Downregulation of 14q32 microRNAs in primary human desmoplastic medulloblastoma

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INTRODUCTION

Medulloblastoma (MB) is one of the most common pediatric cancers, likely originating from abnormal development of cerebellar progenitor neurons. MicroRNA (miRNA) has been shown to play an important role in the development of the central nervous system. Microarray analysis was used to investigate miRNA expression in desmoplastic MB from patients diagnosed at a young age (1 or 2 years old). Normal fetal or newborn cerebellum was used as control. A total of 84 differentially expressed miRNAs (64 downregulated and 20 upregulated) were found. Most downregulated miRNAs (32/64) were found to belong to the cluster of miRNAs at the 14q32 locus, suggesting that this miRNA locus is regulated as a module in MB. Possible mechanisms of 14q32 miRNAs downregulation were investigated by the analysis of publicly available gene expression data sets. First, expression of estrogen-related receptor-γ (ESRRG), a reported positive transcriptional regulator of some 14q32 miRNAs, was found downregulated in desmoplastic MB. Second, expression of the parentally imprinted gene MEG3 was lower in MB in comparison to normal cerebellum, suggesting a possible epigenetic silencing of the 14q32 locus. miR-129-5p (11p11.2/7q32.1), miR-206 (6p12.2), and miR-323-3p (14q32.2), were chosen for functional studies in DAOY cells. Overexpression of miR-129-5p using mimics decreased DAOY proliferation. No effect was found with miR-206 or miR-323 mimics.

Keywords: 14q32 miRNA cluster, desmoplastic medulloblastoma, ESRRG, miR-129-5p, miRNA profile

MATERIALS AND METHODS

PRIMARY MEDULLOBLASTOMA TISSUE SAMPLES

Surgical specimens were obtained from 1 to 5 years old children (n = 10), with desmoplastic MB (Table 1). Of note, microarray analyses were performed with MB samples from children with 1–2 years old. Desmoplastic MBs belong, with rare exceptions, to the SHH molecular subgroup (13–16). All MB samples used in the present study had high mRNA levels of PCH1 and low levels of OTX2 (Figure A1 in Appendix), in comparison to normal cerebellum, which is in keeping with the differential transcriptional profile of SHH tumors (3). Normal cerebellum tissues were
Table 1 | Summary of the medulloblastoma samples included in the study.

| Medulloblastoma | Age at diagnosis | Gender | Histology |
|----------------|-----------------|--------|-----------|
| MB 1<sup>a</sup> | 1               | M      | N/D       |
| MB 2<sup>a</sup> | 1               | F      | N/D       |
| MB 3<sup>a</sup> | 2               | M      | D         |
| MB 4<sup>a</sup> | 2               | M      | N/D       |
| MB 5<sup>a</sup> | 1               | M      | N/D       |
| MB 6<sup>a</sup> | 2               | M      | N/D       |
| MB 7           | 5               | M      | N/D       |
| MB 8           | 5               | M      | D         |
| MB 9           | 4               | M      | D         |
| MB 10          | 5               | M      | N/D       |

<sup>a</sup> Samples used in Affymetrix miRNA microarray analysis; F = female; M = male; D = desmoplastic; N/D = nodular/desmoplastic.

Table 2 | Summary of the normal cerebellum tissues.

| Normal cerebellum | Gestational age | Gender | Diagnosis |
|-------------------|-----------------|--------|-----------|
| C1                | 37              | M      | Bilateral renal agenesis |
| C2<sup>a</sup>    | 39              | M      | Hydropsy   |
| C3<sup>a</sup>    | 22              | –      | –          |
| C4<sup>a</sup>    | 31              | M      | NM         |
| C5<sup>a</sup>    | 36              | –      | NM         |
| C6<sup>a</sup>    | 24              | M      | NM         |
| C7<sup>a</sup>    | 30              | –      | Cardiopathy|
| C8<sup>a</sup>    | 26              | M      | NM         |

<sup>a</sup> Samples used in Affymetrix miRNA microarray analysis; F = female; M = male; NM = no malformation and no aneuploidy.

obtained from 22 to 39 weeks old fetal and newborn (NW) autopsy (n = 8) (Table 2). Ethical approvals were obtained from the Ethical Research Committee of the Faculdade de Ciências Médicas (n°656/2009), CAISM (n°064/2010), the Ethical Research Committee of Centro Infantil Boldrini (n°1.90-030710), and National Committee of Ethics in Research (CONEP) n°0005.0.144.146-09. Subtyping of MB was obtained by histological analysis.

TOTAL RNA ISOLATION AND ANALYSIS OF GLOBAL miRNA EXPRESSION

Total RNA was extracted by Trizol<sup>™</sup> (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions, with an additional overnight precipitation step at −20°C with isopropanol (Merck). RNA quantification was carried out in a Qubit Quantitation Platform (Invitrogen) and RNA quality was analyzed via gel electrophoresis. Five hundred nanograms of RNA from 12 samples (6 MB and 6 fetal cerebellum) were labeled with the 3′-DNA FlashTag Biotin HS kit (Genisphere, Hatfield, PA, USA) and hybridized to GeneChip miRNA Array 1.0 (Affymetrix Inc., Santa Clara, CA, USA), which comprises 847 human miRNAs. Data was acquired using a GeneChip Scanner 3000 7G (Affymetrix).

VALIDATION OF miRNA DEREGULATION BY QUANTITATIVE REAL-TIME PCR

Reverse transcription (RT) and quantitative real-time RT-PCR (RT-qPCR) analysis were carried out using commercially available TaqMan microRNA assays (Applied Biosystems, Foster City, CA, USA) and a 7500 Real-time PCR System (Applied Biosystems). RT reactions (50 ng of total RNA) were performed in a 15 µl final volume containing specific stem-loop primers for each miRNA (129-5p, 206, 323-3p, 495, and internal control small RNA, RNU6B), 10× RT Buffer, dNTPs, reverse transcriptase, RNase inhibitor, and water in 96-well plates. Thermal cycling included 15 min at 16°C, 30 min at 42°C, and a final step of RT inactivation for 5 min at 85°C. PCR reactions were performed in a 10 µl final volume containing 5 µl TaqMan Universal Master Mix II, without UNG (Applied Biosystems), 3.5 µl water, 0.5 µl TaqMan microRNA assay, and 1 µl cDNA. Thermal cycling included an initial step of 10 min at 95°C for Taq activation followed by 40 cycles of 15 s denaturation at 95°C and 1 min of annealing/extension at 60°C. Each reaction was performed in triplicate and the miRNAs expression levels were normalized against RNU6B. The threshold cycle numbers (Ct) were calculated by relative quantification using the 2<sup>−ΔΔCt</sup> method, as described by Livak and Schmittgen (17). One of the control samples was chosen as calibrator.

CELL LINES

Four human MB cell lines were utilized: DAOY (HTB 186), D283 Med (HTB185), and D431 Med (HTB-187) were obtained from American Type Culture Collection (ATCC). The MB cell line, MEB-Med-8A, was kindly provided by Prof. T. Pietsch (18). The MB cell lines DAOY, D283 Med, and MEB-Med-8A were maintained in High Glucose Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 1 mM sodium pyruvate (PAA), L-glutamine, 1% penicillin/streptomycin (Invitrogen, Karlsruhe, Germany), and 10% fetal bovine serum (FBS, Invitrogen). The MB cell line D341 Med was maintained in DMEM with L-glutamine supplemented with 1 mM sodium pyruvate, 1% penicillin/streptomycin, and 10% Human Serum (HS, PAA, UK).

TRANSIENT TRANSFECTION OF miRNAs

DAOY cells (1.5 × 10<sup>5</sup>) were seeded in six-well plates in 2 ml of RPMI-1640 medium (Cultilab, Campinas, Brazil) supplemented with 10% FBS (Sigma-Aldrich, St. Louis, MO, USA) and penicillin/streptavidin (Cultilab). Transfection of miRVana miRNA mimics (Invitrogen Ambion, Austin, TX, USA) of miR-206, miR-129-5p, miR-323-3p, or miRVana miRNA mimic negative control #1 (referred to as scrambled) was carried out 24 h after seeding, in a final concentration of 3 nM, using Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer’s recommendation. Efficiency of transfection was evaluated 24 post-transfection by RT-qPCR using total RNA.

CELL VIABILITY: MTS ASSAY

Cell survival/proliferation after the transfection with mimic-miRNAs was evaluated by using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Wallisellen, Switzerland), a colorimetric [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)]-2H-tetrazolium inner salt (MTS) assay. Briefly, mimic-miR-206, mimic-miR-129-5p, and mimic-miR-323-3p or mimic-negative control #1 transected...
### Table 3 | Deregulated miRNA in desmoplastic medulloblastoma compared to normal cerebellum.

| Our miRNA profile (84) | Chromosomal localization | Fold change | Reference |
|------------------------|---------------------------|-------------|-----------|
| **DOWNREGULATED**      |                           |             |           |
| hsa-miR-206            | 6p12.2                     | −7.53       | (23)      |
| hsa-miR-219-2-3p       | 9q33.3                     | −6.64       | (52)      |
| hsa-miR-383            | 8p22                       | −6.56       | (12, 55, 56) |
| hsa-miR-138            | 16q13.3/3p21.32            | −5.16       | (12, 14)  |
| hsa-miR-323-3p         | 1q43.2                     | −4.96       | (12, 52)  |
| hsa-miR-122            | 18q21.31                   | −4.82       |           |
| hsa-miR-105            | Xq28                       | −4.66       |           |
| hsa-miR-129-5p         | 11p11.2/7q32.1             | −4.56       | (23)      |
| hsa-miR-935            | 19q13.43                   | −4.53       | (52)      |
| hsa-miR-329            | 14q32.2                    | −4.48       |           |
| hsa-miR-129-3p         | 11p11.2/7q32.1             | −4.43       |           |
| hsa-miR-850            | 22q11.21                   | −4.19       |           |
| hsa-miR-184            | 15q24.3                    | −4.14       |           |
| hsa-miR-370            | 14q32.2                    | −3.99       | (12)      |
| hsa-miR-433            | 14q32.2                    | −3.96       | (23)      |
| hsa-miR-138-2*         | 16q13.3/3p21.32            | −3.91       |           |
| hsa-miR-487b           | 14q32.2                    | −3.82       | (23)      |
| hsa-miR-487a           | 14q32.2                    | −3.78       |           |
| hsa-miR-756            | 14q32.2                    | −3.65       |           |
| hsa-miR-485-5p         | 14q32.2                    | −3.60       |           |
| hsa-miR-138-1*         | 18q13.3/3p21.32            | −3.55       |           |
| hsa-miR-392            | 14q32.2                    | −3.53       | (12, 29)  |
| hsa-miR-504            | Xq26.3                     | −3.45       | (52)      |
| hsa-miR-128            | 2q21.33/22q23.3            | −3.43       | (12, 14, 51, 59) |
| hsa-miR-490-5p         | 7q33                       | −3.42       |           |
| hsa-miR-770-5p         | 14q32.2                    | −3.35       |           |
| hsa-miR-410            | 14q32.2                    | −3.30       | (23)      |
| hsa-miR-432            | 14q32.2                    | −3.29       |           |
| hsa-miR-495-5p         | 7q33                       | −3.02       |           |
| hsa-miR-381            | 14q32.2                    | −2.98       |           |
| hsa-miR-377**          | 14q32.2                    | −2.73       | (12)      |
| hsa-miR-7              | 15q25.3/19p13.3/8q21.32    | −2.72       | (12, 14)  |
| hsa-miR-124            | 20q23.1/8q12.3/8p23.1      | −2.71       | (12, 14, 29, 48, 49) |
| hsa-miR-323-5p         | 14q32.31                   | −2.69       | (12)      |
| hsa-miR-873            | 9q21.11                    | −2.65       |           |
| hsa-miR-129*           | 11p11.2/7q32.1             | −2.63       |           |
| hsa-miR-338-5p         | 17q25.3                    | −2.61       | (14)      |
| hsa-miR-409-5p         | 14q32.2                    | −2.61       |           |
| hsa-miR-874            | 5q31.2                     | −2.46       |           |
| hsa-miR-495            | 14q32.2                    | −2.46       | (52)      |
| hsa-miR-885-5p         | 3p25.3                     | −2.45       |           |
| hsa-miR-378c           | 14q32.2                    | −2.43       | (52)      |
| hsa-miR-299-5p         | 14q32.2                    | −2.41       |           |
| hsa-miR-539            | 14q32.2                    | −2.40       | (52)      |
| hsa-miR-127-5p         | 14q32.2                    | −2.35       | (12, 29)  |
| hsa-miR-127-3p         | 14q32.2                    | −2.35       | (52, 59)  |
| hsa-miR-411*           | 14q32.2                    | −2.30       |           |

(Continued)
APOTOPSIS ASSAY
DAOY cells transfected with miR-206, 129-5p, 323-3p, or scramble mimics were cultured for 24 h in serum-free RPMI-1640 (Culti-lab), harvested and part of it was resuspended in the appropriate binding buffer, stained with FITC-conjugated Annexin V (BD Biosciences, San Jose, CA, USA) and propidium iodide at room temperature for 15 min, and subsequently analyzed by flow cytometry in a FACS Canto II (Becton Dickinson). The remaining cells were replated in six-well plates for another 24 h culture period in serum-free RPMI-1640 (Culti-lab) and harvested 48 h post-transfection for Annexin V labeling.

STATISTICAL ANALYSIS AND BIOINFORMATICS METHODS TO SIGNALING PATHWAY PREDICTION
MicroRNA expression was analyzed in R environment1 using the packages Affy and RankProd from Bioconductor (19–21). The MB miRNA profile was compared to the cerebellum profile. Differentially expressed miRNAs were selected according to the fold change ≥2.00 and p-value ≤0.05. Heat maps were created using tools of the MetaboAnalyst 2.0.2. Signaling pathways were prospected by DIANA-miRPath (microT-v4.0, beta version)3. The input dataset enrichment analysis was performed by Pearson’s chi-squared test and each pathway was represented by the negative natural logarithm of the P-value (−ln P). The Ingenuity Pathway Analysis (IPA) software4 was used to identify possible pathways associated to differentially expressed miRNAs.

Comparisons of RT-qPCR values from MB versus normal cerebellum were performed by the Mann–Whitney test. Cell proliferation results, at each time point, from mimic miRNA transfection versus mimic-negative control #1 were analyzed by the two-tailed unpaired t-test. Alpha error of P = 0.05 was tolerated. The GraphPad Prism 5 software was used throughout.

RESULTS
IDENTIFICATION OF DIFFERENTIALLY EXPRESSED miRNAs IN DESMOPLASTIC MBs OF 1–2 YEARS OLD CHILDREN
Global miRNA profiles were generated for primary MB of the desmoplastic subtype and most likely SHH molecular subgroup (n = 6), and normal fetal/NW cerebellum (n = 6). Eighty-four miRNAs (64 miRNAs downregulated and 20 miRNAs upregulated) were considered to be differentially expressed (fold change ≥2.0, p ≤0.05) in MB in comparison to normal fetal/NW cerebellum (Table 3; Figure 1). Among these 84 miRNAs, 46 had been previously described as deregulated in human primary MB (Table 3), and only 8 were previously validated by functional assays (Table 4). Upregulation of miRNAs from the miR-17 ~ 92 cluster (in this work miR-18a, 19a, and 92a-1) and downregulation of miR-324-5p were previously described in human MB of the SHH subgroup (12, 13). Of especial note, 32 of the 64 downregulated miRNAs belong to a large cluster on human chromosome 14q32 (Figure 1; Table 3).

SIGNALING PATHWAYS ANALYSIS BY DIANA
Signaling pathways putatively altered by MB deregulated miRNA were depicted by DIANA-miRPath. The list of the top 20 pathways is shown in Table 5. The Ribosome pathway was only pointed by the list of downregulated miRNAs. Adherens junction, oxidative phosphorylation, and TGF-beta signaling pathways showed higher enrichment when the list of downregulated miRNAs was used in the analysis. On the other hand, the MAPK pathway and genes associated to cancer showed higher enrichment when upregulated miRNAs were used in the analysis.

Interestingly, oxidative phosphorylation, TGF-beta signaling pathway, and ubiquitin mediated proteolysis were enriched in the list of 14q32 miRNAs.

INGENIETY PATHWAY ANALYSIS
Network analysis by IPA identified two networks as putative targets for 73 out of the 84 MB miRNAs. Networks were prospected considering only relationships that were experimentally observed. Interestingly, both networks were enriched with miRNAs belonging to the 14q32 cluster.

Network 1 (Figure 2A) included 13 miRNAs of the 14q32 cluster (also known as miR-154 cluster), which were all downregulated in MB samples (miR-154, 323-3p, 323-5p, 369-5p, 377*, 381, 382, 409-5p, 410, 485-3p, 487a, 487b, 539) and were depicted by IPA as having direct interactions with BCL2LI1, JUN, BIRC5, MAP2K4, and NR0B2. BIRC5 and BCL-2 have anti-apoptotic roles, and are expected to be at increasing levels in MB as all miRNAs connecting to these genes were found downregulated (Figure 2A). NR0B2 and JUN were suggested in this network as candidate genes controlling the expression of the 14q32 miRNA cluster.

Again, most miRNAs shown in Network 2 (Figure 2B) belong to the 14q32 cluster (miR-127-3p, 127-5p, 134, 379, 411, 432, 433, 495, and 758). In this case, NR0B2 and estrogen-related receptor γ (ESRRG) were suggested as candidate genes controlling the expression of the 14q32 miRNA cluster. Insulin appeared in Network 2 as indirectly controlling the expression of miR-206, 324-5p, 432, and 95.

RT-qPCR VALIDATION OF SOME DEREGULATED miRNAs
miR-323-3p and 495, both belonging to cluster 14q32, were chosen for validation by RT-qPCR. In addition, miR-206 and miR-129-5p were chosen for analysis because of their high fold change (see Table 3), lack of previous functional studies and possible oncogenic role. miR-206 expression was reported to inhibit cell proliferation in breast cancer cells (22). miR-129 is reported to be significantly downregulated in pediatric brain tumors compared to normal tissues (23). Most importantly, miR-129 downregulation is associated to SOX4 overexpression in endometrial and gastric cancers (24, 25). SOX4 is upregulated and has prognostic impact in MB (26, 27).

Real-time RT-qPCR analysis were performed with all samples used in the microarray analysis (n = 6 MB and n = 6 cerebellum) plus four other samples of MB (Table 1) and two new fetal/NW cerebellum controls (Table 2). As expected, miR-206 (p = 0.0001; Mann–Whitney test), miR-129-5p (p = 0.002), miR-323-3p (p = 0.014), and miR-495 (p = 0.054), had lower expression in MB in comparison to normal cerebellum (Figure 3), thus confirming our microarray findings. Expression of miR-206,

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1. www.r-project.org
2. www.metaboanalyst.ca
3. http://diana.cslab.ece.ntua.gr/pathways/index_multiple.php
4. http://www.ingenuity.com/
129-5p, 323-3p, and 495 were also investigated in a representative panel of MB cell lines. Compared with normal human cerebellum, miR-206, 129-5p, and 323-3p expression were found to be downregulated in all MB cell line tested (Figure 3).

**UPREGULATION OF miR-206, 129-5p, AND 323-3p IN DAOY CELLS**

As a first approach to investigate the functional significance of miRNAs downregulation in MB, DAOY cells were transiently transfected with mimics of miR-206, 129-5p, 323-3p, or negative control #1. Transfection efficiency was confirmed by RT-qPCR (Figure A2 in Appendix). Twenty hours post-transfection, cells were collected and seeded in 96-well plates in serum-free medium. DAOY cells survive and even proliferate in serum-free medium for a short period of time. As shown in Figure 4, no consistent differences in proliferation were found in transfections with miR-206 and miR-323-3p. On the contrary, transfections with miR-129-5p...
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Table 4 | miRNAs validated by functional assay in human and mouse medulloblastoma.

| miRNA | Deregulation | Cells | Functional assay | Target genes | Reference |
|-------|--------------|-------|------------------|--------------|-----------|
| 124   | Down         | Primary human MB, cell lines | ↑ Cell cycle progression at G1, ↓ Cell proliferation | CDK6 | (48, 49) |
|       |              |       |                  | SLC16A1      |           |
| 324-5p| Down         | Primary human and mouse MB, cell lines | ↓ Cell proliferation | SMO | (50) |
| 326   | Down         | Primary human and mouse MB, cell lines | ↓ Cell proliferation | GLI1 |           |
| 125p  | Down         | Primary human MB, cell lines | ↑ Apoptosis, ↓ Cell proliferation | Trkc | (12) |
| 199b-5p| Up          | Primary human and mouse MB, cell lines | ↑ Cell cycle progression at G1, ↓ Cell proliferation | HES1 | (28) |
| 128   | Down         | Primary human and mouse MB | ↓ Cell proliferation, ↑ Cell senescence | BMI-1 | (61) |
| 21    | Up           | Primary human MB, cell lines | ↓ Cell proliferation | PDCD4 | (31) |
| 935   | Down         | Primary human MB, cell lines | – | KIAA0232, SLC5A3, TBC1D9, ZFAND6 | (52) |
| 344   | Down         | MB cell lines | ↑ Apoptosis, ↑ Cell cycle progression at S/phase and G2/M, ↑ Cell proliferation, ↑ Cell senescence | MAGE-A, Dll1, Notch1, Notch2 | (30, 53) |
| 512-5p| Down         | Primary human MB, cell lines | – | MYCC | (64) |
| 383   | Down         | Primary human MB, cell lines | ↑ Apoptosis, ↑ Cell cycle progression at G1, ↓ Cell proliferation | PRDX3 | (55, 56) |
| 183~96~182| Down     | MB cell lines | ↑ Cell cycle progression at G0/G1 and G2, ↓ Cell migration, ↓ Cell proliferation | AKT | (57) |
| 218   | Down         | MB cell lines | ↓ Cell migration, ↓ Cell proliferation | CDK6, REST | (58) |

Underlined miRNAs are miRNAs also found deregulated in the present study;
*results of ectopic expression or knockdown assays; MB = medulloblastoma.

resulted in a significant decrease in DAOY cell proliferation, as evaluated by the MTS assay (Figure 4). Similar experiments were conducted to evaluate cell survival and apoptosis by the Annexin V and propidium iodide staining methodology. No significant differences were found on cell viability or apoptosis after miR-206, miR-129-5p, and miR-323-3p transfections in comparison to control (Figure 4; Figure A3 in Appendix, respectively).

**DISCUSSION**

We investigated the expression profile of miRNAs in primary human MB of desmoplastic histology and SHH molecular subgroup, in comparison to normal fetal/newborn cerebellum. Eighty-four miRNAs were found to be differentially expressed in MB. The majority of these differentially expressed miRNAs were downregulated in comparison to normal cerebellum, corroborating previous studies. Most upregulated miRNAs identified in our study (12 out of 20) had been previously described in MB (12–14, 23, 28–32). On the contrary, 31 out of the 64 downregulated miRNAs are here described for the first time in association to MB (Table 3). Differences may be explained by the fact that a more comprehensive version of Affymetrix miRNA microarray was used in the present study. Moreover, previous studies included different subtypes of MB and a mix of children and adults cerebellum samples in their analysis (12, 13, 29). We believe that analysis on more uniform groups of both cancer and control samples may have helped us in detecting some smaller but consistent differences between groups.
Table 5 | Top 20 pathways predicted by DIANA-miRPath analysis.

| Pathway signaling | All deregulated miRNAs | Downregulated miRNAs | Upregulated miRNAs | 14q32 miRNAs |
|-------------------|------------------------|-----------------------|--------------------|--------------|
| P-value            |                        |                       |                    |              |
| Ribosome          | 30.03                  | 25.34                 | –                  | 17.78        |
| Axon guidance     | 24.96                  | 19.98                 | 17.95              | 17.7         |
| Wnt signaling path| 18.6                   | 19.34                 | 17.71              | 16.99        |
| Focal adhesion    | 16.65                  | 17.02                 | 15.13              | 14.73        |
| Adherens junction | 16.23                  | 20.37                 | 12.18              | 16.6         |
| Oxidative phosphorylation | 14.95 | 14.76                 | 7.34                | 14.45        |
| ErbB signaling pathway | 14.8     | 11.03                 | 9.14                | 9.02         |
| Metabolism of xenobiotics by cytochrome P450 | 14.06 | 15.33                 | 3.15                | 10.01        |
| Renal cell carcinoma | 13.48     | 13.21                 | 9.35                | 11.64        |
| TGF-beta signaling pathway | 12.4 | 14.11                 | 5.64                | 17.56        |
| Regulation of actin cytoskeleton | 12.25 | 11.52                 | 7.86                | 10.63        |
| Chronic myeloid leukemia | 12.06 | 11.02                 | 8.14                | 10.54        |
| MAPK signaling pathway | 11.86 | 9.81                  | 17.06              | 12.4         |
| Colorectal cancer | 11.72                  | 13.24                 | 9.79                | 16.92        |
| Glioma            | 10                     | 8.47                  | 16.21              | 7.23         |
| Pancreatic cancer | 9.69                   | 9.61                  | 13.18              | 5.83         |
| Melanogenesis     | 9.4                    | 9.32                  | 9.07                | 10.59        |
| Ubiquitin mediated proteolysis | 9.28 | 10.09                 | 6.63                | 11.11        |
| Prostate cancer   | 9.1                    | 7                     | 18.2                | 6.06         |
| Insulin signaling pathway | 9     | 8.54                  | 5.86                | 4.65         |

*The negative natural logarithm of the enrichment P-value calculated for the specific pathway. Underlined shows higher enrichment in downregulated, upregulated, or 14q32 miRNAs lists.

A computational analysis was performed to predict the network and signaling pathways collectively targeted by the 64 downregulated and 20 upregulated miRNAs. Downregulated miRNAs in MB were predicted to target genes related to the ribosome, adherens junction, oxidative phosphorylation, metabolism of xenobiotics by cytochrome P450, and transforming growth factor-beta (TGF-β) signaling pathways. Axon guidance, TGF-β, WNT, insulin signaling pathways are known to play an important role in neurulation, CNS development, and/or MB pathogenesis (3, 33, 34). Since miRNA act as negative regulators of gene expression, a simpler interpretation of these findings is that MB has increased activation of these pathways in comparison to normal cerebellum.

Most importantly, half (32/64) of downregulated miRNAs reported in our study were found to belong to the cluster at 14q32 locus (also known as miR-154 cluster). This is in keeping with a previous study in a mouse model of MB, reporting that activation of SHH signaling leads to downregulation of the miR-154 cluster (35). Moreover, previous publications with primary MB found downregulation of some 14q32 miRNAs in MBs of the molecular subgroups WNT, SHH, and C as compared to normal cerebellum and MBs of subgroup D (13, 29). However, this is the first time that so many 14q32 miRNAs are shown to be downregulated in MB, thus suggesting a co-regulatory control of this cluster’s expression.

Deletions at locus 14q32 would be one possible explanation to the decreased 14q32 miRNAs expression. The recent analysis of somatic copy number aberrations in 1,087 MB samples report significant losses of chromosome arm 14q in the SHH subgroup of MBs, though not restricted to 14q32 (36). Alternative explanations are discussed below. Our Ingenuity pathways analysis pointed to nuclear orphan receptor NR0B2 (also known as Small Heterodimer Partner, SHP) and (ESRRG) as possible controllers of 14q32 mRNA cluster expression. There is indeed experimental evidence in mouse showing that NR0B2 is a repressor while ESRRG is and activator of a miRNA cluster in chromosome 12, which is ortholog to the 14q32 cluster in humans (37). Our analysis of microarray mRNA expression data for 64 primary human MB samples, accessible through GEO Series accession number GSE28245 (38) in NCBI’s Gene Expression Omnibus (39) revealed that NR0B2 is not expressed in MB. Interestingly, ESRRG expression was found to be relatively high in MB of the molecular subgroup D, intermediate in MB of the WNT and C subgroups, and very low or absent in MBs of the SHH subgroup (Figure A4A in Appendix), thus reflecting 14q32 miRNAs abundance in each of the MB subgroup. These findings were confirmed by the analysis of gene expression data of an independent cohort of 90 primary MB samples (accession number GSE21166) deposited by Northcott et al. (13) (data not shown). ESRRG suppress cell proliferation in prostate cancer cells (40) and the estrogen receptor beta agonist diarylpropionitrile (DPN) exhibit a pro-apoptotic and anti-proliferative effect on MB (41). Experiments are warranted to investigate a possible causal connection between ESRRG and 14q32 miRNA cluster expression in MB.

The miRNA cluster at 14q32 lies within a parentally imprinted chromosomal area spanning genes Dlk1, Meg3, Rtl1, Meg8, and Dio3 (42). Dlk1, Rtl1, and Dio3 are paternally-, whereas Meg3 and Meg8 are maternally expressed transcripts (43). Imprinting of 14q32 is regulated, to some extent, by two intergenic differentially methylated regions known as IG-DMR and MEG3-DMR (44, 45).
Deletions of the regulatory regions and/or epigenetic modifications may in theory cause aberrant 14q32 silencing in cancer. The recent 1,000 genome study of somatic copy number aberrations shows no recurrent focal deletions at locus 14q32 in MB (36). However, our analysis of public mRNA microarray expression data GSE28245 (38) revealed that MEG3 is downregulated in MB in comparison to normal cerebellum. MBs of the molecular group C and WNT have the lowest expression, SHH has intermediate
FIGURE 3 | Validation of miR-206, 129-5p, 323-3p, and 495
downregulation by reverse transcriptase-quantitative PCR.
Expression levels of miR-206, 129-5p, 323-3p, and 495 were
performed in MB, normal fetal/newborn cerebellum and four different
MB cell lines. Expression of miR-495 was not performed in D341 cell
line. ○, DAOY; ▼, Meb-Med-8A; x, D283Med; ♦, D341. Comparisons
of MB versus normal cerebellum were done by the Mann–Whitney
test.

FIGURE 4 | Effect of miR-206, 129-5p, and 323-3p overexpression in
DAOY cells proliferation. DAOY cells were transiently transfected with
miRNAs mimic and replated after 20 h at a 1,500 cells/well density. Cell
proliferation was evaluated by the MTS assay at 4, 28, and 52 h after
re-plating, thus after 24, 48, and 72 h post-transfection. Results are
representative of three independent experiments. Mean ± SE are shown;
** P < 0.01 according to two-tailed t-test; NC, negative control miRNA
mimic (scrambled).
levels while group D have MEG3 levels closer to normal cerebellum (Figure A4B in Appendix). Thus MEG3 expression seems to correlate with the expression of 14q32 miRNAs among the different MB molecular groups, suggesting that the 14q32 miRNA locus may be under epigenetic regulation in MB. However, a genome-wide analysis of promoter methylation on four primary MB samples showed no consistent methylation of 14q32 gene promoters (46). Although higher number of samples should be analyzed, this result corroborates findings in osteosarcoma, a tumor also presenting with downregulated 14q32 miRNAs expression and with no consistent changes in the methylation patterns at 14q32. Instead, silencing of 14q32 miRNA in osteosarcoma seems to be mediated by histone modification(s) (47).

Preliminary functional studies were performed in DAOY cells by ectopic expression of miR-129-5p, 206, and 323-3p mimics. Mimics for miR-206 and 323-3p had no significant effect on DAOY cells. miR-129-5p overexpression resulted in decreased cell proliferation, which may suggest a tumor suppressor role in MB.

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APPENDIX

FIGURE A1 | Expression of OTX2 and PTCH1 in the different primary medulloblastomas (MB), medulloblastoma cell lines (named), and normal cerebellum (C). Total RNA (100 ng) was reverse transcribed and gene expression was measured by qPCR, in triplicates, using the SYBR method. Primers are available upon request to the authors. Values were normalized to the HPRT endogenous control gene. Relative expression values were calculated using the $2^{-\Delta\Delta C_T}$ method.

FIGURE A2 | Transfection efficiency of miR-206, miR-129-5p, and miR-323-3p in DAOY cells as evaluated by RT-qPCR. Cells transfected with the scrambled mimic were included as negative control (NC). Values were normalized to the RNU6B endogenous control RNA. Relative expression values were calculated using the $2^{-\Delta\Delta C_T}$ method. Bars represent mean ± SD. Comparisons were performed by the Mann–Whitney test.
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Propidium Iodide

Annexin V

0.5% 3.0%
86.3% 10.3%
1.5% 3.5%
91.8% 3.2%
0.7% 4.1%
83.8% 11.3%
0.9% 3.4%
83.5% 12.2%

NC miR-206 miR-129-5p miR-323-3p

24 hours post-transfection

48 hours post-transfection

NC miR-206 miR-129-5p miR-323-3p

FIGURE A3 | Apoptosis analysis of DAOY cells at 24 and 48 h post-transfection with miR-206, 129-5p, 323-3p, or negative control mimics, as evaluated by Annexin V and propidium iodide staining and FACS analysis. DAOY cells grown in serum-free RPMI-1640 medium. The percentage of necrotic (Q1), late apoptotic (Q2), viable (Q3), and early apoptotic (Q4) cells are shown in the corresponding quadrants. NC, negative control (scrambled mimic).
FIGURE A4 | Gene expression graph for different microarray probes of interest. Data from 64 primary human MB samples accessible through GEO Series accession number GSE28245 (38) in NCBI’s Gene Expression Omnibus. (A) probe sets for ESCRG. (B) Probe sets for MEG3. Samples were grouped according to the molecular MB subgroups into WNT, SHH, C, and D. SHH desmoplastic was separated from SHH classic.