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Pleiotropic effects of miR-183~96~182 converge to regulate cell survival, proliferation and migration in medulloblastoma

Shyamal Dilhan Weeraratne¹, Vladimir Amani¹, Natalia Teider¹, Jessica Pierre-Francois¹, Dominic Winter², Min Jeong Kye^{1,2}, Soma Sengupta¹, Tenley Archer¹, Marc Remke^{3,4}, Alfa H.C. Bai³, Peter Warren², Stefan M. Pfister^{3,4}, Judith A.J. Steen^{1,2}, Scott L. Pomeroy¹, and Yoon-Jae Cho^{1,5,*}

¹Department of Neurology, Children's Hospital Boston and Harvard Medical School, Boston, MA 02115, USA

²Department of Pathology, Children's Hospital Boston, Boston, MA 02115, USA

³German Cancer Research Center (DKFZ), Heidelberg, Germany

⁴Heidelberg University Hospital, Department of Pediatric Hematology and Oncology, Heidelberg, Germany

⁵Department of Neurology and Neurological Sciences and Department of Neurosurgery, Stanford University School of Medicine, Stanford, CA 94305, USA

Abstract

Medulloblastomas are the most common malignant brain tumors in children. Several large-scale genomic studies have detailed their heterogeneity, defining multiple subtypes with unique molecular profiles and clinical behavior. Increased expression of the miR-183~96~182 cluster of microRNAs has been noted in several subgroups, including the most clinically aggressive subgroup associated with genetic amplification of *MYC*. To understand the contribution of miR-183~96~182 to the pathogenesis of this aggressive subtype of medulloblastoma, we analyzed global gene expression and proteomic changes that occur upon modulation of miRNAs in this cluster individually and as a group in *MYC*-amplified medulloblastoma cells. Knockdown of the full miR-183~96~182 cluster results in enrichment of genes associated with apoptosis and dysregulation of the PI3K/AKT/mTOR signaling axis. Conversely, there is a relative enrichment of pathways associated with migration, metastasis and epithelial to mesenchymal transition, as well as pathways associated with dysfunction of DNA repair in cells with preserved miR-183 cluster expression. Immunocytochemistry and FACS analysis confirm induction of apoptosis upon knockdown of the miR-183 cluster. Importantly, cell-based migration and invasion assays verify the positive regulation of cell motility/migration by the miR-183 cluster, which is largely mediated by miR-182. We show that the effects on cell migration induced by the miR-183 cluster are coupled to the PI3K/AKT/mTOR pathway through differential regulation of AKT1 and AKT2 isoforms. Furthermore, we show that rapamycin inhibits cell motility/migration in

*Corresponding Author: Yoon-Jae Cho, MD, Stanford University School of Medicine, Department of Neurology and Neurological Sciences, 1201 Welch Road, MSLS Bldg, Rm P213, Stanford, CA 94305, yjcho1@stanford.edu.

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medulloblastoma cells and phenocopies miR-183 cluster knockdown. Thus, the miR-183 cluster regulates multiple biological programs that converge to support the maintenance and metastatic potential of medulloblastoma.

Keywords

medulloblastoma; microRNA; miR183; miR182; miR96; EMT; AKT; mTOR; migration; apoptosis; DNA repair

Introduction

Medulloblastomas are aggressive tumors of primitive neuroectodermal origin. They are a major cause of cancer-related mortality in children [7]. Several genomic studies have revealed that medulloblastomas are heterogeneous tumors comprised of distinct molecular subgroups [4,10,25,41,52,44,45]. These studies have also shown the differential expression of microRNAs (miRNA) across various disease subtypes [10,40]. Notably, the miR-183~96~182 cluster is highly expressed in specific medulloblastoma subgroups, including one associated with *MYC* amplification and a particularly aggressive clinical course involving high rates of metastasis and overall poor survival [10].

miR-183~96~182 is a highly conserved cluster of miRNAs residing on an intergenic region of human chromosome 7q. Chromosome 7 is commonly gained in medulloblastoma and follows isochromosome 17q as the second most common recurrent large-scale genetic lesion observed in medulloblastomas [10]. The miR-183 cluster has largely been described in sensory organ development, particularly in retina and in hair cells of the inner ear [46,57,58]; but their expression has also been documented in several cancers including breast, ovarian, endometrial, bladder, prostate, lung, hepatocellular and thyroid [1,17,18,28,34,47,53,59,62,35,37]. Additionally, various components of the miR-183 cluster have been shown to regulate target genes such as FOXOs, BRCA1, EZRIN, ITGB1 and KIF2A [16,29,30,36,38,48,54]. However, the role of this miRNA cluster in tumor biology remains unclear. While most studies suggest that miR-183~96~182 has pro-tumorigenic/pro-metastatic effects in cancers, some have reported an inhibition of these effects [31,54,63]. Given the likelihood that thousands of genes are coordinately targeted by a single miRNA, only a fraction of which are expressed in any given normal or cancerous tissue, these transcriptional discrepancies highlight the importance of considering cellular and disease context in the study of miRNAs.

Here we examine the role of miR-183~96~182 in the context of *MYC*-amplified medulloblastoma. We base our investigations on cell lines that preserve high expression of miR-183~96~182 and importantly, the genetic and transcriptional signatures consistent with the clinically aggressive 'c1/*MYC*' medulloblastomas defined in our prior studies [10,51]. We employ a 'top-down' systems-level approach, documenting the global transcriptional and proteomic changes taking place upon modulation of the collective miR-183 cluster and also of its individual components. We verify our computational analyses with standard assays measuring cell viability, apoptosis, DNA repair and migration of medulloblastoma cells in vitro. Overall, we identify miR-183~96~182 as pro-tumorigenic in *MYC*-driven

medulloblastoma through inhibition of apoptosis, dysregulation of the mTOR pathway and modulation of cell migration and motility. These result in oncogenic processes that converge to sustain and enable the maintenance and progression of this aggressive tumor.

Materials and Methods

Cell culture

Medulloblastoma cell lines R262, R300 UW402 and UW426 were supplied by Dr. Michael Bobola, University of Washington, Seattle. D341, D384, D425, D458, D556 were obtained from Darrell Bigner, Duke University, North Carolina. D283 and DAOY medulloblastoma cell lines and HEK 293T cell line were purchased from American Type Culture Collection (Manassas, VA). Cells were maintained in Dulbecco's Modified Eagle Medium with F-12 nutrient mixture (Invitrogen, Carlsbad, CA) supplemented with 10% FBS, 2mM L-Glutamine, 1% penicillin and streptomycin (all from Gibco, Carlsbad, CA) at 37°C in a humidified chamber of 5% CO₂.

RNA extraction

Total RNA from cell lines was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA). RNA samples enriched for small RNA molecules were further enriched in the samples by using the Nirvana RNA Isolation kit (Ambion, Austin, TX) according to the manufacturer's protocol. RNA concentration was measured using a NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE). Total RNA for adult cerebellum and fetal cerebellum were purchased from BioChain (Hayward, CA).

Scratch assay

UW426 or DAOY cells were seeded at 200,000 per well in a six-well dish and transfected with LNAs or lentiviral overexpression constructs respectively. The confluent cell monolayer was scratched across the full well diameter with a sterile 200 µl pipette tip and dislodged cells were washed with PBS and replaced with fresh medium. In some cases 50nM rapamycin (Sigma) or control DMSO were added to the medium. Plates were then returned to the incubator for 24 hours. Cells were imaged under 10× magnification by a Nikon Eclipse TE 2000-S inverted microscope (Nikon Instruments, Inc., Melville, NY) at 0 and at 24 hours. Migration into the wounds was measured in an unbiased manner based on pixel intensity with Adobe Photoshop software.

Transwell matrigel invasion assay

100,000 transfected cells in 1% serum were seeded into upper chamber of a pre-hydrated Matrigel Invasion Chamber (BD Biosciences, Franklin Lakes, NJ). The cells were allowed to migrate to the underside of the membrane for 24 hours. After that, non-invading cells were removed by wiping the upper side of the membrane, and the invaded cells were fixed with methanol and stained by using crystal violet (Sigma, St Louis, MO, USA). Invaded cells were imaged by a Nikon Eclipse TE 2000-S inverted microscope at 10× magnification. To quantify the invaded cells, the matrigel matrix was dissolved in 10% acetic acid and the absorbance at 560nm was measured. All experiments were performed in triplicate.

Immunocytochemistry

All steps were carried out at room temperature as previously described [56]. Briefly, cells were plated on poly-D-lysine coated glass cover slips 72 hours post-transfection and were fixed in 4% (w/v) paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 1 hour, washed in phosphate-buffered saline (PBS, Invitrogen) (6 times, 5min each), then incubated for 1 hour in blocking buffer (PBS, 0.8% Triton X-100, 10% normal goat serum), followed by an overnight incubation in blocking buffer with the primary antibodies. The antibodies were purchased from the following companies: Anti-BAX, Anti-BAD, anti-RAD51 and anti-AKT1 (Santa Cruz Biotechnology, Santa Cruz, CA); AKT2 (Cell Signaling Technology, Danvers, MA); Anti-BAK1 and anti-BRCA1 (Calbiochem, Darmstadt, Germany); anti-Vimentin (BD Biosciences). The cells were washed in PBS (6 times, 5 min each) before the addition of fluorescent goat anti-rabbit and goat anti-mouse secondary antibodies (Alexa Ig488 [green] or Alexa Ig565 [red], Invitrogen) at 1:250 for 60 min. The cells were washed in PBS (6 times, 5 min each) and visualized with a Zeiss LSM510 META 2-Photon confocal microscope or a LSM700 confocal microscope (Zeiss, Oberkochen, Germany).

Cell Cycle analysis

Cells were harvested 72 hours post-transfection, washed in PBS and fixed with 70% ethanol overnight at 4 °C. Cells were treated with 100µg/ml RNase A (Roche Diagnostics, Mannheim, Germany) and 50µg/ml propidium iodide (Sigma) for 30 minutes at 37°C in the dark. Propidium iodide fluorescence was monitored with FACSCalibur flow cytometry (BD Biosciences). At least 10,000 cells were collected and analyzed with Cell Quest Pro Software (BD Biosciences) and FlowJo software version 4.6.1 (TreeStar, Ashland, OR).

Western blots

Membranes were blocked in PBS-Tween 5% milk and probed with primary antibodies at manufacturer recommended concentrations. Anti-β-actin (Abcam, Cambridge, MA) was used as the protein loading control. The HRP-conjugated secondary antibodies were used at 1:10,000 (Jackson ImmunoResearch Laboratories, West Grove, PA). Blots were developed using the SuperSignal West PICO Chemiluminescent Detection System (Pierce Biotechnology, Rockford, IL). Digital images were recorded using the Fuji Image 3000 Chemiluminescence Detection System (Fuji Film, Tokyo, Japan).

Senescence assay

To investigate senescence, cells were harvested 72 hours post transfection, fixed and stained using a Senescence associated β-galactosidase kit (Cell Signaling Technology) according to the manufacturer's instructions. Cells were visualized and captured with a Nikon E800 Epifluorescence microscope.

Real-time quantitative reverse transcription-PCR

The miRNA sequence-specific reverse transcription-PCR (RT-PCR) primers and assays for miR-96, miR-182, miR-183, miR-34a and endogenous control RNU6B were purchased from Applied Biosystems (Carlsbad, CA). TaqMan Gene Expression assays for NeuroD2 and

NeuroG1 were also purchased from Applied Biosystems. Analysis was carried out using the Applied Biosystems 7300 q-RT-PCR System. The gene expression dCT values of miRNAs in each sample were calculated by normalizing with the internal control RNU6B and relative quantification values were plotted. All reactions were done in triplicate and at least 3 independent experiments were performed to generate each data set.

Transfections

For the knockdown of miRNAs, Antisense Locked Nucleic Acid (LNA) oligonucleotides (Exiqon, Denmark) for miR-96, miR-182, miR-183 and the scrambled control were transfected at a final concentration of 100nM using Lipofectamine 2000 reagent (Invitrogen). For the pooled knock-down of the cluster, LNAs for miR-96, miR-182 and miR-183 were used in equal amounts for a final concentration of 100nM. For the overexpression of miRNAs, precursor sequences of miR96, miR182, miR183 (Sanger Institute, IDT) were cloned into the lentiviral vector pLVTHM (Addgene) and transiently transfected. In addition, human pre-microRNA Expression Construct Lenti-miR-183+96+182 (System Biosciences, Mountain View, CA) was used for overexpressing the entire miRNA cluster.

Lentiviral production and transduction

miRZip-anti-96, miRZip-anti-182, miRZip-anti-183 and the miRZip control vector (System Biosciences) were transfected into 293T cells and pseudovirus particles were generated according to manufacturer's instructions. Virus particles were concentrated using PEG-*it* Virus Precipitation Solution (System Biosciences). Subsequently, D458, D425 and D556 target cells were infected at a MOI of 2 in the presence of 5µg/ml polybrene (Sigma). Cells were FACS sorted for GFP positive cells and used for downstream assays.

Cell proliferation assay

Cells were monitored for cluster formation 120 hours post-infection. Clusters were visualized and captured by a Nikon Eclipse TE 2000-S inverted microscope and counted in 5 random fields for all the conditions tested. Bar graphs were generated by combining data from three independent experiments with each condition was plated at least in duplicate.

Comet assay

D458 cells were transfected with the pooled LNAs or the scrambled control LNA and DNA damage was analyzed 72 hours later using a Neutral Comet Assay (Trevigen, Gaithersburg, MD) detecting single and double stranded DNA breaks. The assay was carried out using the manufacturer's recommend protocol. Prior to imaging, 100µL of diluted SYBR® Green 1 was spotted onto each sample and incubated for 30 minutes. Comets were captured at a constant depth of the gel using an LSM700 confocal microscope (Zeiss) at a wavelength of 488nm. CometScore® software (The TriTek Corporation, Sumerduck, VA) was used to calculate the tail moment of the comets.

Gene expression and bioinformatics analysis

Gene expression data was generated from D458 and D556 medulloblastoma cells using Illumina HT12 Bead Arrays. GCT files were then generated from IDAT files using the GenePattern software suite (www.broadinstitute.org/cancer/software/genepattern). Data was further normalized using rank-invariant set normalization (GEO accession number pending). Gene expression and miRNA data from primary human tumors were generated as previously described [10]. Briefly, miRNA profiling was performed using a bead-based detection system containing 582 miRNA probes, representing 435 individual miRNAs as previously described [32]. Raw signal intensities were transformed from log2 to linear values and each miRNA probe was then normalized to the median and median polishing applied when necessary with a threshold cutoff for signal intensities less than 4. miRNA data was pre-processed and visualized using either the Genspring GX7.3.1 software package or GenePattern.

For comparison of transcriptional profiles between cell lines and primary tumors, we performed unsupervised hierarchical clustering of gene expression data from 15 established medulloblastoma cell lines and for comparison, a series of glioma and malignant rhabdoid tumor cell lines. We then utilized a subclass mapping algorithm developed by Hoshida et al. [21] to map clusters identified from the cell line clustering analyses to molecular subgroups of primary human medulloblastomas as described in Cho et al.[10] and Northcott et al.[41]

Gene set enrichment analysis (GSEA) was performed as previously described (www.broadinstitute.org/software/gsea) [49]. Briefly, we performed GSEA using a reference database of 2,163 gene sets: 1,891 gene sets from the MSigDB-C2 database (<http://www.broad.mit.edu/gsea/msigdb>) and 272 additional manually curated gene sets representing oncogene activation/tumor suppressor deregulation (OPAM database v3) [49,3]. To all gene sets we added sets that combined up and down regulated sets derived from the same experimental condition or publication. The final total was 2,599 signatures.

Proteomics

Cell pellets were lysed and proteins extracted and trypsin-digested in solution using optimized procedures (Winter and Steen, submitted). Peptides were labeled using a 6plex TMT kit (Thermo) following the manufacturer's instructions and samples combined and desalted using Oasis HLB cartridges (Waters). The desalted peptides were fractionated in 24 fractions using Offgel electrophoresis[20] and the resulting samples reduced in volume using a vacuum centrifuge. Peptide samples were analyzed using a LTQ-Orbitrap mass spectrometer, the raw files processed to text files, searched against IPI human v3.69 and the results combined.

Results

Characterization of miR-183~96~182 expression and 'group c1/MYC' signatures in medulloblastoma cell lines

We previously profiled the miRNA transcriptome of a series of primary medulloblastomas and revealed the elevated expression of miR-183~96~182 in several subgroups of this

disease [10]. Of particular interest was the high expression of miR-183~96~182 in a subgroup of medulloblastomas with *MYC* amplification and activation of photoreceptor transcriptional programs. These patients have high rates of disseminated disease at diagnosis, a significantly elevated rate of relapse and a very poor response to conventional chemotherapy and radiation therapy, even at maximal doses [10,41,51,43]. Here, we show that miR-183~96~182 expression is preserved in a series of established medulloblastoma cell lines (Figure 1a). A comparative genomic analysis shows that these cell lines share common transcriptional profiles with the 'c1/c5' or 'Group C' subgroups as defined in Cho et al. and Northcott et al., respectively (Figure S1) [10,41]. In addition, SNP array profiling shows that genetic features such as *MYC* amplification and isochromosome 17q are also preserved (data not shown). Our genomic characterization confirms that these cell lines constitute robust models for examining the contribution of this miRNA family in *MYC* amplified medulloblastoma.

miR-183~96~182 inhibits apoptosis and promotes survival in medulloblastoma cells

We utilized locked nucleic acid (LNA)-mediated knockdown of each component of the miR-183 cluster individually and in a pooled manner, followed by gene expression profiling using Illumina Bead Arrays and proteomic profiling using a tandem mass tag (TMT) approach. Given the partial homology between miR-183, miR-96 and miR-182, knockdown of these individual miRNAs results in a 'sponge' effect [14]. Specifically, knockdown of miR-96 also results in a measurable decrease in miR-183. Knockdown of miR-182 also decreases miR-183 levels but not miR-96; and knockdown of miR-183 leads to a decrease of all three miRNAs resulting in the equivalent of a 'pooled' knockdown (Figure 1b).

Using Gene Set Enrichment Analysis (GSEA) of gene expression data, we observe enrichment of gene sets associated with apoptosis pathways upon knockdown of miR-183~96~182 (Table1) [49]. To verify these findings, we assayed cell growth in cells where each individual component of the cluster was stably knocked-down. We note an overall decrease in cell viability and proliferation upon miR-183 cluster knockdown (Figure 1c). miR-96 knockdown (and pooled knockdown) achieves the most pronounced effect on cell viability with miR-182 knockdown having very little effect (see Figure 1c).

We investigated changes to cell cycle kinetics upon cluster knockdown and observe an increase of cells in G0/G1 and decreased transit through S-phase (Figure 2a). Furthermore, there is increased expression of the apoptotic markers BAX, BAK1 and BAD upon miR-183 cluster knockdown (Figure 2b). We also observe an accumulation of cells in G2 upon miR-183 cluster knockdown which suggests that a subset of cells is undergoing cellular senescence (see Figure 2a). Staining with beta-galactosidase confirms an increase in cellular senescence upon cluster knockdown consistent with observations in our cell cycle analysis (Figure S2).

miR-183~96~182 regulates DNA repair pathways in medulloblastoma cells

We identify enrichment of gene sets associated with dysfunction of DNA double strand break repair in scramble control cells relative to miR-183 cluster knockdown, findings consistent with a recent report showing BRCA1 as a direct target of miR-182 (see Table 1)

[36]. Using immunofluorescence staining for BRCA1, we show that knockdown of the miR-183 cluster results in an upregulation of BRCA1 (Figure 3a,b). Notably, BRCA1 staining is predominantly cytosolic and previous studies have shown that cytosolic BRCA1 localizes to mitochondrial membranes where it interacts with BCL2, serving to enhance apoptosis [55,26,23]. In addition, to assess whether the reduction of BRCA1 levels mediated by miR-183~96~182 expression resulted in a deficiency of DNA double strand break repair, we compared COMET assays on medulloblastoma cells with miR-183 cluster knockdown *versus* scrambled control. We identified an increase in the 'tail moment' of the COMET in the scrambled control relative to miR-183 cluster knockdown suggesting an increase in unrepaired DNA double strand breaks (Figure 3c,d).

miR-183~96~182 regulates cell migration and EMT in medulloblastoma cells

We note an overrepresentation of gene signatures associated with invasion and metastases in scramble control cells relative to miR-183~96~182 knockdown, and in particular relative to miR-182 knockdown (see Table 1). This suggests the miR-183 cluster promotes metastasis and invasion in the context of medulloblastoma. To validate these findings, we performed scratch assays and matrigel invasion assays using medulloblastoma cells having either robust or no expression of miR-183~96~182 with knockdown or forced expression of each or all miRNAs in the cluster, respectively. In cells with moderate levels of miR-183, miR-96 and miR-182 expression (UW426 cells), we find efficient closure of a scratch within 24 hours (Figure 4a). Knockdown of individual components of the miR-183 cluster shows that miR-182 knockdown significantly inhibits migration into the scratch area whereas miR-96 knockdown had little to no effect on the ability of these cells to close the insult (see Figure 4a). Invasion assays using a modified Boyden chamber with a matrigel barrier also showed an inhibition of migration/invasion (Figure 4b). In contrast to medulloblastoma cells that have increased expression of the miR-183 cluster, DAOY medulloblastoma cells have little to no expression of miR-183~96~182. In the scratch assay there is little to no closure of the scratch at 24 hours (Figure 4c). However, forced expression of the miR-183~96~182 cluster and of miR-182 in particular, results in a dramatic increase in cell migration and near complete closure of the scratch by 24 hours (see Figure 4c).

Consistent with the migration phenotype mediated by the miR-183~96~182 cluster, gene expression changes associated with epithelial to mesenchymal transition (EMT) are noted. These include upregulation of *Vimentin*, *TGFb2*, *SNAIL1*, *SNAIL2*, *HIF1a*, *COL5a2* and *WNT5a* and downregulation of *LAMA3* in scrambled control cells relative to miR-183 cluster knockdown (Figure 5a,b) [64,60,50,33,11,6]. We also identify enrichment of gene sets associated with VHL activity which, consistent with our results, has been shown to induce an EMT phenotype upon its loss [42,19]. Finally, our proteomics analysis identifies a greater than two-fold decrease in Desmin and NCAM after miR-183~96~182 knockdown, further supporting the role of the miR183 cluster in promoting EMT (Supplementary materials) [61,24,13,39,27,15,2].

Changes to cellular morphology are also observed upon knockdown of the miR-183 cluster consistent with a state change from a mesenchymal to a more epithelial phenotype. Upon miR-183 cluster knockdown, medulloblastoma cells take on a more flattened appearance

with projection of cellular processes indicative of neurite/axonal outgrowth (Figure 5c). Accordingly, we noted increased proneural gene expression in pooled knockdown conditions relative to scramble control (Figure 5d).

miR-183~96~182 regulates the PI3K/AKT/mTOR signaling axis

We identify enrichment of gene sets associated with PI3K/AKT/mTOR pathways upon knockdown of the miRNA cluster. This result was surprising given the increased apoptosis and decreased cell survival observed upon miR-183 cluster knockdown. Upon closer inspection, one of the AKT1 signatures enriched after miR-183 cluster knockdown has been shown to represent AKT1 signaling that is 'independent' of the mTOR pathway (see Table 1) [12]. Related to this observation, we also observe enrichment of a rapamycin gene signature upon knockdown of the miR-183 cluster, indicating that knockdown of miR-183~96~182 has a global transcriptional effect comparable to rapamycin treatment. This suggests that the AKT1 signature observed upon miR-183 knockdown is uncoupled from mTOR signaling and presumably allows for the induction of apoptosis which AKT1 otherwise inhibits.

To investigate the exchange between miR-183~96~182 and PI3K/AKT/mTOR signaling suggested by our GSEA results, we performed Western blot analysis and/or immunofluorescence for various AKT isoforms. We show that pooled knockdown of the miR-183 cluster induces AKT1 expression. Conversely, decreased AKT2 expression is noted upon miR-183~96~182 knockdown (Figure 6a,b). These findings are consistent with reports that have identified divergent roles of the AKT1/2 isoforms in regulating migration and EMT, where AKT1 inhibited migration through phosphorylation of palladin [9] and AKT2 induced migration/EMT [22,8,9]. Thus, the divergent AKT1/AKT2 expression in our experiments is wholly consistent with our results showing the induction of cell migration/invasion by miR-183~96~182.

To investigate whether pharmacological inhibition of the mTOR pathway could also affect the phenotypes seen in our migration and invasion assays, we treated medulloblastoma cells with rapamycin. We show a distinct inhibition in migration upon rapamycin treatment similar to cells in which the miR-183 cluster was knocked down (Figure 6c). Conversely, when we force expression of miR-183 cluster in DAOY cells and treat with rapamycin, we show an inhibition of migration (Figure 6d). These results suggest that the effects of cell migration induced by the miR-183 cluster rely on intact mTOR pathway signaling. In combination, our results suggest that mTOR positively regulates both the upstream factors responsible for miR-183 cluster expression and also the downstream factors that enable miR-183 cluster-mediated migration/EMT.

Discussion

Here we provide evidence that the miR-183~96~182 cluster promotes tumorigenesis in medulloblastoma by regulating multiple biological processes relied upon for maintenance, survival and dissemination of tumor cells. Our data also indicate that each individual miRNA within this enigmatic cluster has specific properties that alone can confer specific phenotypes to cells. This is particularly evident with miR-182 which promotes cellular

migration on forced expression in cells with an otherwise limited capacity for migration; and with miR-96 whose knockdown results in a much more robust growth inhibition than miR-182 or miR-183. But the combined biological effects of all three miRNAs as a unit are especially intriguing as they are co-transcribed/expressed off one primary miRNA transcript, and, in addition, are highly conserved across species.

Our global transcriptional profiling suggests that a predominant function of miR-183~96~182 is to regulate the cellular response to growth/nutritional signals and cellular stress by fine tuning biological actions such as DNA repair, apoptosis, senescence proliferation and migration/motility. Under normal biological circumstances, expression of the miR-183 cluster is largely restricted to ciliated sensory neurons whose longevity and consistent functions are relied upon for survival at the whole organism level. This suggests that the miR-183 cluster is part of an evolutionary mechanism that has been established to support the maintenance and longevity of sensory neurons. Certainly, the neuronal degeneration witnessed when these miRNAs are disrupted in rodents and humans supports miR-183~96~182's critical role in maintaining neuronal health. It may be that cancer usurps the abilities of these miRNAs to its survival advantage.

Interestingly, we were able to counter the pro-migratory effects of the miR-183 cluster with rapamycin treatment. However, we also showed that rapamycin treatment alone decreased the expression levels of miR-183~96~182. Identifying the feedback circuits that link the upstream and downstream regulation of this miRNA cluster will be informative. Indeed, there is marked conservation of the upstream regulatory region of the miR-183 cluster and prior ChIP-on-chip analyses have shown enrichment of this promoter region upon immunoprecipitation of the medulloblastoma oncogene, OTX2 [5] (M. Kool, personal communication). At a minimum, incorporation of mTOR inhibitors as a treatment or adjuvant for medulloblastomas expressing miR-183~96~182 should be considered.

In summary, we have examined the global pathogenetic mechanisms mediated by miR-183~96~182 in medulloblastoma using a combination of computational and bench approaches. Our strategy has taken into consideration the complex, systems-level regulation of biological functions regulated by miRNAs in a context-dependent manner, revealing pathways critical to the survival and maintenance of medulloblastoma and mechanisms by which metastatic dissemination of this disease occurs. Targeting of the upstream and downstream pathways regulating this pro-tumorigenic/ pro-metastatic miRNA cluster could provide a therapeutic avenue for this otherwise lethal childhood disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

miR-183~96~182 is differentially expressed in medulloblastoma cell lines and regulates cell proliferation. **a** Quantitative Real Time PCR (q-RT-PCR) analysis of miR-183, miR-96, and miR-182 expression in medulloblastoma cell lines. Raw data were normalized to the endogenous control *RNU6B*. The errors bars were derived from 3 independent experiments done in triplicate. **b** LNA-mediated knockdown of individual components of the miR-183~96~182 cluster results in a sponge effect. **c** LNA-mediated knockdown of individual or pooled components of the miR-183 cluster results in decreased cluster formation and overall cell viability. Bar graphs reflect combined data from three independent experiments with each condition plated at least in duplicate.

Figure 2.

Knockdown of miR-183~96~182 cluster induces apoptosis and G1 and G2 arrest. **a** Cell cycle analysis of medulloblastoma cells transfected with LNAs directed towards individual components of the miR-183 cluster shows an increase in the G0/G1 and G2 fractions upon miR-183~96~182 pooled knockdown and miR-96 knockdown in particular. **b** Immunofluorescence staining shows an increase of apoptotic markers BAD and BAK1 (red) and BAX (green) after miR-183~96~182 knockdown; all cells counterstained with DAPI (blue). P-values (Fisher's exact test) indicate significance relative to scramble control, *p-value<0.05, **p-value<0.01, ns=not significant.

Figure 3.

miR-183~96~182 inhibits double strand break repair. **a** Immunofluorescence staining and western blot (**b**) of BRCA1 (red) and DAPI counterstain (blue) shows increased, predominantly cytoplasmic BRCA1 staining in D458 cells upon knockdown of miR183~96~182. **c** Representative COMET assay showing decreased 'tail moment' in the miR-183 cluster knockdown compared to the scrambled control. **d** Dot plot indicative of a decreased level of DNA double-strand breaks in miR-183 cluster knockdown relative to scrambled control (***P-value<0.0001).

Figure 4. miR-182 induces cell migration and invasion in medulloblastoma cells. **a** Scratch/wound healing assay in UW426 medulloblastoma cells shows knockdown of miR-182 inhibits wound closure. Notably, knockdown of miR-183 results in a sponge effect-mediated reduction of miR-182 leading to impeded wound healing. **b** Representative images from a transwell matrigel invasion assay shows reduction of cell invasion upon knockdown of miR183~96~182. Migrated cells are quantified in the bar graph reflecting data from three independent experiments. **c** Scratch assay after stable overexpression of individual or pooled miR-183~96~182 cluster components in DAOY cells shows that miR-182 alone is sufficient

to enhance scratch closure. P-values (Fisher's exact test) indicate significance relative to scramble control, ***p-value<0.0001.

Figure 5.

miR-183~96~182 promotes epithelial to mesenchymal transition (EMT). **a** Heatmap showing genes associated with EMT are downregulated upon miR-183~96~182 knockdown in D458 cells. **b** Immunofluorescence staining and Western Blot show decreased expression of vimentin in D458 cells after miR-183~96~182 knockdown. **c** Increased neurite/axonal outgrowth and elevated TUJ1 staining upon knockdown of miR-183~96~182. **d** Knockdown of miR-183~96~182 cluster induces the expression of pro-neural genes NeuroD2 and NeuroG1. P-values (Fisher's exact test) indicate significance relative to scramble control, **p-value<0.01, ***p-value<0.0001.

Figure 6.

miR-183~96~182 cluster regulates the PI3K/AKT/mTOR signaling axis. **a**

Immunofluorescence staining shows elevated AKT1 (red) and decreased AKT2 (green) upon knockdown of the microRNA cluster in D458 medulloblastoma cells. **b** Western Blot showing increased AKT1 and decreased AKT2 in pooled miR-183~96~182 knockdown compared to the scrambled control. **c** Increased migration upon forced expression of miR-183~96~182 cluster in DAOY cells is negated by rapamycin. **d** Impeded migration upon rapamycin treatment in UW426 cells comparable to the effects of miR-183~96~182 knockdown. Bar graphs represent migration into wounds measured in an unbiased manner

based on pixel intensity. P-values (Fisher's exact test) indicate significance relative to scramble control, **p-value<0.01, ***p-value<0.0001.

Table 1

Selected gene sets enriched in D458 and D556 medulloblastoma cell lines treated with LNAs antagonizing miR-96, miR-182 or miR-183/96/182 (pooled). Pathways associated with apoptosis (red), DNA repair (blue), migration/metastasis/EMT (green) and PI3K/AKT/mTOR (yellow) are highlighted.

Gene sets enriched in scrambled control relative to KD			Gene sets enriched in KD relative to scrambled control		
GENE SET NAME	NES*	NOM p-val*	GENE SET NAME	NES*	NOM p-val*
SCHLOSSER_MYC_TARGETS_REPRESSED_BY_SERUM	-1.7850131	0	REACTOME_INTRINSIC_PATHWAY_FOR_APOPTOSIS	1.5348431	0
PUJANA_BRCA_CENTERED_NETWORK	-1.7782083	0	JIANG_HYPOXIA_VIA_VHL	1.4970423	0
KEGG_HOMOLOGOUS_RECOMBINATION	-1.6971726	0	PENG_RAPAMYCIN_RESPONSE_UP	1.4693013	0
EHLERS_ANEUPLOIDY_UP	-1.6958622	0	HU_ANGIOGENESIS_UP	1.4663571	0
PUJANA_BREAST_CANCER_WITH_BRCA1_MUTATED_UP	-1.6822959	0	SCHUETZ_BREAST_CANCER_DUCTAL_INVASIVE_DN	1.4350308	0
MILI_PSEUDOPODIA_HAPTOTAXIS_UP	-1.6714127	0	PARK_APL_PATHOGENESIS_DN	1.4250693	0
PUIFFE_INVASION_INHIBITED_BY_ASCITES_DN	-1.6329579	0	GESERICK_TERT_TARGETS_DN	1.3926878	0.10700389
MILI_PSEUDOPODIA_CHEMOTAXIS_UP	-1.594972	0	BIOCARTA_AKT_PATHWAY	1.3910866	0
WINNEPENINCKX_MELANOMA_METASTASIS_UP	-1.5919378	0	SCHLOSSER_MYC_AND_SERUM_RESPONSE_SYNERGY	1.3682749	0.09215687
SHEN_SMARCA2_TARGETS_UP	-1.5806684	0	OUYANG_PROSTATE_CANCER_PROGRESSION_DN	1.8437006	0
GUENTHER_GROWTH_SPHERICAL_VS_ADHERENT_DN	-1.5804006	0	BIOCARTA_MITOCHONDRIA_PATHWAY	1.644545	0
CLASPER_LYMPHATIC_VESSELS_DURING_METASTASIS_UP	-1.5683563	0	BIOCARTA_DEATH_PATHWAY	1.607138	0
CHANDRAN_METASTASIS_TOP50_UP	-1.5505456	0	REACTOME_AUTODEGRADATION_OF_CDH1_BY_CDH1_APC	1.5974913	0
VERRECCHIA_RESPONSE_TO_TGFB1_C1	-1.5343549	0	BIOCARTA_INSULIN_PATHWAY	1.5460787	0
KAUFFMANN_DNA_REPAIR_GENES	-1.5199604	0	REACTOME_INTRINSIC_PATHWAY_FOR_APOPTOSIS	1.5000709	0
COULOUARN_TEMPORAL_TGFB1_SIGNATURE_UP	-1.5043387	0	BIOCARTA_CASPASE_PATHWAY	1.4638826	0
REACTOME_HOMOLOGOUS_RECOMBINATION_REPAIR	-1.6519243	0			
TANG_SENESCENCE_TP53_TARGETS_DN	-1.6312816	0			
REACTOME_DOUBLE_STRAND_BREAK_REPAIR	-1.5398794	0			
CHIANG_LIVER_CANCER_SUBCLASS_CTNNB1_UP	-1.5113037	0			
ONDER_CDH1_KNOCKDOWN_UP	-1.4969212	0			
ALONSO_METASTASIS_EMT_UP	-1.4837567	0			
VERNELL_RETINOBLASTOMA_PATHWAY_UP	-1.6657789	0	BARRIER_CANCER_RELAPSE_NORMAL_SAMPLE_DN	1.6420163	0
SHEN_SMARCA2_TARGETS_UP	-1.6654809	0	CREIGHTON_AKT1_SIGNALING_VIA_MTOR_UP	1.6079166	0
MILI_PSEUDOPODIA_HAPTOTAXIS_UP	-1.6512823	0	POMEROY_MEDULLOBLASTOMA_PROGNOSIS_UP	1.5818696	0
PUIFFE_INVASION_INHIBITED_BY_ASCITES_DN	-1.6266841	0	PENG_RAPAMYCIN_RESPONSE_UP	1.43558	0
SCHLOSSER_MYC_TARGETS_REPRESSED_BY_SERUM	-1.6057379	0	HENDRICKS_SMARCA4_TARGETS_UP	1.3748474	0
BIOCARTA_PDGF_PATHWAY	-1.5912979	0	JIANG_VHL_TARGETS	1.3583958	0
WELCSH_BRCA1_TARGETS_1_UP	-1.584182	0	SA_CASPASE_CASCADE	1.8422506	0
PUJANA_BRCA_CENTERED_NETWORK	-1.5646061	0	BIOCARTA_INSULIN_PATHWAY	1.7745755	0
RAMALHO_STEMNESS_UP	-1.5606577	0	LEE_TARGETS_OF_PTCH1_AND_SUFU_UP	1.6877975	0
SCIAN_CELL_CYCLE_TARGETS_OF_TP53_AND_TP73_DN	-1.348773	0	BIOCARTA_NGF_PATHWAY	1.6625682	0
			BIOCARTA_IGF1R_PATHWAY	1.6512883	0
			WELCSH_BRCA1_TARGETS_1_DN	1.523696	0.07802875
REACTOME_FANCONI_ANEMIA_PATHWAY	-1.7325499	0	WILLIAMS_ESR1_TARGETS_UP	1.7721083	0
SCHLOSSER_MYC_TARGETS_REPRESSED_BY_SERUM	-1.7144573	0	SCHLOSSER_MYC_AND_SERUM_RESPONSE_SYNERGY	1.5386548	0
REACTOME_DNA_REPAIR	-1.6950321	0	WALLACE_JAK2_TARGETS_UP	1.5234002	0
MILI_PSEUDOPODIA_HAPTOTAXIS_UP	-1.6289537	0	DAIRKEE_TERT_TARGETS_UP	1.4899685	0
SHEN_SMARCA2_TARGETS_UP	-1.6064231	0	SCHUETZ_BREAST_CANCER_DUCTAL_INVASIVE_DN	1.4655753	0.08974359
PUIFFE_INVASION_INHIBITED_BY_ASCITES_DN	-1.5878445	0	CREIGHTON_AKT1_SIGNALING_VIA_MTOR_UP	1.4366912	0
QUELLET_CULTURED_OVARIAN_CANCER_INVASIVE_VS_LMP_UP	-1.5698702	0	PARK_APL_PATHOGENESIS_DN	1.3963195	0
KARAKAS_TGFB1_SIGNALING	-1.5482111	0	KEGG_REGULATION_OF_AUTOPHAGY	1.3795631	0
KYNG_DNA_DAMAGE_UP	-1.5304508	0	JIANG_VHL_TARGETS	1.3662833	0
ALONSO_METASTASIS_UP	-1.5295339	0	AMIT_SERUM_RESPONSE_480_MCF10A	1.3607559	0
LEE_METASTASIS_AND_RNA_PROCESSING_UP	-1.6389884	0	REACTOME_INTRINSIC_PATHWAY_FOR_APOPTOSIS	1.3417903	0.08917198
FIRESTEIN_CTNNB1_PATHWAY	-1.6293223	0			
STANELLE_E2F1_TARGETS	-1.5891911	0			
WNT_SIGNALING	-1.5705578	0			
CHIANG_LIVER_CANCER_SUBCLASS_CTNNB1_UP	-1.4873686	0			
TANG_SENESCENCE_TP53_TARGETS_DN	-1.4816539	0			

*NES = normalized enrichment score; Nom p-val = nominal p-value