

Micro-RNA-128 (miRNA-128) down-regulation in glioblastoma targets ARP5 (ANGPTL6), Bmi-1 and E2F-3a, key regulators of brain cell proliferation

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Abstract High density micro-RNA (miRNA) arrays, fluorescent-reporter miRNA assay and Northern miRNA dot-blot analysis show that a brain-enriched miRNA-128 is significantly down-regulated in glioblastoma multiforme (GBM) and in GBM cell lines when compared to age-matched controls. The down-regulation of miRNA-128 was found to inversely correlate with WHO tumor grade. Three bioinformatics-verified miRNA-128 targets, angiopoietin-related growth factor protein 5 (ARP5; ANGPTL6), a transcription suppressor that promotes stem cell renewal and inhibits the expression of known tumor suppressor genes involved in senescence and differentiation, Bmi-1, and a transcription factor critical for the control of cell-cycle progression, E2F-3a, were found to be up-regulated. Addition of exogenous miRNA-128 to CRL-1690 and CRL-2610 GBM cell lines (a) restored ‘homeostatic’ ARP5 (ANGPTL6), Bmi-1 and E2F-3a expression, and (b) significantly decreased the proliferation of CRL-1690 and CRL-2610 cell lines. Our data suggests that down-

regulation of miRNA-128 may contribute to glioma and GBM, in part, by coordinately up-regulating ARP5 (ANGPTL6), Bmi-1 and E2F-3a, resulting in the proliferation of undifferentiated GBM cells.

Keywords ARP5 (ANGPTL6) · Bmi-1 · Cell-cycle control · Differentiation · Differentiation regulated transcription factors (DRTFs) · E2F-3 · Glioblastoma multiforme (GBM) · Micro-RNA array · miRNA-128 · Neural stem cell (NSC) · NSC renewal

Introduction

Glioblastoma multiforme (GBM) represents a class of high-grade malignant neoplasm which rapidly proliferates, invades and destroys surrounding brain tissue. The median survival of patients first diagnosed with GBM is much less than 1 year, and the current prognosis for GBM patients remains dismal [1–3]. Molecular-genetic, array-based gene profiling analysis of GBM and gain- or loss-of function studies are beginning to reveal that specific alterations in messenger RNA (mRNA) and micro RNA (miRNA) complexity strongly associates with glioma and GBM development, and also with tumor grade [1–9]. Indeed, miRNAs, small non-protein-coding RNAs that function through the negative control of gene expression at the post-transcriptional level, exert important regulatory controls on cancer cells, and are emerging as critical players in the control of apoptosis, inflammation, cell-cycle regulation, neural stem cell (NSC)-like behavior and proliferative neuropathology during cellular transformation and tumorigenesis [6–13].

This study examined, using miRNA array panels (LC Sciences, Houston TX), confirmatory Northern and

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Western analysis, miRNA abundance and pathogenic gene expression in (a) six ATCC (American Type Culture Collection) glioma and GBM tumor cell lines CRL-2610 (LN-18), CRL-2020 (DBTRG-05MG), CRL-1690 (T98G), HTB-138 (HS683), CRL-2365 (M059K), and CRL-2366 (M059J), in (b) six WHO grade I and II glioma tissues, and in ten WHO grade IV GBM tissues, obtained from human brain biopsy [9, 10]. Both miRNA array and Northern analysis revealed in glioma and GBM a significant down-regulation of miRNA-128, a small brain-enriched RNA that was found to be particularly reduced in abundance in advanced GBM [6–8, 14]. Using miRBASE miRNA database (Version 10.1; Sanger Institute, Cambridge UK) and microrna.org database (Memorial Sloan-Kettering Cancer Center, New York, NY USA) search algorithms, miRNA-128 was found to be strongly complementary to several members of a family of GBM-related messenger RNA (mRNA) targets, including high affinity targets for the ARP5 (ANGPTL6), Bmi-1 and E2F-3a mRNA 3' un-translated regions (UTRs) [15, 16]. These regulatory proteins, known to support NSC renewal and regulate cell-cycle progression, were found to be significantly up-regulated in glioma and in GBM. While ARP5 (ANGPTL6) normally regulates cell regeneration and proliferation, Bmi-1 normally promotes NSC renewal by acting as a polycomb silencing complex that alters chromatin structure, and thereby inhibits the expression of known tumor suppressor genes involved in senescence and differentiation [17–19]. Members of the E2F gene family play a crucial role in the control of cell cycle and are also targets for the transforming proteins of small DNA tumor viruses [15, 20–23]. Taken together, these studies suggest that a single under-expressed miRNA-128 species up-regulates a pathogenic gene family that includes ARP5, Bmi-1 and E2F-3a, and thereby contributes interactively to the maintenance of an undifferentiated self-renewing state of brain cells and altered brain cell-proliferating functions.

Materials and methods

Reagents and antibodies

All reagents were purchased from commercial suppliers and were used without further purification. Glioma and GBM cell culture reagents, Western reagents and small RNA isolation reagents such as isopropanol, ethanol, diethyl pyrocarbonate (DEPC) water, RNase-free plastic reaction vials, RNase inhibitors and disposable mini-homogenizers were nucleic acid grade and purchased from Ambion (Houston, TX) or Invitrogen (Carlsbad, CA) and were utilized as previously described [9–11]. Western immunoblots were performed using human-specific primary antibodies

directed against the control marker β -actin (3598-100) and ARP5 (WH0083854M7; Sigma–Aldrich Chemical Company St. Louis, MO), Bmi-1 (C-20; sc-8906) and E2F-3a (C-18; sc-878; Santa Cruz Biotechnologies, Santa Cruz, CA) [9].

Human glioma and GBM cell lines

Human glioma and GBM tumor cell lines CRL-2610 (LN-18), HTB-138 (Hs683), CRL-2020 (DBTRG-05MG), CRL-1690 (T98G), CRL-2365 (M059K), and CRL-2366 (M059J) were purchased from the American Type Culture Collection (ATCC, Rockville MD) and cultured according to the supplier's specifications. These multinucleated polyploid cells, analyzed at about 30–40% confluence, were found to be a good source of high quality total RNA and protein and were assayed for β -actin, and Bmi-1 and transcription factor E2F-3 gene expression levels (mRNA and protein) using methods previously described [9–11, 14, 16].

CRL-2365 (M059K) and CRL-2366 (M059J) GBM and miRNA-128 cell studies

To study the effects of exogenously added miRNA-128 levels on ARP5, Bmi-1 and E2F-3a protein levels and cell division in CRL-1690 (T98G) and CRL-2610 (LN-18) GBM cell lines, transfection of miRNA-128 oligo (25–75 nmol/l; Ambion) was performed with Lipofectamine 2000 (Invitrogen) and were compared to transfection with a standard control miRNA (mimic-miRNA-1) as previously described [14]. Proliferation of CRL-1690 (T98G) and CRL-2610 (LN-18) GBM cell lines was determined using an EdU (5-ethynyl-2'-deoxyuridine)-Alexa Fluor® alkyne-azide cell proliferation assay (Invitrogen).

Human brain biopsy tissues

Human glioma/GBM biopsy samples ($N = 6$ glioma, $N = 10$ GBM; West Jefferson Medical Center, Marrero LA) were analyzed for total RNA and protein as previously described [9–11, 14, 16]. All tissue samples were from frontal lobe or parietal lobe tumors; the 6 gliomas studied [World Health Organization (WHO) tumor grade I or II] had a mean age of 43.5 ± 16.5 years and the 10 GBM studied (all WHO tumor grade IV) had a mean age of 47.5 ± 16.5 years. Two different kinds of human brain cell controls were used; (1) control human neural cells in primary culture, derived from human neural progenitor cells (CC-2599; Lonza, Walkersville MD) as previously described [5] and (2) control short post-mortem interval (<3 h) human brain tissues from either the frontal or parietal lobe [9, 11]. All human brain tissues were used in accordance with the ethical standards and Institutional

Review Board protocols and guidelines of the LSUHSC [9–11, 14, 16].

RNA isolation, quality control and northern analysis

Lightly packed cultured cells, or human biopsied tissue samples of 100 mg wet weight were rapidly processed into total RNA. RNA quality was assessed using an Agilent Bioanalyzer 2100 (Lucent Technologies; Murray Hill, NJ) [9–11]. In a typical analysis 10 ug total RNA samples from control, glioma or GBM cells or tissues were screened for 911 small RNA and miRNA abundance using miRNA array panels (LC Sciences, Houston, TX). Control total RNAs were obtained from adult human neural cells in primary culture or from control human brain [9, 11, 14, 16]. Northern analysis using radiolabelled antisense 21-mer probes were performed as previously described by our group [9–11, 14, 16].

Protein isolation and western analysis

Total cellular proteins were isolated from the same sample using TRIzol reagent (Invitrogen) and concentrations were determined using dotMETRIC microassay (sensitivity 0.3 ng protein/ml; Chemicon) [9, 14]. Signals were detected with an anti-IgG fluor-linked secondary antibody and an ECL+ Western immune blotting system (RPN2132/PA45007; Amersham Bioscience, Piscataway, NJ).

Bioinformatics and statistical analysis

Specific brain mRNA candidates, containing high affinity (<−14.6 kcal/mole) targets in their 3′ un-translated regions for miRNA-128-specific binding, were obtained using the miRBASE miRNA database search algorithms (Version 10.1; <http://microrna.sanger.ac.uk/targets>; Sanger Institute, Cambridge UK), or the microrna.org website (<http://www.microrna.org/microrna/home>; Memorial Sloan-Kettering Cancer Center, New York, NY USA). All statistical procedures for specific miRNA, mRNA and protein were carried out using programs and procedures in the SAS language (Statistical Analysis Institute, Cary, NC). Only *P* values of less than 0.05 were considered to be statistically significant.

Results

miRNA, total RNA and protein extraction and quality control

Control, glioma and GBM cell and tissue samples all yielded total RNA samples with 28S/18S ratios >1.4 and

single, sharp protein bands for β -actin, ARP5, Bmi-1 and E2F-3 with no evidence of protein sub-banding or degradation. Each miRNA sample from control, glioma or GBM passed stringent quality control analyses using an Agilent Bioanalyzer 2100 and yielded high quality abundance data after analysis on miRNA panels or Northern analysis (Fig. 1) [9–11, 14, 16].

miRNA-128 levels and mRNA targets in cultured cells and biopsied tissues

Both glioma and GBM cell lines exhibited a significant down-regulation of miRNA-128 ranging from an average of 0.24- to 0.54-fold compared to primary human neural cell controls (Fig. 1). Similarly, glioma and GBM biopsied tissues exhibited a down-regulation of miRNA-128 ranging from 0.63- (grade I–II glioma) to 0.27-fold (grade IV GBM) when compared to controls. There were no significant changes in the high brain abundance miRNA-125b or miRNA-127 species in any of the control or glioma/GBM samples analyzed (Fig. 1a; data not shown) [9–12, 14, 16]. Using miRBASE miRNA database search algorithms miRNA-128 displayed significant interactions with the 3′UTR of ARP5 (ANGPTL6), Bmi-1 and E2F-3a (Fig. 2).

ARP5 (ANGPTL6), Bmi-1 and E2F-3a mRNA and protein levels in glioma and GBM

Glioma and GBM cells and tissues exhibited increases in ARP5 (ANGPTL6), Bmi-1 and in E2F-3a gene expression at both the mRNA and protein level. ARP5 mRNA was found to be expressed at 2.1- and 3.6-fold over control levels in glioma and GBM cells, respectively, Bmi-1 mRNA was found to be expressed at 2.2- and 4.2-fold over control levels in glioma and GBM cells, respectively, and E2F-3a mRNA abundance was found to be increased from 3.1- and 4.7-fold over controls, respectively, in glioma and GBM cells (Fig. 3). In glioma tissues, ARP5 (ANGPTL6), Bmi-1 and E2F-3a mRNA were found to be increased 2.2-, 2.3- and 2.7-fold, respectively, and in GBM tissues, ARP5 (ANGPTL6), Bmi-1 and E2F-3a mRNA were found to be increased 2.2-, 3.3- and 4.9-fold, respectively. In support of these observations of mRNA increases, glioma and GBM cells exhibited significant up-regulation of a major 47 kD ARP5 (ANGPTL6) protein ranging from 1.4- to 2.2-fold of human neural cell controls, a major 37 kD Bmi-1 protein and ranging from 1.4- to 2.4-fold of controls, and up-regulation of a major 58 kD E2F-3a protein ranging from 1.7- to 2.7-fold over human neural cell controls, and these results were highly significant, especially in GBM (Fig. 4). In glioma tissues, ARP5 (ANGPTL6), Bmi-1 and E2F-3a protein were found to be increased 1.2-, 1.4- and 1.7-fold over controls, respectively, and in GBM tissues, ARP5

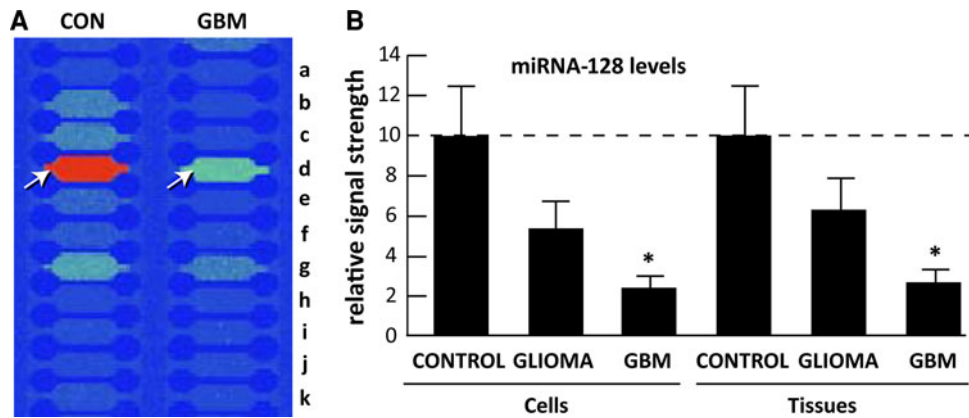


Fig. 1 Specific down-regulation of miRNA-128 in GBM tissues compared to controls **a** using high density, differential display miRNA fluorescent panels (only the miRNA-128 region shown; LC Sciences, Houston TX), or **b** using Northern dot-blot analysis; data expressed in bar graph format. By convention red signals (wavelength 650 nm) indicate high expression of that particular miRNA and blue-green signals (wavelength 475–510 nm) indicate relatively lower

expression of that same miRNA (arrows). Signals from miRNA arrays containing in total 911 control, small RNA and miRNA targets (**a**) were further electronically quantified for bar graph display format in (**b**). A control miRNA-127, also enriched in the brain, showed no such increases in either glioma or GBM (Fig. 1a first column position (**f**); data not shown); see text for further details; $N = 10$; significance compared to control, * $P < 0.05$; ** $P < 0.01$ (ANOVA)

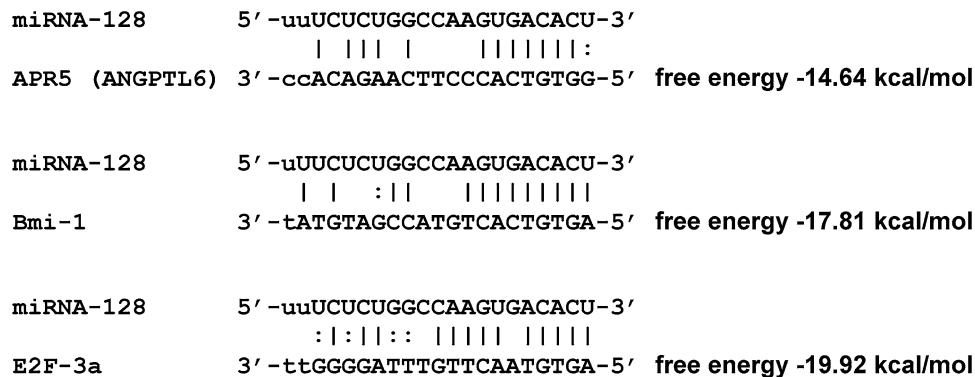


Fig. 2 Potential interaction of miRNA-128 with the 3' un-translated region (3'UTR) of ARP5 (ANGPTL6), Bmi-1 and E2F-3a using miRBASE miRNA database search algorithms (Version 10.1; <http://microrna.sanger.ac.uk/targets>; Sanger Institute, Cambridge UK). Solid vertical lines between miRNA-128 sequence and 3'UTR targets indicate hydrogen bonding; two vertical dots indicate potential for partial hydrogen bonding. In all 3 cases 7–10 nucleotide miRNA-128-3'UTR 'seed sequences' are enriched in the 3' end of

miRNA-128 [6, 7, 28]; free energy of association is shown for each miRNA-128-3'UTR species. Interestingly, the free energy of association is inversely correlated to the degree of mRNA and protein up-regulation for all 3 species (Figs. 3, 4). Other details of the ARP5 (ANGPTL6), Bmi-1 and E2F-3a 3'UTRs including their location in the mature mRNA can be found at the EMBL-EBI website: <http://www.ebi.ac.uk/enright-srv/microcosm/cgi-bin/targets/v5/search.pl>

(ANGPTL6), Bmi-1 and E2F-3a protein were found to be increased 2.1-, 2.3- and 2.7-fold over controls, respectively, and again these results were highly significant, especially in GBM (Fig. 3).

ARP5 (ANGPTL6), Bmi-1 and E2F-3a levels in miRNA-128-transfected CRL-2610 cells

CRL-2610 cells are derived from the temporal lobe of a 65 year old Caucasian male suffering from a grade IV GBM (ATCC). Thirty-five percent confluent CRL-2610 cells were treated with a pre-miR-128 (30 nM) instilled into the cell culture medium and cells were extracted for

total protein and analyzed 4.5 days after initial miRNA-128 treatment. ARP5 (ANGPTL6), Bmi-1 and E2F-3a protein levels were found to be decreased 55, 51 and 49%, respectively after miRNA-128 treatment when compared to controls, and the results were highly significant (Fig. 5).

Effects of miRNA-128 on glial cell division in CRL-1690 and CRL-2610 cell lines

Thirty-five percent confluent CRL-1690 or CRL-2610 cell lines showed reductions in cell proliferation of 64 and 53% age-matched control cells, respectively, and again the results were highly significant (Fig. 6). The small

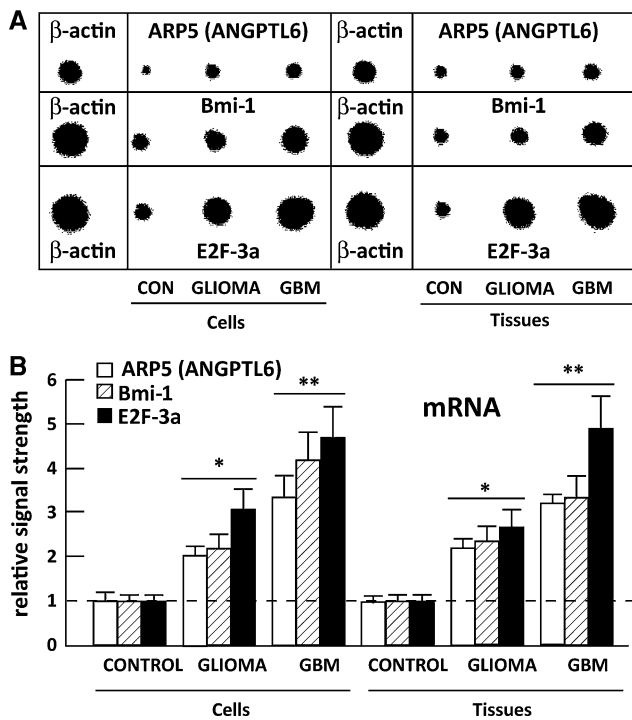


Fig. 3 Comparison of ARP5, Bmi-1 and E2F-3a mRNA levels in glioma (GLIOMA; $N = 3$) and GBM ($N = 3$) ATCC cell lines and in glioma (GLIOMA; $N = 6$) and GBM ($N = 10$) tissues obtained from brain biopsy using Northern dot-blot analysis. Representative results of pooled total RNA samples are shown in (a); controls were human neural cells in primary culture (cell control) or a pool of control frontal lobe total RNA preparation from pathology-free short post-mortem interval human brains (tissue control) as previously described [9, 11]. Data were expressed as fold-changes over β -actin mRNA signal in the same cells or tissue controls arbitrarily set to 10.0 (dashed horizontal line) (b). Small horizontal line over both Bmi-1 and E2F-3a bars indicate the significance of Bmi-1 and E2F-3a up-regulation over their respective levels in control cells or tissues, $N = 5$; * $P < 0.05$; ** $P < 0.01$ (ANOVA)

RNAs miRNA-1, miRNA-125b, miRNA-127, miRNA-132, miRNA-146a, 5S RNA or a scrambled miRNA-128 (containing the same nucleotide composition but in a random order) showed no such effects.

Discussion

Micro RNA-128 in GBM

Micro-RNA-128 (miRNA-128; chr 2q21.3; 5'-UUUCUC UGGCAAGUGACACU-3'; Genbank AJ459739), the identical product of 2 distinct genes (miRNA-128-1 and miRNA-128-2), is highly expressed in human brain neocortex and hippocampus, but is significantly down-regulated in human glioma and GBM (Figs. 1, 2) [7, 8, 13–16, 24]. The level of human miRNA-128 has been found to be significantly increased in cytokine- or amyloid beta 42

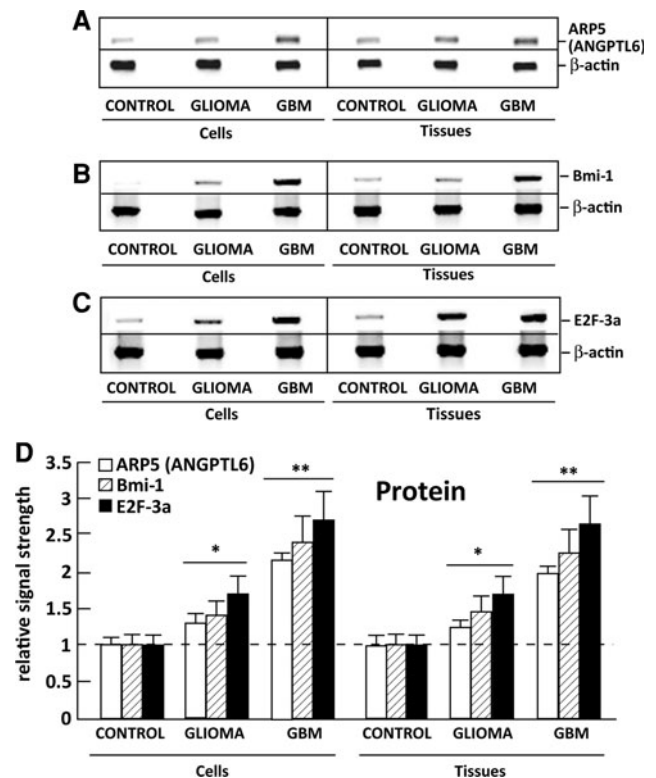


Fig. 4 Immunoblot analysis of ARP5 (47 kD), Bmi-1 (37 kDa) protein (a) and E2F-3a (58 kDa) protein (b) levels, each in relation to β -actin (48 kDa) protein levels in the same sample, in CONTROL ($N = 3$), glioma (GLIOMA; $N = 3$) and GBM ($N = 5$) obtained from ATCC cell lines, and in CONTROL ($N = 3$), glioma (GLIOMA; $N = 6$) and GBM ($N = 8$) tissues obtained from human brain biopsy. To minimize equal sample loading errors, gel membranes were probed with ARP5, Bmi-1 or E2F-3a, stripped, and then probed with β -actin on the same gel membrane [9]. Representative results of pooled protein samples are shown for in ARP5 protein in (a), Bmi-1 protein in (b) and for E2F-3a protein (c); controls were, respectively, whole cell protein extracts from cultured primary HN cells or whole human neocortex obtained from short post-mortem interval brain [5, 9]. Data are expressed as fold-changes over β -actin protein signal arbitrarily set to 1.0 (dashed horizontal line) (d). Small horizontal line over ARP5, Bmi-1 and E2F-3a bars indicate the significance of their up-regulation over their respective levels in control cells or tissues, $N = 5$; * $P < 0.05$; ** $P < 0.01$ (ANOVA)

(A β 42)-peptide-stressed primary human neural cells, in Alzheimer’s disease, in prion-induced neurodegeneration in mice, and in glioma and GBM, but not in short post-mortem interval, age-matched healthy control human brain tissues, or in the short post-mortem neocortex obtained from other neurological disorders such as amyotrophic lateral sclerosis, depression, Huntington’s disease, Parkinson’s disease, or schizophrenia [12, 16, 24, 25; unpublished observations]. Currently, the promoters of the human pre-miRNA-128 genes are not known, neither is the identity of transcription factor binding proteins, chromatin structures or epigenetic factors that interact with the miRNA-128 regulatory regions [14, 16, 24]. The observation that

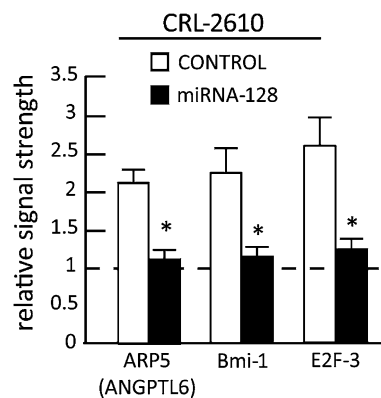


Fig. 5 Effects of exogenously added miRNA-128 (30 nM) on ARP5, Bmi-1 and E2F-3 gene expression levels in CRL-2610 cultured GBM cells 4 h after initial miRNA-128 treatment compared to transfection using a standard control miRNA (mimic-miRNA-1; Invitrogen) and as previously described [14]. Controls showed basal abundance of E2F-3a > Bmi-1 > ARP5; horizontal dashed line at 1.0 is shown for ease of comparison of down-regulated ARP5, Bmi-1 and E2F-3a; $N = 4$; * $P < 0.05$ (ANOVA)

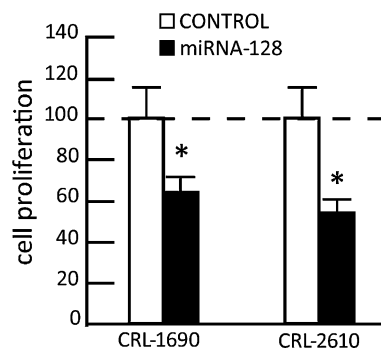


Fig. 6 Effects of exogenously added miRNA-128 (30 nM) on cell proliferation in CRL-1690 and CRL-2610 GBM cells compared to transfection using a standard control miRNA (mimic-miRNA-1), using an EdU (5-ethynyl-2'-deoxyuridine)-Alexa Fluor® alkyne-azide cell proliferation assay (Invitrogen); $N = 4$; * $P < 0.05$ (ANOVA)

miRNA-128 was significantly less abundant in grade IV GBM versus grade I–II glioma suggests that expression of this unique, and normally highly abundant, miRNA species may either be progressively depleted, or related to the more advanced stages of GBM-type pathology. Our results of specific miRNA-128 decreases coupled to Bmi-1 increases in GBM are in agreement with a recent report showing miRNA-128-mediated regulation of the “stem cell-like” characteristics of glioma [1–3, 18]. Over-expression of miRNA-128 in glioma cells has also been shown to inhibit cell proliferation [15, 18]. This is the first report of a down-regulated miRNA-128-mediated up-regulation of ARP5 (ANGPTL6; see below). Interestingly, human miRNA-128, which is unusually depleted in AU or UA dinucleotide ‘instability’ elements, was recently found to have a relatively long half-life, well in excess of 6 h, in both cultured

human neural cells and in human brain tissues [16]. The in vivo manipulation in the abundance of miRNA-128, and other oncogenic miRNAs (‘oncomirs’), by either using miRNA anti-sense or specific transcription factor activation strategies, hold promise in future therapeutic approaches for glioma and GBM treatment [4, 5, 14, 24].

ARP5 (APLP-6; ANGPTL6)

Angiopoietin-related protein 5 (ARP5; also known as angiopoietin-related growth factor, AGF; angiopoietin-like protein 6, APLP-6; ANGPTL6) is a 470 amino acid fibrinogen-domain containing polypeptide encoded on human chromosome 19p13.2 [26]. A brain-enriched ARP5 (ANGPTL6) is predicted to play multiple roles in cell proliferation, remodeling and regeneration, and has been further implicated in neovascularization and stem cell expansion [26, 27]. The simultaneous up-regulation of ARP5 (ANGPTL6), Bmi-1 and E2F-3a suggests the participation of a coordinately expressed gene program in driving glioma and GBM pathology; other genes and gene control mechanisms may be expected to play ancillary roles [15, 18].

Bmi-1

The Bmi-1 proto-oncogene, first identified as a common pro-viral integration site (B lymphoma Mo-MLV insertion region-1) on chromosome 10p11.23, encodes a 324 amino acid nuclear protein that has key functions in the self-renewal of NSCs [13, 17–19]. While self-renewing pluripotent NSCs persist throughout life, why certain NSCs in the brain self-renew more extensively than others remains unclear. Studies have shown that the polycomb factor Bmi-1 represses cell-cycle inhibitors p16, p19, and p21 that are necessary for NSC self-renewal, and that Bmi-1 enhancement of NSC self-renewal is significantly greater with increasing age and NSC passage [18–20]. Importantly, when Bmi-1 is over-expressed in adult forebrain, NSCs expand dramatically and continue to proliferate aggressively [17, 19]. Bmi-1 was one of the first examples of a miRNA-regulated NSC self-renewal factor that may function, in part, by silencing certain target genes through epigenetic chromatin modification [18, 19]. Taken together these findings indicate that miRNAs not only regulate normal brain cell growth and developmental gene expression, but also contribute to the “NSC-like” characteristics of brain cancers that contribute to cellular proliferation and tumorigenesis [18, 19].

E2F-3a

Located on human chromosome 6p22, the E2F-3 gene encodes multipartite ~465 amino acid members of the

E2F gene family of transcription factors. The E2F-3 gene locus encodes two primary isoforms, E2F-3a and E2F-3b, which differ in their amino-termini. E2F-3a contains a DNA binding domain, a dimerization domain which determines interaction with differentiation regulated transcription factors (DRTFs), a transactivation domain enriched in acidic amino acids, a tumor suppressor protein association domain embedded within the trans-activation domain, and an additional cyclin binding domain. E2F-3a, as a transcriptional activator, binds DNA cooperatively with DRTFs through a common E2F recognition site, 5'-TTTC[CG]CGC-3' found in the promoter region of a number of genes whose products are involved in histone acetylase–deacetylase-mediated G(1)/S-specific gene expression, DNA replication or cell proliferation [18, 19]. These signaling cascades are complex as various combinations of DRTFs with E2F-3a (and other E2Fs) appear to form heterodimers that variably regulate cell-cycle progression [18–20]. Specific up-regulation of E2F-3a strongly correlates with the cellular proliferation, tumor stage, histological grade and size, at least in hepatocellular carcinoma [19], however this is the first report of a specifically coupled up-regulation of Bmi-1 and E2F-3 in several diverse glioma and GBM cell lines, and in 16 randomly sampled biopsied tissues (Figs. 3, 4). A recent report shows that over-expression of E2F-3a can partly rescue the proliferation inhibition caused by decreased abundance of miRNA-128 [15]. Further study of the transcriptional regulation of pre-miRNA-128, the coordinated oncogenic roles of ARP5 (ANGPTL6), Bmi-1 and E2F-3a in regulating apoptosis and cellular proliferation in brain cancer, and the mode of E2F-3a's dimerization with other DRTF family members should yield important mechanistic information into their roles in altered cell-cycle regulation and proliferative aspects of the GBM process.

In summary, these studies show a significant decrease in the expression of miRNA-128 in glioma and GBM cells and tissues that are not seen in cultured control cells and tissues, nor in other common human brain diseases. Decreases in the normally high abundance miRNA-128, coupled to significant increases in the expression of ARP5 (ANGPTL6), Bmi-1 and the transcription factor E2F-3a may help explain the undifferentiated, self-renewing state of brain cells, and de-regulated cell-cycle signaling pathways that support cellular proliferation in glioma and GBM. Importantly, miR-128 targeting of Bmi-1 and E2F-3a has already been shown using the appropriate constructs, but this has not yet been formally established for the ARP5 (ANGPTL6) gene. Dissection of the molecular-genetic mechanisms responsible for down-regulated miRNA-128 and coordinated up-regulation of ARP5 (ANGPTL6), Bmi-1 and E2F-3a, may provide novel therapeutic targets, including anti-miRNA or specific

transcription factor blocking strategies, useful for the clinical management of this lethal and devastating neurological disorder.

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