and Croce, 2009). The dimeric RNA complexes are then further acted upon by Argonaute proteins which unwind the double stranded RNA and incorporate the microRNA into the RNA-induced silencing complex (RISC) (Esquela-Kerscher and Slack, 2006; Iorio and Croce, 2009; Pang et al., 2009; Singh and Mo, 2013). The RISC-microRNA complex is then directed to complementary sequences of a target messenger RNA (mRNA) where interaction occurs through base pairing, most often at the 3' untranslated region (UTRs) resulting in target mRNA degradation or translational inhibition (Pillai et al., 2007; Singh and Mo, 2013). In addition, microRNAs may bind to other regions such as within exons, resulting in similar translation inhibitory effects. MicroRNAs have also been shown to occasionally enhance translation instead of inhibition through binding to the 5'UTR in some organisms (Orom et al., 2008).

As mechanistic insight has accumulated, the number of micro-RNAs has grown to hundreds, with over 1500 potential micro-RNA coding sequences within the human genome now identified (Iorio and Croce, 2009; Pang et al., 2009). These unique double stranded RNA molecules are now thought to represent 1–3% of the human genome and regulate approximately 30% of all gene expression (Iorio and Croce, 2009). With our evolving understanding of the roles of microRNAs in normal cellular development, proliferation,

differentiation and apoptosis in a variety of different cellular subtypes, interest has developed regarding the potential role of microRNAs in pathological cellular processes including cancer. Since many cellular aberrations that occur during malignant transformation result from activation or inactivation of specific transcripts, it follows from a biological perspective that microRNAs may play an indispensable role in these processes (Iorio and Croce, 2009; Pang et al., 2009). Interestingly, approximately 50% of microRNAs are located within malignancy-associated sites and loci of genomic instability (Pang et al., 2009). Indeed, early observations in the history of microRNAs pointed toward a potential role in human cancer with the earliest discovered microRNAs in *C. elegans* shown to control apoptosis and cellular proliferation (Bartel, 2004; Chan et al., 2005; Fire et al., 1998; Lee et al., 1993). Evidence has also demonstrated malignant cells to exhibit widespread dysregulation of microRNAs compared to normal cells (Chan et al., 2005; Ciafre et al., 2005). A large number of subsequent studies have demonstrated a role for microRNAs in tumorigenesis (Chan et al., 2005; Ciafre et al., 2005; Esquela-Kerscher and Slack, 2006; He et al., 2005; Koo et al., 2012; Lavon et al., 2010).

MicroRNAs in cancer

The first insight with regard to a possible role for microRNAs in malignancy came from studies by Calin et al. (2004) investigating the chromosomal deletion observed in CLL and down-regulated expression of two microRNAs proposed to target the anti-apoptotic factor B cell lymphoma 2 (BCL2), miR-15 and miR-16 (Calin et al., 2004). Analysis of miR-15 and miR-16 expression in blood samples from patients with CLL showed that both microRNAs were absent or downregulated in the majority (68%) of cases compared to normal tissue or lymphocytes (Calin et al., 2004). These findings indicated that down-regulation of miR-15 and miR-16 resulted in elevated levels of Bcl-2 and thereby contributed to tumorigenesis in CLL. Thus, these microRNAs may indeed act as tumor suppressors. These were the first findings to suggest a causal relationship between microRNAs and the pathogenesis of a cancer, chronic lymphocytic leukemia. Following this report, other studies identified other specific tumorigenic microRNAs (Ciafre et al., 2005; Gaur et al., 2007; Kefas et al., 2008; Lavon et al., 2010; Zhang et al., 2009a; Zhi et al., 2010). Two separate studies provided mechanistic insight regarding microRNAs and carcinogenesis (He et al., 2005; O'Donnell et al., 2005). He et al. (2005) and O'Donnell et al. (2005) independently demonstrated a relationship between mir-17-92 and the c-myc oncogene (He et al., 2005; O'Donnell et al., 2005). He et al. (2005) demonstrated that miRNAs from the *mir-17-92* cluster were overexpressed in lymphoma cell lines carrying this amplification, and expression levels correlated with gene copy number of the *mir-17-92* locus (He et al., 2005). They demonstrated that additional expression of the *mir-17-92* cluster in a mouse model of B cell lymphoma overexpressing c-myc accelerated c-myc-induced tumorigenesis. O'Donnell and colleagues independently found mir-17-92 to be regulated by c-myc (O'Donnell et al., 2005). Interestingly, they showed that c-myc was responsible for regulating mir-17-92 expression which in turn regulated E2F1, suggesting a novel regulatory mechanism for "fine-tuning" gene expression (O'Donnell et al., 2005). Further, a separate key study elucidated a connection between let-7 and the proto-oncogene RAS and was the first microRNA demonstrated to modulate the expression of a proto-oncogene (Johnson et al., 2005). Importantly, RAS is a commonly over-expressed oncogene in human malignancies,

present in 15–30% of all cancers. Of note, RAS over-expression is common in lung cancer and Johnson et al. (2005) reported that RAS protein expression in lung cancer corresponded to reduced let-7 microRNA levels (Johnson et al., 2005).

Widespread genomic loss of microRNA levels have also been documented in human malignancies (Johnson et al., 2005; Nan et al., 2010; Pang et al., 2009; Singh and Mo, 2013). Lu et al. (2005) were the first to demonstrate a global reduction in microRNA levels compared to normal tissues (Lu et al., 2005). Here they investigated 334 human cancers, cell lines and non-malignant cells analyzing 217 microRNAs, and demonstrated that malignancies exhibited significantly reduced microRNA levels compared to non-malignant cells (Lu et al., 2005). With support for a role of microRNAs in cancer, many subsequent studies have investigated the role of microRNAs in malignancies including retinoblastoma, breast, prostate, lung, pancreatic, colon cancers and many others (Catania et al., 2012; Drakaki and Iliopoulos, 2013; Kim and Kim, 2013; Li et al., 2013a; Lu et al., 2005). Gliomas are one such malignancy in which the role of microRNAs has demonstrated continued and increasing clinical implications (Catania et al., 2012; Ciafre et al., 2005; Lavon et al., 2010). Since the initial description of aberrations in microRNA expression in pituitary adenomas and glioblastomas in 2005, there have been increasingly more publications regarding microRNA dysregulation in gliomas (Esquela-Kerscher and Slack, 2006; He et al., 2005; Nan et al., 2010; Rao et al., 2010).

Glioblastoma multiforme (GBM)

Gliomas represent a heterogeneous group of malignancies of the central nervous system arising from glial cells. This clinical entity is classified based upon cell type, grade and location within the central nervous system. Gliomas are named as a result of the suspected glial cell of origin and further characterized by the World Health Organization (WHO) grading system ranging from low grade (WHO grade II) to high grade (WHO grade III–IV). Gliomas account for 70% of human malignant primary brain tumors - the most frequently diagnosed adult glioma is GBM (WHO grade IV).

GBM (GBM) remains the most common malignant adult primary brain tumor, with more than 17,000 cases diagnosed each year in the United States alone (Grossman et al., 2010). GBMs affects all ages with a peak incidence between 50 and 60 years of age and a slight male predominance (Grossman et al., 2010; Stewart, 2002). Biologically, GBMs are highly aggressive, often with diffuse infiltration of the brain parenchyma making complete surgical resection difficult (Grossman et al., 2010; Mizoe et al., 2007; Stewart, 2002). Despite advances in radiation therapy, chemotherapeutics and surgical interventions, the median survival for patients with newly diagnosed GBM is only 14.6 months (Stupp et al., 2005). The majority of patients will experience disease recurrence with estimated 72% recurrence by 17 months per one study (Milano et al., 2010). The currently accepted treatment for GBM is chemoradiotherapy with temozolomide after maximal safe surgical resection (Stupp et al., 2005). As data has accumulated in the arena of causality in malignancy, a number of studies have highlighted the potential role of cancer stem cells in this setting.

Cancer Stem Cells

Consisting of only a small percentage of the total cell population, cancer stem cells (CSCs) are a unique subset of tumor cells thought to play a critical role in the initiation and progression of carcinogenesis (Singh et al., 2003). The first convincing data to support the presence of this population came in 1997 when Bonnet and Dick identified a subpopulation of leukemic cells that were CD34⁺/CD38⁻ and highly efficient at initiating leukemia when re-implanted into NOD/SCID mice compared to any other subset of leukemic cells tested (Bonnet and Dick, 1997). This subset of cancer stem cells has subsequently been shown to express consistent proliferation, self-renewal, and differentiation properties (Guo et al., 2006). Due to their unique replicative properties, CSCs are broadly hypothesized to initiate and drive tumor growth and recurrence, while other non-CSC bulk tumor cells are largely incapable of such extended expansion. Since the initial studies proposing the presence of this unique subpopulation, a number of reports have subsequently demonstrated the presence of cancer stem cells responsible for tumorigenesis in various malignancies such as breast, colon, ovarian, pancreatic, melanoma, multiple myeloma and brain cancers (Guo et al., 2006; Lang et al., 2009; Li et al., 2007; Matsui et al., 2004; O'Brien et al., 2007; Schatton et al., 2008; Schmidt et al., 2011). With regard to GBM, the neural stem cell marker CD133 has been used to enrich for a highly tumorigenic sub-population with cancer stem cell-like characteristics (Bao et al., 2006a; Singh et al., 2004).

Due to the known aberrant expression of microRNAs in cancer, and data suggesting a significant role for these microRNAs in stem cells, normal and cancer, various studies have demonstrated the presence of microRNA aberrations in GBM and GBM cancer stem cells (Chan et al., 2005; Ciafre et al., 2005; Luan et al., 2010; Pang et al., 2009; Papagiannakopoulos et al., 2008; Rao et al., 2010; Silber et al., 2008). In this review we will summarize the current findings regarding microRNAs in GBM and the implications of this data.

MicroRNAs in GBM

The advent of global genomic profiling techniques has enabled high throughput assessment of microRNA expression patterns in brain tumors. Utilizing the advantages of this high throughput process, Ciafre et al (2005) and Chan et al. (2005) were the first to document global microRNA expression profiles in glioblastoma human tissue samples (Chan et al., 2005; Ciafre et al., 2005). Ciafre et al. (2005) utilized microarray-based technology to profile the expression of 245 microRNAs in glioblastoma tissue samples. This study found nine microRNAs to be upregulated (miR-10b, miR-130a, miR-221, miR-125b-1, miR-125b-2, miR-9-2, miR-21, miR-25, miR-123) and four to be downregulated (miR-128a, miR-181c, miR-181a, miR-181b) (Ciafre et al., 2005; Zhi et al., 2010). Subsequent northern blot validation identified miR-221 as GBM upregulated and miR-128, miR-181a, miR-181b and miR-181c as GBM downregulated microRNAs (Ciafre et al., 2005). In a similar study reported the same year, Chan et al. (2005) demonstrated five up-regulated (miR-21, miR-138, miR-347, miR-291-5', miR-135) and three down-regulated (miR-198, miR-188, miR-202) microRNAs by analyzing 180 microRNAs in glioblastoma samples with miR-21 found to be the most up-regulated (Chan et al., 2005). In an array of 192 microRNAs utilizing quantitative RT-PCR, Silber et al. (2008) found 13 microRNAs to be

downregulated in anaplastic astrocytoma and GBM samples (miR-101, miR-128a, miR-132, miR-133a, miR-133b, miR-149, miR-153, miR-154, miR-185, miR-29b, miR-323, miR-328, miR-330) and 3 microRNAs to be up-regulated (miR-21, miR-155, miR-210) (Silber et al., 2008). Another study carried out by Godlewski et al. (2008) found eight microRNAs to be upregulated and 11 down-regulated when analyzing 245 microRNAs in the setting of GBM (Godlewski et al., 2008). A microarray based study by Rao et al. (2010) analyzing 756 microRNAs identified another 55 up-regulated and 29 down-regulated microRNAs in primary and secondary glioblastoma and anaplastic astrocytoma in comparison to controls (Rao et al., 2010). This study not only validated the role of several deregulated microRNAs but also provided data for the development of a 23 microRNA signature pattern for distinguishing between anaplastic astrocytoma and glioblastoma (Rao et al., 2010; Sasayama et al., 2009).

miR-21—Expression profiling analyses have revealed that miR-21 is one of the most commonly up-regulated microRNAs in glioblastomas with 44-100% of GBMs demonstrating this aberration (Chan et al., 2005; Ciafre et al., 2005; Conti et al., 2009; Lages et al., 2011; Sasayama et al., 2009). Further confirmation with northern blot and RT-PCR analyses have demonstrated that miR-21 is strongly elevated in glioblastoma tumor samples (Chan et al., 2005; Conti et al., 2009; Zhi et al., 2010). MiR-21 expression has been shown most prominently upregulated in grade IV astrocytomas with high expression correlated with poor patient survival (Zhi et al., 2010). Functional assessment of miR-21 suppression in glioblastoma cell lines demonstrated decreased cell growth, increased apoptosis, reduced invasiveness and tumorigenicity (Conti et al., 2009). Papagiannakopoulos et al. (2008), utilizing a systems based biological approach, developed predicted targets of miR-21 and showed that the over-expression of miR-21 was linked to three major cancer pathways: TGF- β , p53, and mitochondrial initiated apoptosis pathways. The subsequent knock down of miR-21 in glioma cell lines resulted in upregulation of many tumor suppressor proteins including p53, Bax, DAXX, APAF1, p21, TAp63 and TGFBR2 (Papagiannakopoulos et al., 2008). Further studies by Gabriely et al. (2008), have shown that miR-21 is a regulator of matrix metalloproteinase inhibitors (RECK and TIMP3) with implications in tissue invasion (Gabriely et al., 2008). Collectively, these findings provide strong evidence that miR-21 may function as an oncogene in GBM. As such, much work is ongoing to determine the role of miR-21 in the setting of cancer therapeutics.

miR-10b—miR-10b has been demonstrated to be overexpressed in GBM in at least eight reports (Ciafre et al., 2005; Huse et al., 2009; Lavon et al., 2010; Rao et al., 2010; Sasayama et al., 2009; Silber et al., 2008; Sun et al., 2011; Wuchty et al., 2011). A pertinent role for miR-10b has been suggested in glioblastoma progression through correlation between expression levels and WHO grade (Sasayama et al., 2009; Sun et al., 2011). Expression of miR-10b has been directly correlated with those of Ras homolog gene family member C (RhoC) and urokinase receptor (uPAR) and shown to enhance GBM invasiveness (Sasayama et al., 2009).

miR-221 and miR-222—MiR-221 and miR-222 have both been demonstrated to be up-regulated in glioma samples and cell lines (Ciafre et al., 2005; Conti et al., 2009). They are

clustered together on Xp11.3 and have the same target specificity. MiR-221 levels have been demonstrated to rise particularly in high-grade gliomas with increased proliferation rates (Conti et al., 2009). Functional studies show a link between miR-221/222 and cell cycle progression (Medina et al., 2008). Luciferase reporter analyses demonstrated that cyclin dependent kinase 1B/p27 was a direct target of miR-221/222 (Medina et al., 2008). Stimulation of quiescent glioblastoma cell lines revealed that miR-221 and miR-222 were upregulated as cells progressed through G1-S and were found to target cyclin dependent kinase inhibitors p27 and p57 preventing cellular quiescence by causing S-phase entry (Medina et al., 2008). Furthermore Zhang et al. (2009) demonstrated that miR-221/222 knockdown through antisense oligonucleotides reduced glioma cell lines subcutaneous xenograft growth through up-regulation of p27 and radiosensitivity (Zhang et al., 2009a). This resulted in increased apoptosis, reduced Ki-67 staining, increased PUMA and Bax and reduced Bcl-2 expression (Zhang et al., 2009a). The effect of miR-221/222 on apoptosis has been attributed to its ability to target the pro-apoptotic gene PUMA (Zhang et al., 2009a). Studies revealed that the over expression of PUMA reversed the phenotypes caused by the over expression of the miR-221/222 (Medina et al., 2008; Zhang et al., 2009a). Collectively, these data suggest that miR-221/222 may enhance the proliferative potential of tumor cells.

miR-17~92 cluster—The miR-17~92 cluster includes miR-17-3p, miR-17-5p, miR-18a, miR-19a, miR-19b, miR-20a, and miR-92a and is upregulated in glioblastoma cell lines and tumor samples (Ernst et al., 2010; Lavon et al., 2010; Malzkorn et al., 2010). This cluster of microRNAs was shown to possess a number of tumorigenic properties through targeting of regulators of DNA-repair and angiogenesis (CTGF, and POLD2), and anti-proliferative transcripts (TGFBR2, SMAD4, and CAMTA1) (Dews et al., 2010; Ernst et al., 2010; Malzkorn et al., 2010).

miR-128—miR-128, a brain enriched microRNA, is downregulated in glioma cell lines and has been documented to be repressed in GBM cell lines and samples in numerous reports (Lages et al., 2011; Lavon et al., 2010; Li et al., 2011; Rao et al., 2010). Indeed, miR-128 is one of the most commonly downregulated microRNAs in glioblastoma (Ciafre et al., 2005; Godlewski et al., 2008). Studies have demonstrated that while miR-128 is downregulated in WHO grade II–IV tumors, levels are significantly lower in higher grade gliomas (Zhang et al., 2009b). MiR-128 overexpression decreases the proliferative capacity of glioblastoma cell lines in vitro and in GBM xenografts. Mechanistically the 3' UTR of Bmi-1 is bound by miR-128 leading to its downregulation and a subsequent decrease in Akt phosphorylation through overexpression of p21CIP1 (Godlewski et al., 2008). As Bmi-1 is a critical component of normal neural stem cell and glioblastoma self-renewal, loss of Bmi-1 mediated self-renewal has been shown to result from p21CIP1 upregulation (Godlewski et al., 2008). Papagiannakopoulos et al. (2012) demonstrated that the growth factor receptors epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor α (PDGFR α), both of which are typically overexpressed in GBM, are repressed by miR-128 (Papagiannakopoulos et al., 2012). In vitro glioma neurosphere cultures with “stem-like” properties have been utilized to demonstrate that miR-128 possesses the capacity to block glioma cell self-renewal and reduce overall neurosphere number and size (Godlewski et al., 2008). These data suggest that miR-128 downregulation is likely to contribute to glioma

tumorigenesis through promotion of an undifferentiated phenotype via increased expression of Bmi-1. Furthermore, miR-128 is suggested to promote gliomagenesis via deregulation of self-renewal in glioma stem cells.

miR-34a—miR-34a, located on chromosome 1p36, is a transcriptional target of p53, and has been proposed to function as a tumor suppressor. MiR-34a is downregulated in glioblastoma cell lines and tissues compared to normal brain tissues and is significantly reduced in p53-mutant cells compared to cells expressing wild-type p53 (Li et al., 2009; Luan et al., 2010). The many predicted interaction sites for miR-34a within the transcriptome include the MYC, CCND1, CDK6, SIRT1 and c-Met oncogenes (Guessous et al., 2010; Yan et al., 2009). A number of molecular mechanisms may be responsible for the downregulation of miR-34a in gliomas. As 70–85% of oligodendrogliomas and up to 30% of astrocytomas possess 1p deletions this may represent a critical molecular abnormality related to tumorigenesis. Also, p53 mutations are typically found in up to 30% of all gliomas. Other mechanisms to include CpG dinucleotide methylation of the miR-34a promoter may play a role in the dysregulation of this microRNA (Li et al., 2009). MiR-34a has been shown to directly inhibit c-Met and Notch-1/2 in glioma cells and stem cells through direct 3' UTR binding (Guessous et al., 2010). Further, transfection of miR-34a results in significant suppression of cell proliferation, invasion, cell cycle progression, and in vivo glioma xenograft growth (Guessous et al., 2010). Glioma tumorigenesis has further been demonstrated to be inhibited by miR-34a by Luan et al. (2010), as well as reporting silent information regulator 1 (Sirt1) as a negative target of miR-34a in glioma cell lines (Luan et al., 2010). The role of Sirt1 in tumorigenesis as an oncogene has been demonstrated through a role in regulating apoptosis in response to oxidative stress and genomic insults (Luan et al., 2010). Thus miR-34a may possess an important role as a tumor suppressor in gliomas. However, deletion of chromosome 1p has been noted to be a positive predictor of survival in oligodendroglioma, highlighting a complex and potentially different interplay between microRNAs to include miR-34a in the setting of oligodendroglioma compared to GBM.

miR-7—Numerous studies published within the last few years have confirmed the role of miR-7 in GBM through its downregulation (Leber et al., 2011; Wuchty et al., 2011). Kefas et al. (2008) was the first to document a role for miR-7 in GBM revealing EGFR and IRS-2 as direct targets of mir-7 (Kefas et al., 2008). MiR-7 has been shown to inhibit EGFR expression through binding of its 3' UTR and independently to result in a reduction in Akt phosphorylation (Kefas et al., 2008). Since EGFR and Akt activated pathways are known to represent the most common genetic alterations in glioblastoma and proposed to act together in gliomagenesis, it is likely that miR-7 plays a prominent role in this process (Kefas et al., 2008). Another target of miR-7 is p21 activating kinase (PAK1). Data from the literature has demonstrated a role for the PAK family in a number of biological processes to include proliferation and motility (Reddy et al., 2008). In glioblastomas, PAK1 upregulation has been associated with decreased survival, and knockdown studies result in suppressed invasive capacity (Aoki et al., 2007). More recently it has been shown that focal adhesion kinase (FAK) is yet another target of miR-7, and that miR-7 over-expression reduced

invasion and migration (Aoki et al., 2007; Kong et al., 2012). These data suggest that miR-7 could have clinical implications for potential therapeutics.

miR-124/miR-137—miR-124 and miR-137 are markedly downregulated in anaplastic astrocytomas and glioblastomas compared to non-neoplastic brain tissue (Silber et al., 2008). Since miR-137 was initially described in the setting of GBM in 2008, subsequent studies have confirmed the downregulation of miR-137, often in conjunction with other microRNAs such as miR-124 (Silber et al., 2008). Transfection of mir-124 and miR-137 into tumor derived and neural stem cells leads to G1 arrest and reduction in CDK6 expression levels, which is a regulator of the cell cycle and known target of miR-124 and miR-137. Interestingly, the addition of DNA demethylating agents to glioma cell lines leads to activation of miR-137 but not miR-124, suggesting a CpG island methylation based promoter regulation for miR-137 (Silber et al., 2008). In addition, miR-124 was demonstrated to play an important role in neurogenesis. Functional studies have shown that miR-124 promotes the differentiation of neural cells through the regulation of small C-terminal domain phosphatase 1 (SCP1), suppressing an anti-neural pathway (REST), thus modulating neural expansion (Visvanathan et al., 2007). A number of regulation mechanisms in this setting have been proposed and warrant further investigation, with clinical implications in GBM treatment.

miR-181—The miR-181 family of microRNAs are known to be neuron enriched and downregulation of miR-181a and miR-181b has been demonstrated in glioma samples and cell lines (Conti et al., 2009; Shi et al., 2008). Expression profiling showed a reduction in miR-181 family transcript levels in 20–30% of glioblastomas. Studies by Conti et al. (2009) and Shi et al. (2008) both demonstrated reduced expression levels of miR-181 in gliomas (Conti et al., 2009; Shi et al., 2008). Further, transfection of glioblastoma cells with miR-181a and miR-181b induced apoptosis, reduced cell growth, decreased anchorage independent growth, and decreased invasion capabilities. Zhi et al. (2010) found that poor patient survival was associated with miR-181b downregulation (Zhi et al., 2010). Therefore, miR-181b may be useful as a prognostic marker and assist in selection of patients who may benefit from adjuvant therapy. These findings suggest a potential prognostic role for this family of microRNAs.

miR-100—Our group has recently identified mir-100 as a GBM suppressing microRNA. Mir-100 was first identified as downregulated in multiple GBM cell lines as compared to normal neural cell controls. After transfection, mir-100 reduced proliferation and increased apoptosis of multiple GBM lines through inhibition of silencing mediator of retinoid or thyroid hormone receptor-2 (SMRT/NCOR2). Additionally, either via implantation of stable GBM cell lines expressing miR-100 or direct injection of microRNA to already established tumor xenografts, miR-100 decreased proliferation in orthotopic GBM xenografts and extended survival (Alrfaei et al., 2013).

MicroRNAs with variable regulation levels within GBM

miR-451—miR-451 has been shown to be upregulated in glioma cells compared to non-neoplastic brain parenchyma via microarray based profiling (Gal et al., 2008; Nan et al.,

2010). However, contrasting studies have revealed downregulation of this microRNA in glioma cell lines. Nan et al. (2010) demonstrated decreased transcript levels of miR-451 in three glioma cell lines while Gal et al. (2008) have shown overexpression in CD133⁺ GBM cells (Gal et al., 2008; Nan et al., 2010). In the study by Gal et al. (2008), six microRNAs were shown to be overexpressed in the CD133⁺ fraction (miR-16, miR-107, miR-185, miR-425, miR-451, and miR-486). However, both studies demonstrated concordance with the results of endogenous miR-451 overexpression via transfection experiments, which resulted in decreased cell proliferation and viability suggesting a tumor suppressor role for miR-451. It appears that miR-451 may play a unique role as a regulator of the adaptive response observed in glioma cells during metabolic stress and low glucose availability (Godlewski et al., 2010).

miR-145—Another microRNA with conflicting data regarding expression levels in GBM when compared to non-neoplastic tissues is miR-145 (Koo et al., 2012). Koo et al. (2012) demonstrated miR-145 upregulation in a number of highly aggressive GBM cell lines (Koo et al., 2012). This group further reported that the downregulation of miR-145 led to decreased invasive capabilities of these cell lines. Discordant to these findings are data published by Lee et al. (2012), who reported downregulation of miR-145 in GBM cell lines and showed that overexpression of miR-145 led to decreased proliferation and invasion of GBM cell lines (Lee et al., 2012). Findings by Yang et al. (2012) supported the data from Lee et al. (2012), by demonstrating that miR-145 overexpression resulted in decreased “stemness”, migration and xenograft tumor growth (Yang et al., 2012). Oct4 and Sox2 were found to be targets of miR-145 mediating the loss of “stemness.” Further, Yang et al. (2012) reported that overexpression of miR-145 resulted in increased chemo- and radiosensitivity of GBM cell lines (Yang et al., 2012). More data is currently needed to clarify the published discrepancies of miR-145 activity.

MicroRNAs in GBM with limited functional characterization

A number of well characterized microRNA expression patterns and functional studies have been carried out to date. Trends have developed around the investigation of certain microRNAs, which have led to better functional characterization of these targeting transcripts. Continued expression analyses have demonstrated the presence of many more microRNAs with potential functional roles in GBM; however, characterizations at the functional levels have not yet occurred. In all, approximately 95 microRNAs have been reported to be down-regulated and 255 up-regulated in GBM, either tissue samples or cell lines (table 1 and 2). Further work to classify and characterize the functionality of these microRNAs is needed to determine possible therapeutic targets in the setting of GBM.

Of note, global microRNA analysis represents a robust challenge from a statistical standpoint. Parsing out differences of significance need to be correlated with relative and overall changes from baseline controls, and when doing so with large numbers of analyzed transcripts this can often be quite challenging. While findings of initial high throughput screening can serve as the basis for elucidation of “novel” regulatory microRNAs, it is important to further quantify and qualify the role of said microRNAs in target samples. The

use of quantitative PCR, northern blot and western blot may be useful to assess transcript and resultant protein levels in the setting of changes in microRNA expression levels.

Cancer stem cells and microRNAs

Ignatova et al. (2003) were the first to demonstrate the presence of neural stem-like cells from glioma termed glioma stem cells (GSCs) (Ignatova et al., 2002), followed by many other groups with similar findings (Galli et al., 2004; Singh et al., 2003). This sub-population of glioma cells express common markers of neural “stemness” such as Sox2, Nestin, and CD133. GSCs exhibit tumorigenic capacity in rodent xenograft models as well as multi-lineage differentiation potential and increased angiogenic capabilities through higher levels of VEGF expression (Bao et al., 2006b). Interestingly, this subset of tumor stem cells exhibits radiation and chemotherapy resistance (Bao et al., 2006a; Johannessen et al., 2009; Liu et al., 2006).

Many groups have investigated the role of microRNAs in GSCs. Silber et al. (2008) reported reduced levels of miR-124 and miR-137 in high grade gliomas and transfection experiments demonstrating that miR-124 and miR-137 overexpression led to cell cycle arrest, suggested a potential tumor suppressor role in GSCs (Silber et al., 2008). Overexpression of miR-128 resulted in negative regulation of glioma self-renewal in neurosphere cultures (Godlewski et al., 2008). Further, a number of microRNAs have been shown upregulated in neural progenitor cells including mir-17~92, mir-106b-25, mir-106a, the mir-183-182 cluster, the mir-302-367 cluster on chromosome 4q25 and mir-371-373 cluster on chromosome 19q13 (Lavon et al., 2010).

MiR-125b has been found to be significantly downregulated in CD133⁺ glioma stem cells compared to CD133⁻ GSCs (Shi et al., 2012). This microRNA has also been shown to be instrumental in the process of stem cell fission, allowing bypass of the G1/S checkpoint. Further, transfection of mir-125b to CD133⁺ cells decreased the number of actively proliferating cells, inducing arrest through miR-125 mediated downregulation of CDK6 and CDC25A (Shi et al., 2010). Differential expression of miR-125 and miR-29b in CD133⁺ cells compared to CD133⁻ cells may suggest a role for these microRNAs relating to maintenance of stem cell self-renewal.

MiR-34a has been shown to be down-regulated in glioma stem cells (Guessous et al., 2010; Li et al., 2009). Transfection experiments of miR-34a into GSCs inhibited proliferation, cell survival, and migration as well as resulted in the reduction in stem cell markers CD133 and Nestin (Guessous et al., 2010).

Further evidence for microRNAs in GSCs comes from studies assessing miR-451 in CD133⁺ cells, in which miR-451 is down-regulated. Transfection experiments resulted in decreased cell proliferation and viability thus suggesting a tumor suppressive role of miR-451 (Gal et al., 2008). Target sites of SMAD in the upstream promoter region of miR-451 suggest regulation via SMAD. Indeed transfection of SMAD into glioblastoma cells inhibited growth, suggesting possible differentiation of CD133⁺ cells through upregulation of miR-451 (Nan et al., 2010).

Conclusions

Recent and ongoing developments in our understanding of tumor biology continue to contribute to the potential for future therapeutic breakthroughs. The discovery of microRNAs and the subsequent elucidation of their aberrant expression levels in malignancies suggest microRNAs play a role in malignant transformation, progression, invasiveness and response to therapeutic interventions. Further, identification of glioma and glioma stem cell-specific microRNAs led to an appreciation for a role of these unique regulatory transcripts in the setting of brain tumors and specifically GBM. Through extensive molecular characterization of microRNA expression and function, it is clear that aberrantly expressed microRNAs have widespread effects on tumorigenesis, including on critical GBM pathways such as receptor tyrosine kinase signaling, p53 signaling, and cell cycle control (Ciafre et al., 2005; Kim et al., 2011; Papagiannakopoulos et al., 2008; Silber et al., 2012). However, the extent of microRNA dysregulation in GBM and emergence of many microRNA “families” suggest that single microRNAs may exert minimal effect and more global integrative approaches are needed to fully understand the role of microRNAs in GBM tumorigenesis and their therapeutic potential (Barca-Mayo and De Pietri Tonelli, 2014; Sun et al., 2012). Moving forward, it will be important for the facilitation of a timely transition from findings on high throughput analysis in the setting of GBM to thorough functional analyses. Studies documenting expression level changes alone, while useful in highlighting initial transcriptional differences, will not translate into therapeutic gains without clear functional clarification. As highlighted in Tables 1 and 2, much work remains to functionally define the actions of aberrantly expressed microRNAs in GBM before therapeutic benefit can be accomplished.

An initial promising avenue for clinical microRNA use is in biomarker development to improve prediction of prognosis or treatment response for glioma patients. Because of their small size, microRNAs were originally difficult to confidently identify in clinical formalin-fixed paraffin embedded (FFPE) specimens, but advances in *in situ* probe design and labeling methodology have resulted in highly sensitive and specific microRNA identification (Stenvang et al., 2008). Many microRNAs either alone or in signatures have been identified to correlate with patient survival (Zhi et al., 2010). Additionally, global microRNA analysis has been successfully used to subclassify GBM, with potential prognostic and treatment response predictive value (Kim et al., 2011; Rao et al., 2010). Furthermore, microRNAs alone or in combination with already used biomarkers could potentially be used in differentiating malignant and non-neoplastic populations or as biomarkers to better delineate astrocytoma histological grades (Pang et al., 2009; Rao et al., 2010; Sasayama et al., 2009).

As data continues to accumulate in this arena, the potential for therapeutic innovations are promising. MicroRNA-based therapeutics would be desirable on the basis that a single microRNA exerts widespread anti-cancer effects (Guessous et al., 2010; Li et al., 2009). As a cancer, GBM in particular demonstrates extensive therapeutic resistance through intrinsic and **rapidly** acquired mechanisms partially contributing to the failure of tested molecular targeted agents such as epidermal growth factor receptor (EGFR) inhibitors in clinical trials (Huang et al., 2009; Johannessen and Bjerkvig, 2012). MicroRNA-based therapeutics could

potentially overcome these resistances through broad inhibition of tumorigenic pathways or promotion of tumor suppressive networks. However, many hurdles such as efficient tumor delivery remain for successful translation of microRNAs into clinical therapeutics. Successful microRNA delivery has been greatly improved through optimizing RNA scaffold design and incorporating nanotechnology delivery methods such as liposomes or other nanoparticles (Monaghan and Pandit, 2011; Yezhelyev et al., 2008). Some of these platforms are nearing clinical utility and provide potential for microRNA-based delivery to brain tumors.

In summary, the discovery and characterization of microRNAs and RNA-based post-transcriptional epigenetic regulation significantly altered cell and molecular biology paradigms, and microRNAs have now been shown to play a role in regulating almost all normal and pathologic processes, such as proliferation, differentiation, and apoptosis. Although clinical translation will be difficult, microRNA-based therapeutics have the potential to broadly control tumorigenic processes and ultimately improve brain tumor patient survival and quality of life.

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Figure 1.

MicroRNA biogenesis. MicroRNA genes are first transcribed in the nucleus by RNA polymerases as pri-miRNA, and subsequently cleaved by the DROSHA enzyme to an oligonucleotide stem loop pre-miRNA. In an alternate pathway, microRNA genes are transcribed directly as short hairpin pre-miRNA. The pre-miRNA is then pumped into the cytosol by Exportin-5, where it is further processed by DICER to a miRNA duplex. The duplex is then unwound, and the single-strand microRNA is incorporated into the RNA-induced silencing complex (RISC) along with Argonautes and other proteins. The microRNA guides the RISC complex to complementary sites on messenger RNA, leading to epigenetic regulation via mRNA degradation or translation inhibition. Meanwhile, the passenger strand of the duplex miRNA is either degraded or acts as a second active microRNA (generally designated as the miR* or miR-5p counterpart).

Table 1

MicroRNAs over-expressed in GBM.

microRNA	Proposed target(s)	Functions when expressed	Biological effects of knockdown	Reference
mir-21	RECK, TIMP3	↑ proliferation, ↑ invasion, ↓ apoptosis, chemoresistance	↓ proliferation, ↑ apoptosis, ↓ invasion	(Chan et al., 2005) (Ciafre et al., 2005) (Visani et al., 2013) (Gabriely et al., 2008) (Papagiannakopoulos et al., 2008) (Rao et al., 2010) (Visani et al., 2013) (Zhi et al., 2010) (Zhou et al., 2010)
mir-10b		↑ invasiveness		(Ciafre et al., 2005) (Silber et al., 2008) (Sasayama et al., 2009) (Sun et al., 2011) (Visani et al., 2013)
mir-221/222	CDK1B/p27, PUMA	↑ proliferation	↑ apoptosis	(Ciafre et al., 2005) (Conti et al., 2009) (Medina et al., 2008) (Zhang et al., 2009a)
mir-17~92	CTGF, POLD2, TGFBR1, SMAD4, CAMTA1	↑ tumorigenesis, cell cycle progression		(Dews et al., 2010) (Ernst et al., 2010) (Lavon et al., 2010) (Malzkorn et al., 2010)
mir-125b	Bmf, E2F2	↓ apoptosis	↓ proliferation, ↑ sensitivity to ATRA-induced apoptosis	(Ciafre et al., 2005) (Shi et al., 2010) (Shi et al., 2012) (Xia et al., 2009) (Wu et al., 2012)
mir-130a				(Ciafre et al., 2005)
mir-9				(Ciafre et al., 2005)
mir-25				(Ciafre et al., 2005) (Rao et al., 2010)
mir-123				(Ciafre et al., 2005)
mir-138				(Chan et al., 2005)
mir-347				(Chan et al., 2005)
mir-291-5'				(Chan et al., 2005)
mir-135				(Chan et al., 2005)
mir-155				(Silber et al., 2008) (Rao et al., 2010)
mir-210				(Silber et al., 2008)
mir-93	Integrin-β8	↑ tumor growth, ↑ angiogenesis	↑ cell death	(Rao et al., 2010)
mir-23a				(Rao et al., 2010)
mir-16				(Rao et al., 2010)
mir-106b				(Rao et al., 2010)
mir-143		↑ invasiveness		(Koo et al., 2012)
mir-145		↑ invasiveness		(Koo et al., 2012)
mir-196a		↓ patient survival		(Guan et al., 2010)

Table 2

MicroRNAs under-expressed in GBM.

microRNA	Proposed target(s)	Biological effects when re-expressed	References
mir-7	EGFR, PAK1, FAK, IRS-2	↓ invasion, ↓ cell viability	(Kefas et al., 2008) (Kong et al., 2012) (Leber et al., 2011) (Reddy et al., 2008) (Visani et al., 2013)
mir-128	EGFR, PDGFRA ARP5, Bmi1, E2F3a	↓ proliferation, ↓ self-renewal	(Ciafre et al., 2005) (Godlewski et al., 2008) (Lages et al., 2011) (Papagiannakopoulos et al., 2012) (Zhang et al., 2009b)
mir-34a	MYC, CCND1, CDK6, SIRT1, and c-MET, NOTCH1/2	↓ invasion, ↓ proliferation, ↓ cell cycle progression, inhibition of Notch and c-Met	(Guessous et al., 2010) (Li et al., 2009) (Luan et al., 2010) (Silber et al., 2012)
mir-124	ITGB1, LAMC1, CDK6, SCP1, NFATc1, PTBP1, CAMTA1	G1 arrest, ↓ proliferation, ↑ differentiation	(Silber et al., 2008) (Kang et al., 2013) (Fowler et al., 2011) (Visvanathan et al., 2007)
mir-137	CDK6	G1 arrest, ↓ proliferation, ↑ differentiation	(Silber et al., 2008) (Visani et al., 2013)
mir-100	SMRT/NCOR2	↓ proliferation, ↑ cell death	(Alrfaei et al., 2013)
mir-181		↑ apoptosis, ↓ proliferation, ↓ invasion	(Ciafre et al., 2005) (Conti et al., 2009)
mir-188			(Chan et al., 2005)
mir-198			(Chan et al., 2005)
mir-202			(Chan et al., 2005)
mir-101	EZH2	↓ invasion, ↓ proliferation, ↓ angiogenesis	(Smits et al., 2010) (Visani et al., 2013)
mir-132			(Silber et al., 2008)
mir-133a			(Silber et al., 2008)
mir-133b			(Silber et al., 2008)
mir-149			(Silber et al., 2008)
mir-153			(Silber et al., 2008)
mir-154			(Silber et al., 2008)
mir-185			(Silber et al., 2008)
mir-29b			(Silber et al., 2008)
mir-323			(Silber et al., 2008)
mir-328			(Silber et al., 2008)
mir-330			(Silber et al., 2008) (Visani et al., 2013)
mir-451	CAB39	↓ proliferation, ↓ viability	(Gal et al., 2008) (Godlewski et al., 2010) (Nan et al., 2010)
mir-31			(Visani et al., 2013)
mir-218	IKKβ, Bmi1	↓ migration, ↓ proliferation, ↓ self-renewal	(Rao et al., 2010) (Song et al., 2010) (Tu et al., 2013)

microRNA	Proposed target(s)	Biological effects when re-expressed	References
mir-219-5p		↓ colony formation	(Rao et al., 2010)
mir-139	Mcl-1	↑ apoptosis	(Li et al., 2013b)
mir-326	NOTCH1, NOTCH2, PKM2	↓ invasion, ↓ proliferation, ↓ viability, ↑ apoptosis	(Kefas et al., 2009) (Kefas et al., 2010)