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MicroRNA profiling in human medulloblastoma

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Medulloblastoma is an aggressive brain malignancy with high incidence in childhood. Current treatment approaches have limited efficacy and severe side effects. Therefore, new risk-adapted therapeutic strategies based on molecular classification are required. MicroRNA expression analysis has emerged as a powerful tool to identify candidate molecules playing an important role in a large number of malignancies. However, no data are yet available on human primary medulloblastomas. A high throughput microRNA expression profiles was performed in human primary medulloblastoma specimens to investigate microRNA involvement in medulloblastoma carcinogenesis. We identified specific microRNA expression patterns which distinguish medulloblastoma differing in histotypes (anaplastic, classic and desmoplastic), in molecular features (ErbB2 or c-Myc overexpressing tumors) and in disease-risk stratification. MicroRNAs expression profile clearly differentiates medulloblastoma from either adult or fetal normal cerebellar tissues. Only a few microRNAs displayed upregulated expression, while most of them were downregulated in tumor samples, suggesting a tumor growth-inhibitory function. This property has been addressed for miR-9 and miR-125a, whose rescued expression promoted medulloblastoma cell growth arrest and apoptosis while targeting the proproliferative truncated TrkC isoform. In conclusion, misregulated microRNA expression profiles characterize human medulloblastomas, and may provide potential targets for novel therapeutic strategies.

Key words: microRNA; medulloblastoma; miRNA-125a; miRNA-9

Medulloblastoma (MB) is the most frequent brain malignancy observed in childhood and originates from aberrant development of cerebellar progenitor neurons.[1,2] However, the molecular aspects of its tumorigenic pathways are so far poorly understood. MB multimodal treatments (surgical resection, chemotherapy and/or radiotherapy) have improved survival; nevertheless, it is still incurable in about a third of cases and survivors commonly have severe treatment-induced long-term side effects.[3] Novel and risk-adapted therapeutic strategies are thus needed and an appropriate molecular classification is essential to improve assignment to disease risk classes and to identify subsets of patients responsive to targeted therapies. MBs are genetically and epigenetically heterogeneous and are associated with several molecular alterations that can be exploited as prognostic markers.[4] Recently, a high-throughput complementary DNA (cDNA) microarray analysis of human primary MBs suggested different subsets of tumors based on clustered gene-expression patterns.[5] These specific mRNA expression signatures are related with known or still unknown signal transduction pathways, which are potential targets for specific therapies in distinct subsets of tumors.[5]

In addition to protein-encoding genes, a second class of genes producing small noncoding RNAs (*i.e.* microRNAs) has been discovered over the last few years.[6] These short RNAs (18- to 24-nucleotides) bind to cis-regulatory elements mainly present in the 3′ UTR of mRNAs, resulting in translational inhibition or mRNA degradation.[6] MicroRNAs (miRNAs or miRs) have emerged as important regulatory factors involved in developmental processes, such as neural progenitor cell growth and differentiation.[7] The critical role played by miRNAs is also suggested by their altered expression observed in a large number of malignancies.[8] In addition, the ability of some miRNAs to target oncogenes or oncosuppressors indicates their role in tumorigenesis.[8]

Although miRNAs play a crucial role in nervous system development, so far specific changes in their expression patterns have been described only in neural crest-derived neuroblastoma[9] and glial cell-derived gliomas,[10,11] while a few cell lines were analyzed from neuronal tumors of the central nervous system (CNS).[12]

Here, we report the first miRNA expression profiling of human primary MB, a CNS tumor that has an ontogenetic cell lineage different from neuronal crest-derived cells and glial progenitors. We show that specific miRNA signatures distinguish tumors from either adult or fetal normal tissues and identify distinct classes of MB. Typical miRNA expression patterns classify MB histotypes, correlating with tumor molecular features and disease risk stratification. Most miRNAs display overall downregulated expression in MB, suggesting a tumor growth-inhibitory function. This property has been validated for miR-9 and miR-125a, whose rescued expression promotes MB cell growth arrest and apoptosis while targeting the proproliferative truncated TrkC (t-TrkC) isoform. We suggest that miRNA expression signatures identify human MBs and may indicate targets for novel therapeutic strategies.

Material and methods

MB and control tissues

Surgical specimens of primary MBs were collected from 34 patients with Institutional Review Board approval. The clinical

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TABLE I – SUMMARY OF THE CLINICAL, PATHOLOGICAL AND MOLECULAR CHARACTERISTICS OF THE 34 MB SAMPLES INVESTIGATED FOR 86 microRNAs

Variable	Number of cases[1]
Age	
<3 years	19
>3 years	15
Gender	
Male	29
Female	5
Tumor material	
Primary	34
Histology	
Desmoplastic	11
Classic	11
Anaplastic/large cell	12
Stage	
T1–4, M0	30
T1–4, M1	4
Surgery resection	
Complete	29
Partial	5
Molecular features	
C-MYC overexpression	18[2]
ErbB2 overexpression	13[2]
Follow-up, 3 years	
Alive	13
Dead of disease	13
Unknown	8
Risk	
Low-average	18
High	16

[1]T1, T2, T3, T4, T5, T6, T7, T8, T9, T10, T11, T12, T14, T23, T15, T16, T17, T18, T19, T20, T21, T22, T24, T25, T26, T27, T28, T29, T30, T31, T32, T34, T35, T36.–[2]Nine tumors showed both c-Myc and ErbB2 overexpression (T1, T8, T10, T12, T16, T22, T23, T24, T26), while 12 tumors were not overexpressing both c-Myc and ErbB2 (T2, T4, T5, T14, T15, T20, T21, T27, T28, T29, T34, T35).

and histopathology details of patients are summarized in Table I. RNA of normal human cerebellum (9 adult samples from 25- to 70-year-old subjects and 5 samples from 22- to 36-week- old fetuses) was purchased from Biocat (Heidelberg, Germany), Ambion (Applied Biosystems, Foster City, CA) and BD Biosciences (San Jose, CA).

Expression profile analysis of microRNA and quantitative real-time PCR

RNA isolation was performed using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Assays for 248 miRNAs and for 2 small RNAs, RNU6B and RNU66 were carried out according to Applied Biosystems protocols (Foster City, CA), including reverse transcription (RT) and quantitative real-time PCR (Q-PCR). RT reactions (10 ng of RNA, specific stem-loop primers for each miRNA, 1× buffer, dNTPs, reverse transcriptase and RNase inhibitor) were incubated in an Applied Biosystems 9700 Thermocycler in 96-well plate for 30 min at 16°C, 30 min at 42°C, 5 min at 85°C and then held at 4°C. Then, Q-PCR for the miRNA measure were performed using the miRNA-specific TaqMan MGB probe and TaqMan universal master mix in an Applied Biosystems 7900HT PCR system.

The reproducibility of the assay was evaluated by measuring interassay variation. The coefficient of variation was calculated from 4 independent samples analyzed on independent PCR reaction plates and showed a variation in the Ct values of 2.1–3.0%. miRNAs expression levels were normalized to 2 internal control small RNAs (RNU6B and RNU66), obtaining similar results. The comparative threshold cycle method was used to calculate the relative miRNAs expression. The expression level of each miRNA has been compared to the mean of all adult control samples represented as unitary value. To determine a molecular profile of all samples, 1 μg of RNA was reverse transcribed to cDNA, as previously described.[13] RT-Q-PCR analysis of ErbB2, c-MYC, the full-length (fl-TrkC) and the t-TrkC mRNAs was performed on cDNAs using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) with TaqMan Gene Expression Assays (ErbB2, c-Myc) and designed (fl-TrkC, F5′-ccc tca tca tgg tct ttg aat aca-3′, R5′-cca tgg gcc ctg agg aa-3′ and t-TrkC, F5′-ccc agt act tcc gtc agg ga-3′, R5′-ttt gaa aag acc cac gtg tcc-3′) primers. Each amplification reaction was performed with TaqMan Universal PCR Master Mix (ErbB2, c-Myc) or with SYBR Green-based PCR Master Mix (fl-TrkC and t-TrkC) in triplicate, and the average of the 3 threshold cycles was used to calculate the amount of transcript in the sample. mRNA quantification was expressed in arbitrary units as the ratio of the sample quantity to that of the mean values of adult control samples. All values were normalized to 3 endogenous controls, GAPDH, β-actin, and HPRT.

Statistical analysis

Results are expressed as median values with minimal and maximal range values. Statistical differences were analyzed by the Kruskall-Wallis rank sum test using R 2.5.0 (R Foundation for Statistical Computing, Vienna, Austria). Because of the small size of tumor subsets investigated, significance levels were corrected for multiplicity using the MTP function in the multitest package, with the method described by van der Laan *et al.*[14] Adjusted *p*-values are reported. An adjusted *p*-value of less than 0.05 was considered as statistically significant, leading to strong control of the familywise error rate at level 0.05. This implies that among the rejected hypotheses there are no false rejections with probability at least 0.95. For histotype, a Tukey's Honestly Significant Differences (HSD) method at level 0.95 was used to produce confidence intervals for the differences between each pair of means corresponding to the levels of the factor.

Hierarchical clustering analysis was performed on the miRNAs expression levels, representing the values as natural logarithms. In Figure 1*a*, all miRNAs were represented using the UPGMA (unweighted average) clustering method, and correlating by Euclidean distance (Spotfire software, Spotfire AB, Göteborg, Sweden). For Figures 2 and 3, dendrograms represent complete linkage clustering (by Euclidean distance) between sample subsets and selected miRNA expression levels.

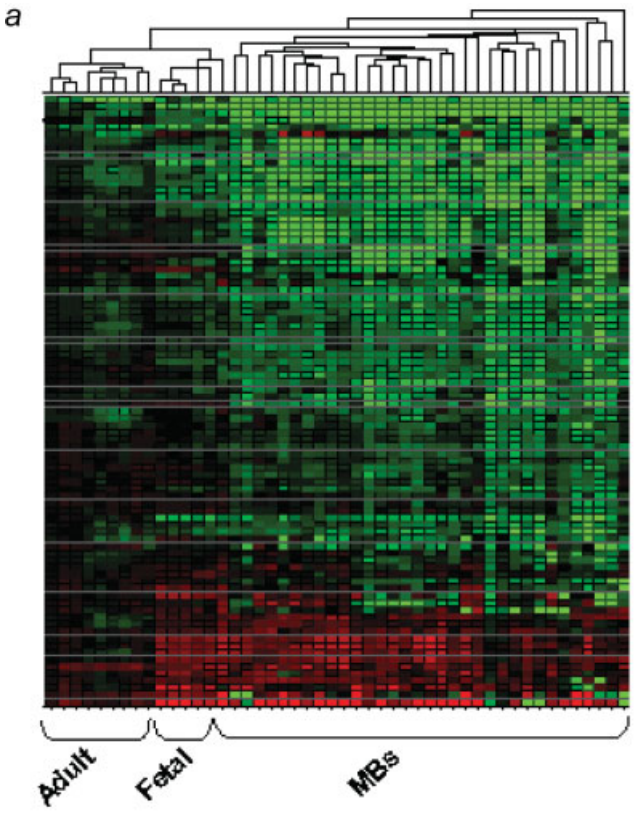
Cloning of miR-9, miR-125a, miR-100 and truncated isoform of TrkC vectors

Genomic fragments containing the pre-miR-9-3 (from −21 to +93 relative to the 5′ end of miR-9), the pre-miR-125a (from −14 to +72) and the pre-miR-100 (from −12 to +68) were PCR-amplified and cloned into a vector carrying the constitutive expression cassette of the snRNA U1 gene, to generate the constructs overexpressing miRNAs.[9] A control plasmid (Ctrl) producing an unrelated 21-nt-long RNA,[9] bearing no homology to any known miRNA or mRNA sequence in human, was used as a control in the transfection experiments. Vectors for the t-TrkC and siRNA t-TrkC were generated as previously reported.[9]

Cell culture, treatments and miRNAs overexpression and knockdown

D283 Med and Daoy human MB cells were cultured in MEM medium (Gibco, Invitrogen), supplemented with 20% or 10% FCS, sodium pyruvate, nonessential aminoacids, penicillin/streptomycin and L-glutamine. All-*trans*-retinoic acid (RA, Sigma, St. Louis, MO) treatments (1, 2 and 5 μM) of D283 Med cells were carried out for 24, 48 and 96 hr.

Vectors were transfected in D283 cells with Lipofectamine 2000, according to manufacturer's instruction (Invitrogen) for Northern blot, proliferation and luciferase assays. Transfections were also performed in Daoy cells with Lipofectamine Plus reagent according to manufacturer's instruction (Invitrogen) for col-



b

MB vs Adult and Fetal		MB vs Adult	MB vs Fetal
Downregulated miRs			
let-7a*	miR-145*	miR-7	miR-9*
let-7b*	miR-149*	miR-29a*	miR-125b*
let-7d*	miR-150	miR-29b	miR-135a
let-7e*	miR-153	miR-187	miR-155*
let-7f*	miR-154		miR-203
let-7g*	miR-181b*		miR-221*
let-7i*	miR-181c*		miR-361
miR-22*	miR-190		miR-372*
miR-26a*	miR-191		
miR-26b*	miR-192		
miR-29c*	miR-194		
miR-30b	miR-212*		
miR-30c	miR-219		
miR-31*	miR-320		
miR-98*	miR-323		
miR-103*	miR-324-3p		
miR-107*	miR-324-5p		
miR-124a*	miR-326		
miR-125a*	miR-328		
miR-127*	miR-330		
miR-128a*	miR-331		
miR-128b*	miR-346		
miR-129	miR-370		
miR-132*	miR-381*		
miR-133b	miR-382*		
miR-134*	miR-383		
miR-138*	miR-425		
miR-143*			
Upregulated miRs			
miR-17-5p*		miR-18a*	
miR-148a		miR-19a*	
miR-199b		miR-20a*	
		miR-21*	
		miR-25*	
		miR-106a*	
		miR-106b*	
		miR-137	

Figure 1 – miRNA profiling of MBs and normal cerebellar tissues. (*a*) Unsupervised hierarchical clustering analysis was performed among the selected miRNAs expressed in MB samples. Dendrogram representing the results of hierarchical clustering analysis distinguishes MBs from adult and fetal normal tissues. miRNAs are in rows and tumor samples are in columns. The relative expression of each miRNA was determined by real-time PCR. A green–red color scale (−9 to +8) depicts normalized miRNAs expression level on a log scale. (*b*) Neuronal miRNAs and/or onco-miRNAs that are either significantly downregulated or upregulated in MBs. Column 1: miRNAs significantly dysregulated in MBs with respect to both adult and fetal controls. Column 2: miRNAs significantly dysregulated compared only to adult controls. Column 3: miRNAs significantly different with respect to only fetal controls. Differentially expressed miRNAs were selected by Kruskall-Wallis rank sum test. Further details on modulation and statistical significance are reported in Supporting Tables III and IV. (*previously described onco-miRNAs).

ony formation and transferase-mediated biotinylated UTP nick end labeling (TUNEL) assays.

For miR-9, miR-125a and miR-100 knockdown, FITC-labeled miRNA specific and scrambled control LNA oligonucleotides (Exiqon, Vedbaek, Denmark) were transfected by hyperfect reagent (Qiagen, Hilden, Germany) in D283 Med cells.

Cell proliferation and apoptosis assays

Cell proliferation was evaluated by bromodeoxyuridine (BrdU) labeling assay (Labeling and Detection Kit I, Roche, Basel, Switzerland) after transfection with miRNA or t-TrkC or its siRNA constructs, together with a 10-fold lower amount of a GFP expression vector to monitor-transfected cells, or LNAs as previously described.[9] Cells were incubated with 10 μM BrdU for 8–12 hr to allow BrdU incorporation, and then were fixed for 20 min at room temperature in 4% paraformaldehyde (Sigma, St. Louis, MO). BrdU labeling was performed following the manufacturer's instructions. Texas red-conjugated goat anti mouse IgG (Jackson ImmunoResearch, West Grove, PA) was used as a secondary antibody. Nuclei were counterstained with Hoechst reagent. The number of BrdU-positive nuclei was recorded analyzing BrdU and GFP or FITC double-positive cells from 3 repeated experiments.

For colony formation assays, 1.4×10^6 Daoy cells were transfected with miRNA-expressing plasmids in 10-cm diameter dishes, and selected with G418 (600 μg/ml Sigma). After 2 weeks of selection, cell colonies were counted following staining in 20% methanol and crystal violet.

For anchorage-independent growth in soft agar assay, 1×10^5 D283 Med cells were transfected with miRNA-expressing plasmids, suspended in culture medium containing 0.3% agarose (Invitrogen) and G418, and plated over a layer of 0.7% agarose in the same culture medium. Twenty-one days after plating, colonies of >50 cells were scored in 10 representative fields in each plate. All assays were performed in triplicate, and the results shown are the average of 3 independent experiments.

Apoptosis was measured in cells 48 hr after transfection, by means of the terminal deoxynucleotidyl TUNEL assay (Cell Death Detection Kit, Roche, Welwyn Garden City, UK), according to the manufacturer's instructions.

Northern blot analysis

Total RNA was fractionated on a 10% polyacrilamide-urea gel and electroblotted to a nylon membrane. DNA-oligos complementary to the sequences of mature microRNAs (a9: 5′-TCATACAGCTAGATAACCAAAGA-3′; a125a: 5′-CACAGGTTAAAGGGTCTCAGGGA-3′; a100: 5′-CACAAGTTCGGATCTACGGGTT-3′) and to U2-snRNA (U2R: 5′-GGGTGCACCGTTCCTGGAGGTAC-3′) were ^{32}P-labeled and used as probes. The expression levels of miRNAs were normalized against the U2 snRNA signals.

Luciferase activity assay

MiR-9 and miR-125a t-TrkC 3′ untranslated region (3′UTR)-luciferase assay was performed as previously described.[9] Briefly,

a

	Desmoplastic			Classic			Anaplastic				distinguishes
miR	med	min	max	med	min	max	med	min	max	p	
Let-7g	0.20	0.05	0.46	0.12	0.05	0.18	0.29	0.12	0.41	0.005	D vs C and A
MiR-106b	4.06	1.87	12.52	1.82	0.83	4.99	8.61	0.82	30.05	0.004	D vs C and A
MiR-191	0.30	0.04	1.48	0.22	0.03	0.36	0.68	0.03	1.71	0.025	D vs A
MiR-19a	7.89	1.38	39.90	3.28	0.81	15.84	29.26	2.79	50.39	0.041	A vs D and C

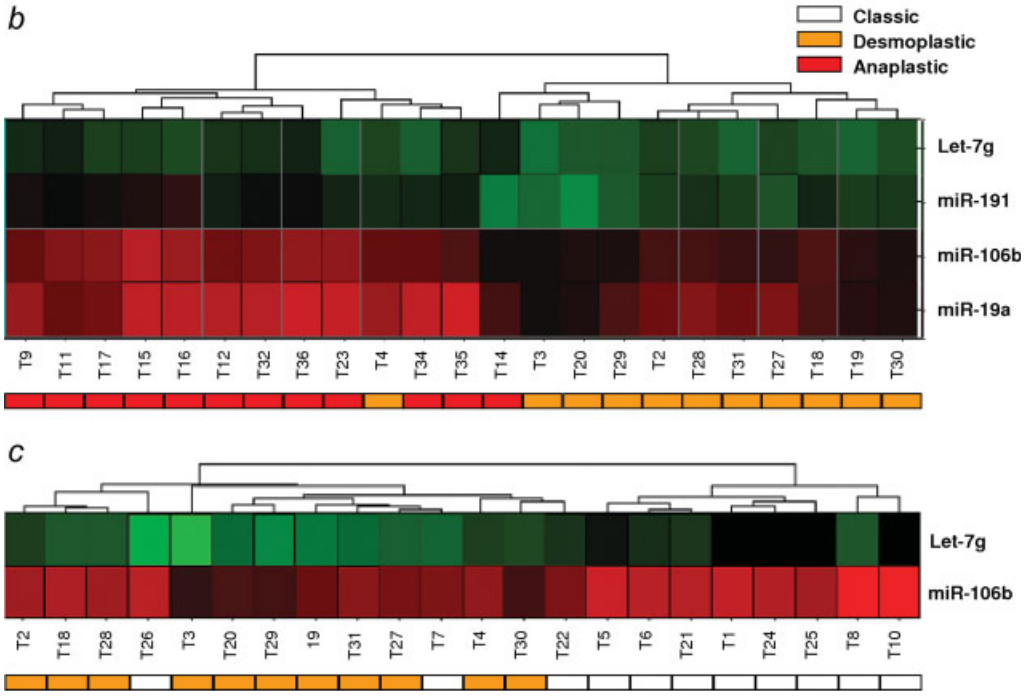


Figure 2 – MicroRNA expression profiles of MBs distinct for histotypes. Hierarchical clustering of desmoplastic, anaplastic and classic MB samples. Hierarchical clustering of tumor samples was performed for each group pair analyzing the expression of miRNA that were found differentially expressed between the two groups. (*a*) Values and statistical analysis of differential microRNAs in desmoplastic, anaplastic and classic tumor samples. Differentially expressed miRNAs were selected by Tukey's HSD analysis. Statistical details and analytical data are reported in Supporting Tables III and IV and in Material and Methods. (*b*) Hierarchical clustering of desmoplastic and anaplastic tumors samples (4 miRNAs differentially expressed). (*c*) Hierarchical clustering of classic and desmoplastic tumors samples (2 miRNAs differentially expressed).

1011 nt-long *t-TrkC* 3′UTR fused to r-luciferase in pRL-TK vector was cotransfected with firefly luciferase pGL3 vector and assayed after 24 hr with the Dual-Luciferase Assay (Promega, Madison, WI).

Results

microRNA profiling of MBs

Expression profiling of miRNAs has not yet been performed in neuronal brain tumors and was only reported in glial malignancies.[10,11] In order to generate MB miRNA profiling, we have preliminarily analyzed the expression of 248 miRNAs in a series of 20 human samples (14 primary MBs and 7 adult and fetal normal cerebellar controls) (Supporting Fig. 1). The 14 tumor samples were representative of MB histopathological variants, clinical features and molecular characteristics (Supporting Table I).

All samples have been clustered by unsupervised hierarchical clustering analysis, generating a dendrogram that shows a clear separation of MBs *versus* controls, suggesting a tumor-specific pattern of miRNA expression (Supporting Fig. 1).

We observed that 215 out of 248 miRNAs screened were expressed in normal cerebellum and/or MB, while 33 were not expressed in any of the samples analyzed (Supporting Table II).

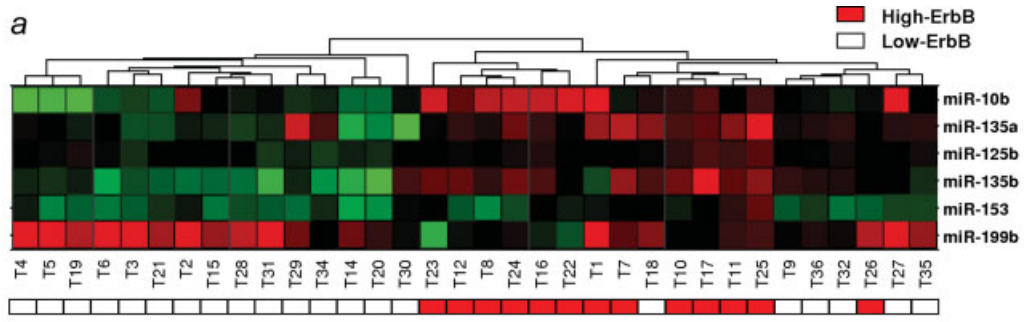
Expression profiles of neuronal and onco-microRNAs

To further validate our results, among the miRNAs identified as expressed in the primary screening, we selected 86 miRNAs previously reported as expressed in neuronal tissues and/or associated with tumors (oncomiRs) (Supporting Table III) and analyzed them in an enlarged series of tumors ($n = 34$) including 14 MB samples previously analyzed (tumors features are summarized in Table I) as well as in an enlarged series of normal controls ($n = 14$, including adult and fetal tissues).

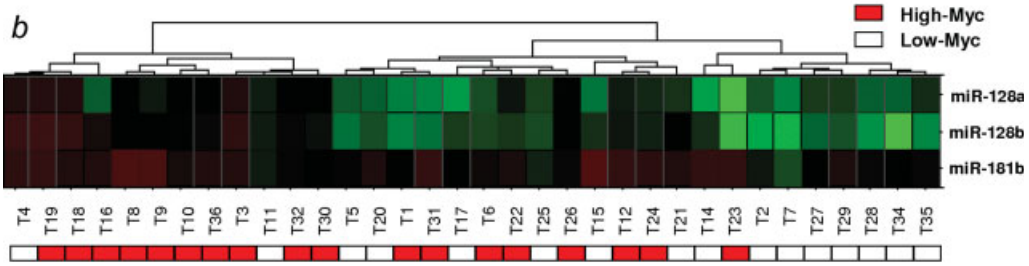
All raw and normalized miRNA expression data are available from GEO publicly accessible server (accession number: GSE12303; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE12303).

Data analysis of the enlarged sample set showed differential expression of 78 miRNAs between tumors and either adult or fetal controls (Fig. 1), with p values derived from the Kruskall-Wallis rank sum test being <0.05 (see Methods for details). miRNAs in MBs were predominantly downregulated, suggesting a tumor suppression function, while only few of them were found upregulated (Figs. 1*a* and 1*b* and Supporting Tables IV and V).

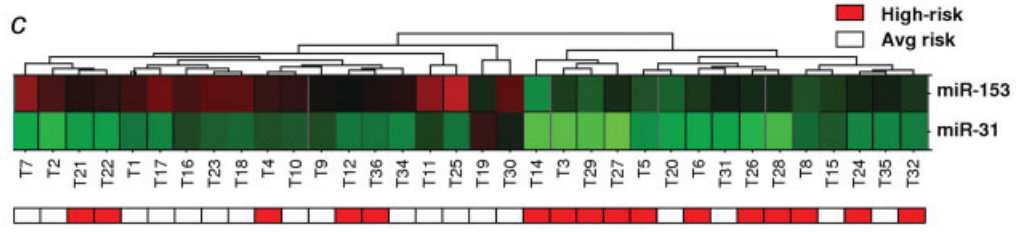
Normal tissue samples, although exhibiting similar expression of several microRNAs, can be further clustered according to developmental stages (adult *vs.* fetal; Fig. 1*a*). In fact miRNAs profiling indicates that a number of miRNAs are differentially



	High ErbB-expressing MB			Low ErbB-expressing MB			
	median	min	max	median	min	max	p
miR-10b	6.947	0.209	122.016	0.142	0.000	9.441	<0.001
miR-135a	3.311	0.338	24.589	0.473	0.000	7.444	0.001
miR-125b	0.916	0.359	3.491	0.427	0.070	1.149	0.026
miR-135b	5.150	0.055	15.571	0.074	0.001	3.073	<0.001
miR-153	0.244	0.013	3.188	0.031	0.003	0.475	0.012
miR-199b	0.732	0.001	56.948	6.534	0.286	51.934	0.018



	High Myc-expressing MB			Low Myc-expressing MB			
	median	min	max	median	min	max	p
miR-128a	0.072	0.001	0.496	0.011	0.001	0.384	0.012
miR-128b	0.092	0.001	0.927	0.006	0.002	0.665	0.015
miR-181b	0.250	0.055	1.444	0.080	0.017	0.826	0.003



	Average Risk-MB			High Risk-MB			
	median	min	max	median	min	max	p
miR-153	0.224	0.008	3.188	0.030	0.003	0.176	<0.001
miR-31	0.006	0.001	0.164	0.002	0.000	0.012	0.026

FIGURE 3 – MicroRNA signature in MBs with distinct molecular features and disease risk stratification. Hierarchical clustering of MB groups based on molecular features. Differentially expressed miRNAs were selected by Kruskall-Wallis rank sum test. Statistical details are also indicated at the bottom of each panel. (*a*) Hierarchical clustering of ErbB2 overexpressing (high) or not (low) tumors samples (6 miRNAs differentially expressed). (*b*) Hierarchical clustering tumors samples overexpressing (high) or not (low) c-Myc (3 miRNAs differentially expressed). (*c*) Hierarchical clustering of MB samples from patients with high risk (HR) and average-low risk (AR) (2 miRNAs differentially expressed).

expressed between normal adult and fetal cerebellum (Supporting Table VI), as previously described in mouse cerebellar development.[7,15,16] Some variability in the expression levels of certain miRNAs (*e.g.* miR-372) was observed among fetal cerebellum samples, probably because of the wide range of ages (from 22 to 36 weeks), corresponding to distinct developmental stages, in analogy to the mouse developing organ.[17]

Some miRNAs were expressed in tumors similarly to both fetal and adult tissues, while some were differentially expressed when compared only with adult controls (Fig. 1*b* and Supporting Table

IV), or with respect to fetal controls only (Fig. 1*b* and Supporting Table V).

These findings highlight that the expression of a subset of miRNAs in MB is reminiscent of the less-differentiated fetal cerebellum, confirming the hypothesis that tumors exhibit some embryonic features.

microRNA expression profiles classify MB histotypes

The dendrogram generated by clustering analysis of 86 miRNAs in 34 tumor samples also suggests the presence of distinct MB subsets characterized by different miRNA expression (Fig. 1*a*). Statistical analysis has been carried out to investigate whether specific miRNA signature characterized tumor histotypes (anaplastic, classic and desmoplastic). Tukey's HSD method comparison indicates different miRNA expression pattern in tumor subsets analyzed as shown in Figure 2*a* and in hierarchical clustering (Figs. 2*b* and 2*c*). Four miRNAs (miR-let7g, miR-19a, miR-106b and miR-191) resulted significantly upregulated in anaplastic *versus* desmoplastic MBs (Figs. 2*a* and 2*b*). Two miRNAs (miR-let7g and miR-106b) were differentially expressed between desmoplastic and classic tumors (Figs. 2*a* and 2*c*), while only miR-19a resulted upregulated in anaplastic *versus* classic MBs (Fig. 2*a*).

microRNAs and molecular markers in human MB

We then asked whether miRNA signatures identify tumor subsets with distinct molecular features.

Since high levels of ErbB2[18] and c-Myc[19,20] distinguish MB subsets that may display different biological features, we analyzed the miRNA pattern of our specimens as a function of the expression levels of both these molecular markers.

Tumor samples were considered as overexpressing ErbB2 or c-Myc if they exhibited mRNA levels that exceeded the mean level found in normal adult cerebella by at least 2 standard deviations. According to these criteria, the 34 MBs were subdivided into either 13 overexpressing and 21 not overexpressing ErbB2 and into 18 overexpressing and 16 not overexpressing c-Myc (Figs. 3*a* and 3*b*). Only a fraction of tumors ($n = 9$) displayed combined overexpression of both ErbB2 and c-Myc, while 12 tumors were not overexpressing both Myc and ErbB2 (Figs. 3*a* and 3*b* and Table I).

A group of 6 miRNAs (miR-10b, miR-135a, miR-135b, miR-125b, miR-153, miR-199b) exhibited significantly different expression in tumors overexpressing or not ErbB2 (Fig. 3*a*, upper panel hierarchical clustering, lower panel statistical analysis).

Similarly, c-Myc overexpressing tumors also exhibited a small group of miRNAs (miR-181b, miR-128a, miR-128b) that were differentially expressed compared to MBs not overexpressing c-Myc (Fig. 3*b*: upper panel hierarchical clustering, lower panel statistical analysis).

microRNA expression signature correlates with disease risk in MB

In order to verify whether a specific miRNA signature distinguishes MBs with poor prognosis, we investigated the association of miRNA expression patterns with disease risk classes, by considering average risk (AR) the patients aged more than 3 years at diagnosis, nonmetastatic and totally or nearly totally resected, while patients not fulfilling these criteria are regarded as high risk (HR), according to literature.[4]

Indeed, lower expression levels of miR-31 and miR-153 were observed in HR *versus* AR group (Fig. 3*c*). Notably, these 2 miRNAs were downregulated in all MBs (Fig. 1*b*) and were significantly more downregulated in HR MB, suggesting that the loss of these miRs is a marker of poor prognosis.

miR-9 and miR-125a promote growth arrest and apoptosis of MB cells

We have described earlier a wide pattern of microRNAs downregulated in MBs *versus* normal controls. These results support the view that downregulated microRNAs may play a role in the loss of signals inhibiting tumor growth.

To verify this hypothesis, we focused on neuronal downregulated miR-9 and miR-125a (Fig. 1*b*), which we recently reported to be downregulated and displaying growth-inhibitory properties in neuroblastoma.[9]

Since the growth of MB cells is reduced by treatment with RA (Fig. 4*a*), we first investigated whether miR-9 and miR-125a were regulated by this drug and may thus be a part of a growth-inhibitory pathway. In keeping with this view, time-course and dose–response assays of the effect of RA-treatment of D283 Med cells revealed an increase of miR-9 and miR-125a expression (Figs. 4*b* and 4*c*), correlating with cell growth arrest (Fig. 4*a*).

To verify the effects of the 2 microRNAs on MB cell proliferation, we transfected D283 Med cells with miR-9 or miR-125a expression vectors. The consequent 2-fold increase of miRNA levels (Fig. 5*a*, upper panel) resulted in a significant decrease of cell proliferation, evaluated by BrdU uptake (Fig. 5*a*, bottom left panel). Conversely, another miRNA (miR-100) that was not misexpressed in MB compared to normal cerebellum did not affect MB cell proliferation (Fig. 5*a*). The role of endogenous miRNAs in the regulation of MB cell proliferation was further clarified by the increased cell proliferation observed after knockdown of miR-9 or miR-125a by LNA-mediated siRNA, while depletion of miR-100 was ineffective (Fig. 5*a*, bottom right panel). The miRNAs' effects on cell growth were confirmed by colony formation assays in an additional MB cell line. Indeed, overexpression of miR-9 and miR-125a significantly reduced the number of Daoy cell colonies formed after G418 selection, while miR-100 was inactive (Fig. 5*b*).

Furthermore, when overexpressed, both miR-9 and miR-125a were able to inhibit the anchorage-independent growth of D283 Med cells, since they significantly reduced the size and the number of colonies grown in soft agar, while miR-100 was inactive (Fig. 5*c*).

In order to investigate whether the growth-inhibitory properties of miR-9 and miR-125a were also due to increased apoptosis, we performed a TUNEL assay on miRNA-transfected cells. Indeed, a significant increase of TUNEL-positive cells was observed after overexpression of miR-9 or miR-125a, but not of miR-100 (Fig. 5*d*).

t-TrkC is targeted by miR-9 and miR-125a, is overexpressed in MB and promotes cell proliferation

The ability of miR-9 and miR-125a to inhibit cell proliferation in neuroblastoma has been reported to be mediated by miR-targeting of the t-TrkC (Fig. 6*a*, upper panel), which displays proliferative capacity in these cells.[9] To confirm these data in MB, we trasfected D283 Med cells with a luciferase reporter vector containing the 3′UTR of *t-TrkC*[9] together with either miR-9 or miR-125a. We observed a decreased luciferase activity (Fig. 6*a* lower panel), indicating that t-TrkC is targeted by the two miRs in MB as well.

In human primary MBs, expression of the proapoptotic fl-TrkC has been described as a good prognosis marker,[21–23] while no information is available on the expression of t-TrkC. Therefore, we analyzed the expression levels of t-TrkC in human primary tumors. Our results showed that t-TrkC was overexpressed in most MBs, and the ratio of t-TrkC/fl-TrkC was significantly ($p < 0.05$) higher compared to normal tissues (Fig. 6*b*), suggesting that the t-TrkC may contribute to tumor growth *in vivo*. Indeed, the proliferative capacity of t-TrkC was confirmed in MB, since either overexpression or siRNA-mediated functional knockdown of t-TrkC resulted in increased or decreased, respectively, proliferation rate of D283 cells (Fig. 6*c*).

Discussion

We provide here the first study on microRNA expression in primary human MB and in human normal cerebellum. Two hundred fifteen microRNAs expressed in these tissues were identified. Among these, 78 displayed downregulated or upregulated expres-

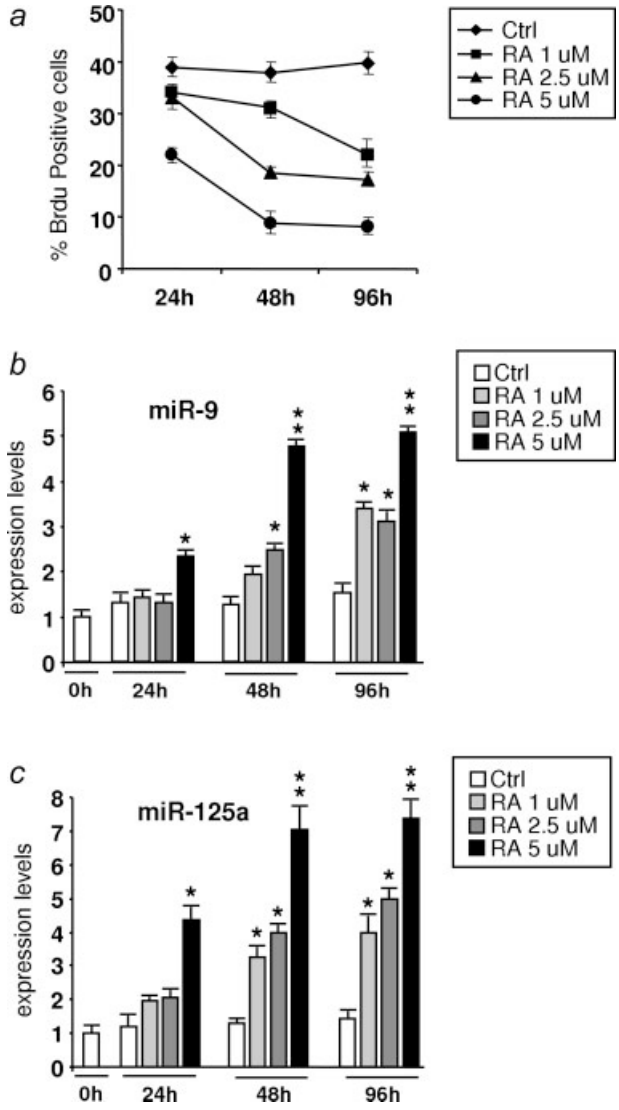


FIGURE 4 – Retinoic acid regulates MB cell proliferation and miR-9 and miR-125a expression. (*a*) BrdU incorporation measured in D283 Med cells treated with increasing concentrations of RA (1, 2 and 5 μM), for 24, 48 and 96 hr. (*b,c*) miR-9 (*b*) and miR-125a (*c*) are upregulated by RA in D283 Med cells treated with RA (1, 2 and 5 μM) for 24, 48 and 96 hr. MiR-9 and miR-125a modulation is quantified by means of RT-Q-PCR. $*p < 0.05$ and $**p < 0.01$.

sion in MB *versus* controls. Furthermore, some of the miRNAs that display developmentally regulated expression from immature (*i.e.* fetal) to differentiated (*i.e.* adult) cerebellum, maintained levels of expression in MB similar to those observed in fetal tissue, suggesting that these tumors exhibit some embryonic features.

Impaired expression of neuronal miRNAs in MB

We observed a deregulated expression of several neuronal miRNAs that have not been so far described (Fig. 1*a*). Our findings suggest that at least some of these miRNAs might be involved in MB tumorigenesis.

Of note, reduced expression of miR-128a, 128b and miR-181b as well as upregulation of miR-21 were observed in MB, similar to what was previously reported in glioblastoma,[10,11,24] thus supporting the general role of these miRNAs as tumor suppressors or oncogenes in neuronal and glial-derived tissues.

Furthermore, among the miRNAs recently reported as downregulated in tumoral CNS cell lines,[12] 11 (let-7d, -g, -i, miR-103, miR-124a, miR-128a, miR-128b, miR-134, miR-138, miR-149, miR-181b) also resulted in downregulation in our series of primary MBs.

Finally, we observed a reduced expression of both miR-9 and miR-125a, as recently reported in neuroblastoma,[9] thus suggesting that significantly lower abundance of these 2 miRNAs is shared in malignant peripheral nervous system (PNS) and CNS tumors.

Taken together, these findings highlight a number of neuronal miRNAs that may contribute to the development of different kinds of nervous system malignancies.

Onco-miRs expression in MB

Most miRNAs differentially expressed in MB were also reported to be altered in other types of cancer, being therefore classified as shared "oncomiRs." These include the antiproliferative let-7 family members, which were observed to be decreased in lung and breast cancer,[25–27] miR-107 (reduced in human promyelocytic leukemia),[28] miR-143 and miR-145 (downregulated in lung, breast, colon-rectal and ovarian cancers),[26,27,29–31] and miR-125b (downregulated in breast and ovarian cancers).[27,31]

An interesting observation from our study is that most of the miRNAs dysregulated in our tumor samples display downregulated expression, suggesting their prevalent role as tumor suppressors. This is consistent with a recent report demonstrating that the widespread reduction of microRNA expression detected in human cancer and in experimentally induced tumors (by impairing miRNA processing) enhances cell transformation and tumorigenesis.[32]

In the present work, we also show a small number of upregulated oncomiRs (*e.g.* miR-106b and miR-25 cluster), consistent with what was reported for lung cancer and T-cell leukemia[26,33] as well as miR-17–92 cluster (miR-17-5p, miR-20a and miR-19a), according with previously reported observations on lung cancer and lymphomas.[26,34]

Deregulated miRNAs target genes involved in MB development

It is tempting to speculate that the misregulated expression of oncomiRs plays a critical role in malignancy and in the control of tumor behavior, by targeting specific gene products involved in MB tumorigenesis. Indeed, let-7 miRNAs inhibit Ras oncogene expression,[35] which has been reported to determine MB aggressive metastatic behavior.[36] Of note, no mutations of Ras have been described in MB, suggesting that alternative mechanisms are responsible for RAS/MAPK pathway activation in this disease,[37] which might involve downregulation of specific let-7 miRNAs.[35] Likewise, members of the miR-17–92 cluster (miR-17-5p, miR-20a and miR-19a) modulate E2F1 and cooperate with c-Myc for tumorigenesis.[38] Myc oncogene is frequently amplified and overexpressed in MB and plays a crucial role in cerebellar tumorigenesis.[19,20]

There are additional genes involved in MB development that can be a consequence of the altered miRNA expression observed in our study. One of these genes is Rb1, since miR-106a, which targets Rb1[39] is upregulated in MB, and Rb1 inactivation has been previously reported to induce MB in mice.[40]

Correlation of miRNA signatures with biological and prognostic features

In order to correlate dysregulated miRNAs expression profiles with biological and clinical features of MBs, we performed statistical analysis that revealed specific miRNAs signatures for distinct classes of tumors.

Of note, a subgroup of miRNAs (miR-let7g, miR-106b, miR-191 and miR-19a) was upregulated in more aggressive MB anaplastic histotype with respect to classic and/or desmoplastic tumors, some of them (miR-19a and miR-106b) being reported to be overexpressed also in other cancers.[41] A specific expression pattern of 9 miRNAs also clustered with ErbB2- and/or c-Myc-

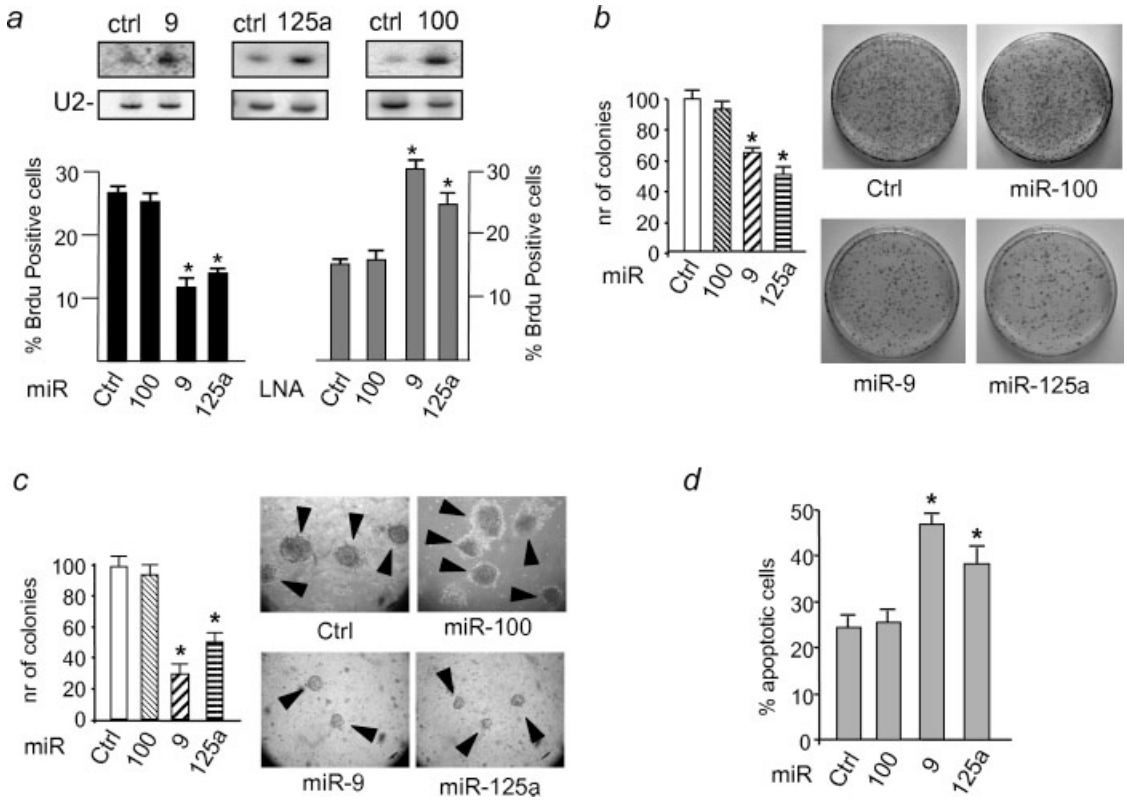


Figure 5 – miR-9 and miR-125a function in MBs cells. (*a*) Northern blot showing the levels of the ectopically expressed miR-9, miR-125a and miR-100 with respect to control plasmid (upper panel). Bottom panel, BrdU incorporation assay in D283 Med cells transfected with miR-9, miR-125a or miR-100 (left panel) or with anti-miR-9, anti-miR-125a or anti-miR-100 LNAs (right panel) with appropriate control plasmid or scrambled LNA (Ctrl). *$p < 0.01$ *versus* controls. (*b,c*) Colony formation assay on Daoy cells (*b*) and soft agar colony formation in D283 Med cells (*c*) after transfection with miR-9 or miR-125a or miR-100 or control plasmid. On the left, panels indicating the relative number of colony formed. Values have been normalized attributing score 100 to the number of colonies grown from control transfected cells (Ctrl). On the right, panels showing representative pictures of the experiments are presented. (*$p < 0.05$). Arrows indicate representative colonies containing >50 cells. (*d*) Percentage (%) of apoptosis (TUNEL assay) in Daoy cells transfected with miR-9 or miR-125a or miR-100 or control plasmid (Ctrl), *$p < 0.05$. All the experiments shown in this figure were done in triplicate. Mean ± SD values from 3 independent experiments are reported.

overexpressing tumors. In addition to the differential expression of miR-128a, b and miR-181b in c-Myc overexpressing *versus* not overexpressing MBs, ErbB2-overexpressing MBs display a miRNA signature characterized by higher expression of miR-135a and b, miR-10b, miR-125b and miR-153, while miR-199b displays lower expression.

Finally, our analysis of human MB revealed a tumor-specific miRNA signature consistently correlated with patient disease risk stratification. These include a lower expression of miR-153 and miR-31 in HR *versus* AR patients, suggesting that their loss is an indicator of poor prognosis.

Taken together, although based on a limited number of tumor samples, because of the rarity of MB disease, our findings suggest that each of the MB subsets we have considered has distinct miRNA signatures, highlighting the molecular heterogeneity and complexity of the signaling pathways that are regulated by miRNAs and potentially involved in tumor behavior.

Antiproliferative role for downregulated miRNAs in MB

Interestingly, miR-9 and miR-125a are downregulated in MB. Both microRNAs are upregulated by RA, a treatment that inhibits tumor growth, suggesting that both microRNAs belong to a growth-inhibitory pathway. Accordingly, overexpression or knocking down of miR-9 and miR-125a decreases or increases, respectively, proliferation of MB cells, while miR-100, whose expression is not misregulated in MB, is ineffective. Both miR-9 and miR-125a also promote MB cell apoptosis. These findings underscore a significant tumor growth-inhibitory function for these miRNAs in MB. We have recently described in neuroblastoma that the tumor growth-inhibitory activity of miR-9 and miR-125a is mediated by targeting the truncated isoform of the neurotropin receptor TrkC that lacks the TK domain, while the full-length isoform is insensitive.[9] We have observed that TK-defective t-TrkC enhances cell proliferation in both neuroblastoma[9] and MB (this study) and is overexpressed in most tumors with respect to normal tissue, with a MB-specific increased ratio with respect to fl-TrkC. Indeed, both microRNAs repress the expression of t-TrkC receptor, and this downregulation is critical for inhibiting neuroblastoma and MB cell growth by misbalancing the ratio between the full-length and the truncated isoforms.[9] Like in neuroblastoma, the expression of the fl-TrkC also correlates with a favorable prognosis of human MB and reduces the growth *in vitro* of MB cells.[21–23] These findings strongly suggest that miR-9 and miR-125a influence MB by targeting t-TrkC receptor, according to our observations on neuroblastoma.[9]

A further miR-9 target in MBs may be the REST/NRSF (RE1 silencing transcription factor/neuron-restrictive silencer factor) complex, a transcriptional silencer that controls neuronal identity.[42,43] REST complex components and REST itself are also targeted by miR-124.[42,44] REST is overexpressed in MB cells and promotes cerebellar tumorigenesis by blocking neuronal differentiation.[45–47] Interestingly, REST reciprocally downregulates the expression of miR-124, providing a double feedback loop maintaining undifferentiated stage.[48] Therefore, the significant downregulation of miR-124 and miR-9 we have observed in MB with

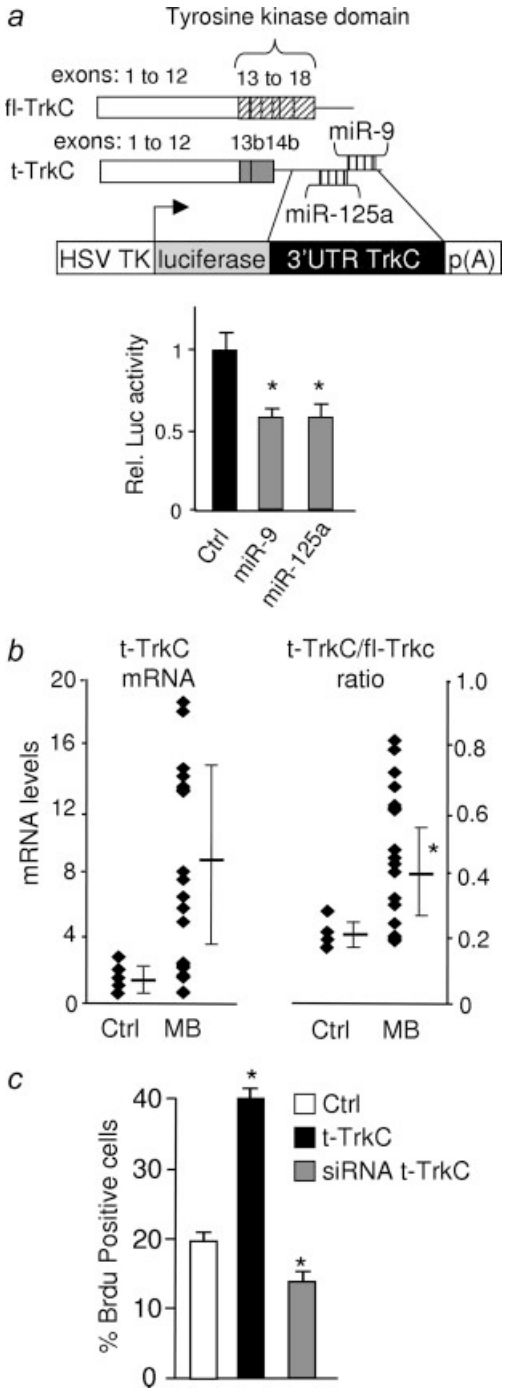


Figure 6 – Truncated TrkC targeted by miR-9 and miR-125a is overexpressed in MB and promotes cell proliferation. (*a*) On the top of the panel, the structures of full-length (fl-TrkC) and truncated (t-TrkC) TrkC, in which the tyrosine kinase domain is deleted by alternative splicing, are shown. Schematic representation of the construct generated for luciferase assay in which miR-9 and miR-125a binding sites are indicated. The levels of luciferase activity of the t-TrkC 3′UTR-luciferase reporter after transfection with miR-9 or miR-125a is indicated in the histogram (bottom of the panel) (mean ± SD from 3 independent experiments, $*p < 0.01$ *versus* empty vector-transfected controls, Ctrl). (*b*) Expression of mRNA for truncated TrkC (t-TrkC) and t-TrkC/full length-TrkC ratios in human primary MB samples, compared with normal adult cerebellum, $*p < 0.05$. (*c*) Proliferation rate in D283 Med cells transfected with t-TrkC, with t-TrkC siRNA or with appropriate control plasmid or scrambled siRNA (Ctrl). BrdU incorporation was normalized to transfected cells and is indicated as the mean ± SD from 3 independent experiments. $*p < 0.01$ *versus* controls.

respect to normal tissue is consistent with the enhanced expression and activity of REST previously reported in these tumors. REST inactivation has been reported to inhibit MB growth,[45,46] suggesting that the reduction of miR-9 and miR-124 we have observed in MB, may play a role in cerebellar tumorigenesis.

In conclusion, miRNA expression signatures described here suggest a series of altered expressed miRNAs in human MB that may represent novel oncogenic molecules involved in tumor formation. Interestingly, several molecular events whose deregulation in MB are responsible for tumor development and progression (*i.e.* Ras, Rb1, c-Myc, TK-defective TrkC, REST/NRSF) appear to be targeted by some of the dysregulated miRNAs described here. Consequently, these miRNAs could provide a better understanding of the mechanisms responsible for the development of human MB and may represent targets for novel therapeutic strategies for this disease.

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