Research article

SIRPα is transcriptionally downregulated by epigenetic silencing in medulloblastoma

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Abstract

Signal regulatory protein α (SIRPα) is a transmembrane protein that is commonly expressed in cells of the hematopoietic system and brain. Its function is not fully understood but it includes tumor suppressor properties and effects on differentiation. SIRPα may play a role in the development of medulloblastoma (MB), a WHO grade IV brain tumor, which is the most common malignant brain tumor in childhood. The aim of the current study was to determine the possible role of SIRPα in MB cells. Interestingly, in contrast to normal cerebellum, SIRPα mRNA was strongly downregulated in MB and its protein was not detectable in MB tissues. This down-regulation in MB cells was associated with transcriptional silencing of SIRPα via CpG island promoter hypermethylation. Furthermore, Oncomir cluster miR17-92 and miR-106a were correlated with SIRPα gene silencing in MB tumor specimens and cell lines. Histone modification and inhibition of DNA methylation using TSA (20 nM) for 24 hrs and 5-AZA (5 μM) and DZnep (2.5 μM) for 72 hrs, respectively, increased SIRPα expression 25-40 fold and resulted in 90% cytotoxicity of MB tumor cell lines D283-med and D458-med. Remarkably, forced upregulation of SIRPα by viral transduction in MB cell lines did not affect cell growth. In conclusion, SIRPα is epigenetically silenced in MB cells and tumor specimens by promoter hypermethylation and possibly by miRNA expression. SIRPα hypermethylation in MB might reflect the precursor cell state of these cells, rather than being a tumor-specific event, since SIRPα overexpression did not influence MB cell viability. The mechanism of the anti-MB action of epigenetic therapy requires further investigation since our findings indicate that this effect is independent of SIRPα upregulation.

Keywords

Medulloblastoma; SHP-1; SHP-2; SIRPα; Epigenetics; Methylation; MiRNA

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1. Introduction

Medulloblastoma (MB), a WHO grade IV tumor, constitutes 20% of all brain tumors in children and is the most common malignant brain tumor in childhood. MB arises in the cerebellar vermis, invading the cerebellum, occasionally metastasizing leptomeningeally via the cerebrospinal fluid. Aggressive therapy, which nowadays consists of chemoradiotherapy after initial surgery and maintenance chemotherapy has improved survival over the last three decades and has resulted in an 10-year overall survival of 42-70% in high-risk patients, depending on metastasis stage, and up to 91% in non-metastasized, standard-risk patients [1]. The downside to this improved survival is the therapy associated occurrence of late effects, such as endocrine and neurocognitive sequelae, mainly attributable to tumor localization and radiation therapy. There is an obvious need to define tumor specific targets, enabling tumor-targeted drug therapy to further ameliorate survival and increase the quality of life by reduction of late effects. In order to identify druggable targets, it is essential to decipher the molecular mechanism(s) on which MB cells rely to sustain their tumor growth. Hence, in our present study, we investigated signal regulatory protein α (SIRPα) (PTPNS1, SHPS-1, CD172a, p84, gp93, MFR or BIT) in MB, as it has previously been associated with growth and differentiation of other cancers [2–8]. SIRPα is a transmembrane protein, which has three extracellular immunoglobulin superfamily (IgSF) domains and a cytoplasmic tail containing four immunoreceptor tyrosine-based inhibitory motifs (ITIMs). Ligation with CD47 and other ligands, such as thrombospondin-1 (THSP-1), leads to phosphorylation of these ITIMs, resulting in recruitment, phosphorylation and activation of tyrosine phosphatases SHP-1 and SHP-2, producing largely inhibitory and tumor-suppressive signals via SHP-2, mainly involving ERK and Akt signaling pathways [9–11].

SIRPα is an important protein in neural development, especially

facilitating neuronal differentiation, maturation and synaptogenesis. Active neurons are able to shed a soluble fragment from the extracellular domain of the SIRPα protein to generate a synaptogenic signal, thereby governing presynaptic neurons. In addition, SIRPα is involved in inducing new synapses [12, 13] and synapse maturation [14, 15]. In cerebellar Purkinje cells, SIRPα associates with dendrites and growing parallel fibers. Upon interaction of SIRPα with its ligand CD47, abundantly expressed in brain, downstream signaling is mediated by the Src family of kinases, promoting the development of axons and dendrites in hippocampal neurons [12, 13]. Thrombospondins interacting with SIRPα are also involved in this process [16]. SIRPα was found to induce apoptosis in acute myeloid leukemia (AML) cells upon ligation with an agonistic antibody [7]. In analogy to AML, and with its role in neuronal development, the aim of the current study was to investigate the expression and anti-tumor activity of SIRPα in MB.

2. Materials and Methods

2.1. In silico analysis

mRNA expression of several genes involved in SIRPα signal transduction was determined using R2, a microarray analysis and visualization platform, provided by the Department of Human Genetics of the Academic Medical Center, Amsterdam, The Netherlands (http://r2.amc.nl). In a large MB dataset provided by the Division of Pediatric Neuro-oncology, German Cancer Research Center DKFZ, Heidelberg, Germany, we analyzed the mRNA expression of SIRPα(SHPS1; 202897_at), CD47 (226016_at), SHP-1 (PTPN6; 206687_s_at) and SHP-2(PTPN11; 212610_at) using MAS5.0 normalized data of the previously defined four MB subgroups [17, 18]. Whole genome bisulfite sequencing data were used to analyze methylation profiles of the SIRPα promoter region in a series of MB samples, comprising Wnt group MB (n = 5), Shh group MB (n = 6), Group 3 (n = 11) and Group 4 (n = 12) MB samples, with normal fetal (n = 4) and adult (n = 4) cerebellum samples as controls, collected within the International Cancer Genome Consortium (ICGC) PedBrain Tumor Project (Heidelberg cohort). Matching SIRPα mRNA expression array data (Affymetrix U133 plus 2.0) were used to correlate SIRPα methylation profiles with expression.

2.2. Immunohistochemistry

SIRPα immunohistochemistry was conducted on an independent MB tissue microarray (TMA) with tumors from 87 patients obtained from the bio-bank of the Department of Neuropathology of the Academic Medical Center. General informed consent was obtained for the use of brain tissue and for access to medical records for research purposes. Normal cerebellum, hippocampus and neocortex were used as control tissues. Tissue sections (5 µm), mounted on superfrost slides (Menzel, Braunschweig, Germany) had undergone dewaxing and rehydration, after which endogenous peroxidase activity was blocked for 30 min in methanol containing 0.3% hydrogen peroxide. Slides were then washed with distilled water and phosphate-buffered saline (PBS; 10 mM, pH 7.4). For antigen retrieval, the slides were placed in sodium citrate buffer (10 mM, pH 6.0) and heated in a microwave oven at 99°C for 10 min, and cooled to room temperature. The sections were washed in PBS and pre-incubated with 10% normal goat serum (NGS) diluted in PBS 30 min prior to incubation with a SIRPα-specific antibody

(1:1000, ab8120, Abcam, Cambridge, UK) for 1 hr. The sections were washed with PBS and incubated at room temperature for 30 min with Dako REAL™ EnVision™ /HRP, Rabbit/Mouse (Dako, Glostrup, Denmark). Peroxidase activity was detected using Dako REAL™ 3,3-diaminobenzidine-tetrachloride (DAB)+ Chromogen (Dako, Glostrup, Denmark), for 10 min in the dark. All sections were counterstained with hematoxylin, covered with a coverslip and examined under the microscope.

2.3. Western blot analysis

To determine protein (SIRPα, SHP-1, SHP-2) levels in MB cell lines, Western blot analysis was used. Whole cell lysates were prepared using RIPA lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP40, 0.5% Na-deoxycholate, and 0.05% SDS) supplemented with 1mM pefabloc (Sigma-Aldrich, Zwijndrecht, The Netherlands). Protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad, Veenendaal, The Netherlands). Proteins (100 µg) were resolved by 7.5% SDS-PAGE and transferred to PVDF membrane (Millipore, Amsterdam, The Netherlands). Subsequently, the membrane was blocked with TBST (Tris-buffered saline with 0.1% Tween 20) + 5% non-fat milk for 1 hr at room temperature (RT) and incubated with rabbit polyclonal anti-SIRPα (1:500; ab8120, Abcam, Cambridge, UK), rabbit SH-PTP2 (1:500, sc280, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and β-actin monoclonal mouse antibody (1:2500; Millipore, Temecula, CA, USA). Protein lysates from tumor cell lines HL60 and CCRF-CEM were used as positive controls for immunoblots of SIRPα (HL60) and SHP-1 and SHP-2 (both HL60 and CEM), respectively. Thereafter, the membrane was incubated for 1 hr at RT with goat anti-mouse-HRP (Dako, Glostrup, Denmark) or goat anti-rabbit-HRP (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA), diluted 1:2500 in 1% blocking buffer. Subsequently, the membrane was washed three times with TBST + 0.5% milk and SIRPα, SHP-1, SHP-2 protein and β-actin was visualized on hyperfilm using an ECL plus system (Amersham Bioscience, England).

2.4. Tumor cell lines

The human MB tumor cell lines D283-med, D425-med, D458-med and D556-med (kindly provided by Dr. Darrell Bigner, Duke University, NC, USA) were routinely cultured in suspension in Dulbecco's Modified Eagle Medium (DMEM; PAA Laboratories GmbH, Pasching, Austria) containing glutamine, supplemented with 1% penicillin/streptomycin (PAA Laboratories GmbH, Pasching, Austria) and 10% fetal bovine serum (FBS; PerBio Science Nederland B.V., Etten-Leur, The Netherlands). HL-60 (human promyelocytic leukemia) and CCRF-CEM cells (human pediatric acute lymphoblastic T-cell leukemia) were routinely cultured in RPMI-1640 medium (Gibco Laboratories, Irvine, UK) supplemented with 10% fetal calf serum (Integro BV, Dieren, the Netherlands).

2.5. Real-time Quantitative PCR

Real-time Quantitative PCR (RT-qPCR) analysis was carried out on cDNA obtained from 1 µg total RNA, isolated using Trizol (Life Technologies) with the Omniscript RT kit (Qiagen, Venlo, The Netherlands) following the manufacturer's procedures. Validated Quantitect primers for SIRPα and the housekeeping gene GAPDH were purchased from Qiagen.

For the RT-qPCR analysis of mature microRNA clusters miR-17-92, miR-106a-25 and 106b-363, small RNAs were isolated from normal cerebellum and MB tissues, further characterized in Table 1, using the Mirvana kit, following the manufacturer's instructions. Validated primers for hsa-miR-17, hsa-miR-20a, hsa-miR-106a, hsa-miR-106b and hsa-miR-363, and control microRNA miR-20b and housekeeping gene RNU48 were purchased from Life Technologies. MiR-20b was employed as control micro-RNA, predicted not to interact with SIRPα. Per 10 μl RT-qPCR reaction containing FastStart Universal SYBR Green Master reaction mixture (Roche), 0.25 μl cDNA were added. RT-qPCR was performed using the ABI7500 real time thermal cycler (Life Technologies). The delta-delta CT method [19] was applied to obtain relative SIRPα mRNA levels. Results are presented as fold change of SIRPα mRNA and these miRNAs to reference sample 9 (Table 1, 18-year old female, normal cerebellum).

Table 1. Characteristics of normal cerebellum and medulloblastoma tissue samples and medulloblastoma cell lines.

Sample#	Age	Sex	Histology
Normal cerebellum			
1	33 GW	Female	
2	37 GW	Male	
3	1 y	Female	
4	2 y	Female	
5	9 y	Male	
6	10 y	Male	
7	15 y	Male	
8	17 y	Female	
9	18 y	Female	
10	31 y	Male	
Medulloblastoma			
1	1 y	Male	MB NOS
2	3 y	Female	Classical MB
3	4 y	Male	Desmoplastic Nodular MB
4	7 y	Male	MB NOS
5	8 y	Male	Classical/Desmoplastic MB
6	13 y	Male	Desmoplastic MB
7	15 y	Male	Large Cell Anaplastic MB
8	31 y	Male	Desmoplastic MB
9	6 y	Female	Desmoplastic MB
10	9 y	Male	Desmoplastic MB
11	5 y	Female	MB NOS
12	31 y	Female	Desmoplastic MB
13	8 y	Male	Desmoplastic MB
Medulloblastoma cell lines			
A	D283-med		
B	D458-med		
C	D556-med		

2.6. Methylation Specific PCR (MSP)

For methylation specific PCR, DNA was isolated using Trizol. DNA was then subjected to bisulfite treatment using the EZ DNA Methylation Gold Kit (Zymo Research) following the manufacturer's instructions. DNA methylation patterns in the CpG island of the PTPNS1 gene (encoding SIRPα; accession AL117335) were determined by PCR with specific primers designed for methylated, bisulfite-treated DNA. Two primer sets for MSP were designed to target two regions within the promoter CpG island (named R1 and R2), R1(F) 5'-AGGGTTAAAAGGTAGACGTTTTTTC-3', R1(R) 5'-ACGCTACGAATAACTACATCGTC-3' and R2(F) 5'-GTTCGTTTAGTTTCGAGTTTTAGC-3', R2(R) 5'-CACCTTCTCTACGAACGAACG-3'. PCR conditions for R1 were: 94°C 3 min (1 cycle); 94°C 30 s, 60°C 30 s (40 cycles); 72°C (1 cycle), for R2: 94°C 3 min (1 cycle); 94°C 30 s, 56°C 30 s (40 cycles); 72°C (1 cycle). A PCR for the bisulfite converted housekeeping gene β-actin (ACTB-Fw: TGGTGATGGAGGAGGTTTAGTAAGT, ACTB-Rv: AACCAATAAAACCTACTCCTCCCTTAAA) was performed as a reference. For RT-qPCR mRNA expression experiments, snRNP was used as a housekeeping gene. MSP was performed on an ABI 7500 real-time PCR system (Applied Biosystems).

2.7. Demethylation experiments

DNA methyltransferase inhibitor 5-aza-cytidine (5-Aza), lysine methyltranferase enhancer of zeste 2 (EZH2) inhibitor 3-deazaneplanocin A (DZN) and histone deacetylase (HDAC) inhibitor trichostatin A (TSA) were purchased from Sigma Aldrich (St Louis, MO, USA). For demethylation experiments, cells were seeded (0.5 × 10^5/ml) and incubated in 5% CO_2 humidified atmosphere at 37°C to obtain logarithmic growth. After 48 hr, cells were treated with 50 nM-5 μM AZA or 50 nM-2.5 μM DZnep or vehicle for 72 h, and 20 nM TSA or vehicle during the last 24 hr. Growth medium containing demethylating agents was renewed daily.

2.8. Lentiviral inducible SIRPα overexpression

To ascertain the impact of SIRPα overexpression in MB cells, a sequence verified, lentiviral vector containing a full-length human SIRPα construct (pLenti6.3 - SirpBIT IRES NGFR) was kindly provided by Dr. T van den Berg (Department of Blood Cell Research, Sanquin Research and Landsteiner Laboratory, Academic Medical Centre, University of Amsterdam, Amsterdam, the Netherlands). The SirpBITconstruct was cloned into a pLenti4/TO/V5-DEST vector, using the ViraPower™ HiPerform™ Gateway® Vector Kit (Life Technologies) according to manufacturer's instructions. D458-med cells were transduced to stably express a tetracycline (tet) repressor (Tet-on), using the ViraPower™ HiPerform™ T-REx™ Gateway® Vector Kit. Subsequently, these cells were transduced with the vector containing the SirpBIT construct, allowing for inducible expression of SIRPα upon tetracycline treatment.

2.9. Statistics

For the *in silico* analysis of SIRPα (SHPS1; 202897_at), CD47 (226016_at), SHP-1 (PTPN6; 206687_s_at) and SHP-2(PTPN11; 212610_at) in datasets of normal cerebellum and MB subgroups, one-way analysis of variance (ANOVA) was used to compare expression between these datasets. A p-value < 0.0005 was considered statistically significant.

To determine the correlation between SIRPα mRNA expression and CpG island methylation, and SIRPα mRNA and micro-RNA expression, Pearson's correlation coefficient (r) was calculated. For analysis of correlation of micro-RNA and SIRPα mRNA expression, Spearman's rank correlation coefficient(ρ) calculation was used. Student's t-test was used to compare miRNA expression levels between normal cerebellum and MB or MB cell lines.

3. Results

3.1. *SIRPα mRNA expression is downregulated in medulloblastoma compared to normal cerebellum*

SIRPα mRNA expression in MB and non-malignant cerebellum was determined *in silico* in four subgroups of MB: Wnt group MB (n = 54), Shh group MB (n = 116), Group 3 MB (n = 94) and Group 4 MB (n = 169). SIRPα mRNA expression was found to be strongly downregulated in all of these four subgroups when compared to normal cerebellum (Fig. 1A).

To determine expression of SIRPα-interacting genes, expression of the SIRPα ligand CD47 and SIRPα ITIM binding tyrosine phosphatases SHP-1 and SHP-2 were also determined in these normal and MB tissues. CD47 showed differential expression in MB, comparable to that observed in normal cerebellum (Fig. 1B). For the expression of tyrosine phosphatases SHP-1 and SHP-2 in MB, downregulation of the former (Fig. 1C) and upregulation of the latter (Fig. 1D) was observed as compared to normal cerebellum.

3.2. *Absence of SIRPα protein in medulloblastoma tumor specimens and tumor cell lines*

SIRPα protein levels were evaluated by immunohistochemistry in non-malignant cortex (Fig. 2A), hippocampus (Fig. 2B) and cerebellum (Fig. 2C) and compared to a tissue array of MB, composed of 276 pediatric MB tissue specimens obtained from 87 patients (example pictures depicted in Fig. 2D-2F). Subgroup information for these tumors was obtained previously by immunohistochemistry using antibodies for the subgroup-specific protein markers β-catenin (WNT), DKK1 (WNT), SFRP1 (SHH), NPR3 (Group 3), and KCNA1 (Group 4) as previously described [17]. The patients' clinical and tumor characteristics are summarized in Table 2.

A strong SIRPα protein signal was detected in neuronal synapses in the cortex, hippocampal dentate gyrus and the molecular layer of the cerebellum. Most strikingly, immunohistochemical analysis of the MB tissue arrays, showed no detectable levels of SIRPα protein in any of these MB tissues (Fig. 2D and 2E), in agreement with the downregulation of SIRPα mRNA *in silico*. In one patient sample that included adjacent normal cerebellar tissue (molecular layer), the cerebellar tissue stained positively for SIRPα, whereas tumor cells were negative (Fig. 2F). Consistent with the MB tissue staining, no SIRPα protein was detected in the MB tumor cell lines D283-med, D458-med and D556-med, whereas its downstream ligand tyrosine phosphatase SHP-2 was expressed. In addition, SHP-1 was also not expressed in these MB tumor cells (Fig. 3).

3.3. *The promoter region of SIRPα is hypermethylated in medulloblastoma compared to normal cerebellum*

To determine the molecular mechanism underlying SIRPα silencing in MB, whole genome bisulfite sequencing analysis was used to investigate *in silico* whether CpG island hypermethylation contributes to the observed downregulation; the results of this analysis are depicted in Fig. 4A. Whole genome bisulfite sequencing data from the four MB subgroups versus adult and fetal normal cerebellum revealed hypermethylation (red) of the promoter region (indicated by red bar*) in most MB samples and, partially, in normal fetal cerebellum, whereas in normal adult cerebellum hypomethylation (blue) was observed. CpG island hypermethylation of SIRPα was irrespective of MB subtype. Interestingly, a similar pattern of hy-

permethylation in MB and fetal cerebellum, and hypomethylation in adult cerebellum was observed in the region just downstream of the transcription start site (green bar**).

To validate the observed *in silico* SIRPα promoter hypermethylation in MB, methylation specific PCR was performed on bisulphite treated DNA of an independent set of normal cerebellum tissues and MB (characteristics in Table 2). Two regions (R1 and R2) in the CpG island within the SIRPα promoter were investigated and the results are depicted in Fig. 4B. A similar pattern was observed as was found *in silico*. For R1, methylation was observed in 7/13 MB tissues, versus 2/10 in normal cerebellum tissues (one fetal and one adult tissue sample). R2 was methylated in 5/13 MBs and in 0/10 normal cerebellar tissues. For MB cell lines D283-med (A), D458-med (B) and D556-med (C), hypermethylation of both regions was observed.
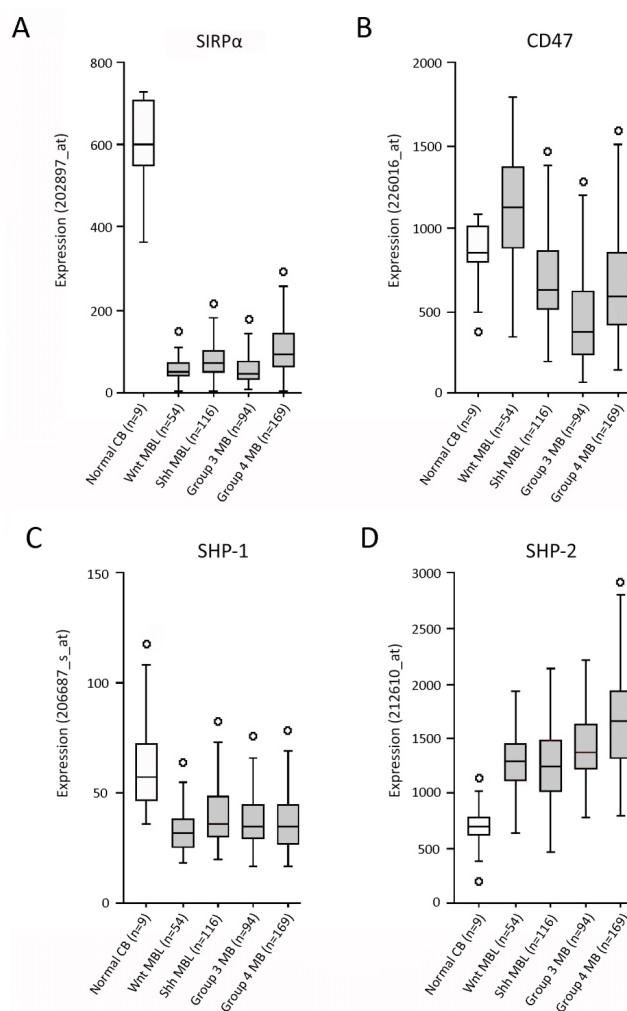

Fig. 1. SIRPα, CD47, SHP-1 and SHP-2 mRNA expression. *In silico* analysis using R2 analysis software on public datasets of non-neoplastic normal adult cerebellum (white) versus datasets of Wnt group, Shh group, Group 3 and Group 4 MB (grey), depicting (A) SIRPα (202897_at), (B) CD47 (226016_at), (C) SHP-1 (206687_s_at) and (D) SHP-2 (212610_at) mRNA expression. Outliers are indicated by "o". All MB subgroups showed significant down-regulation of SIRPα and SHP-1 mRNA expression relative to the normal cerebellum ($p < 0.00001$), whereas SHP-2 was significantly overexpressed in MB. CD47 mRNA was not differentially expressed in MB.
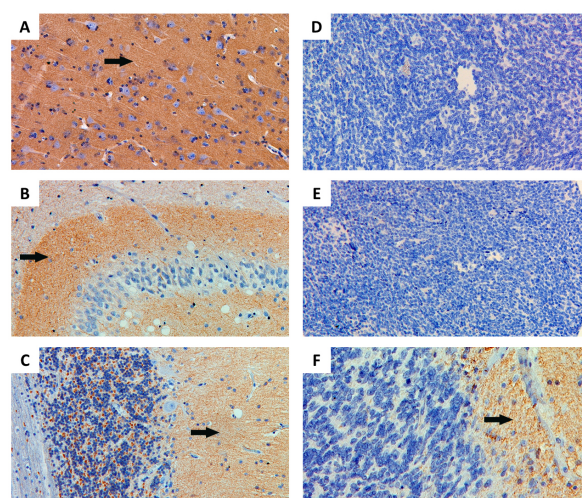
Fig. 2. SIRPα immunohistochemistry on paraffin-embedded sections. (A) Representative images of cytoplasmatic SIRPα staining in neuronal synapses in non-malignant cortex, (B) dentate gyrus of hippocampus and (C) molecular layer of cerebellum. (D,E) No SIRPα protein expression was observed in MB tissues. (F) Positive SIRPα staining in normal cerebellar tissue adjacent to MB tumor tissue. Arrows indicate positive staining.

Table 2. Patient/tumor characteristics of medulloblastoma series on tissue microarray.

Characteristics (N=87)	Number (%)
Gender	
Male	27 (31)
Female	12 (14)
Unknown	48 (55)
Age at diagnosis (median 7.0, range (1-53)	
Infants (<4)	7 (8)
Children (4–16)	19 (22)
Adults (>16)	15 (17)
Unknown	46 (53)
Histology	
Classic	23 (26)
Large cell/anaplastic	3 (4)
Nodular/desmoplastic	8 (9)
Unknown	53 (61)
Molecular subgroups	
WNT	7 (8)
SHH	18 (21)
Group 3	14 (16)
Group 4	33 (38)
Unknown	15 (17)
Metastatic stage	
M0	35 (40)
M+	6 (7)
Unknown	46 (53)
Survival	
Alive	61 (70)
Diseased	26 (30)

To ascertain the correlation between SIRPα methylation and expression in MB and cerebellum, the extent of CpG island methylation

of the SIRPα gene was plotted against SIRPα mRNA expression, using *in silico* analysis of expression and methylation data (Fig. 4C). SIRPα CpG island methylation was found to be in a strong negative correlation with SIRPα mRNA levels in normal adult and fetal brain and the four MB subgroups (Wnt, Shh, Group 3 and 4), with a Pearson's correlation coefficient (r) of -0.76 ($p < 0.000005$).

3.4. Epigenetic modulation induces SIRPα expression and reduces MB cell viability

To overcome the CpG island hypermethylation-based SIRPα gene silencing, MB cell lines D458-med and D556-med were treated for 72 hr with epigenetic modulators as single drugs or as a combination of drugs, and the results are depicted in Fig. 5. All drugs were able to induce SIRPα expression, and a synergistic effect was observed when cells were treated with a combination of the three drugs. In particular, treatment with 5-AZA and DZnep resulted in severe cytotoxicity, even up to 95% when these agents were combined (data not shown). To investigate mRNA expression without extensive cell death, the optimal epigenetic modulator drug exposures were 50 nM, 50 nM, 20 nM for 5-AZA, DZnep and TSA, respectively for 24 hrs. We found SIRPα mRNA upregulation although it did not affect cell viability (shown in Supplementary Fig. S1).

3.5. SIRPα overexpression does not influence cell viability in medulloblastoma cells

These data suggest that the antitumor activity of these epigenetic modulating drugs is independent of SIRPα expression; low concentrations of epigenetic modulating drugs does not affect cell viability, while SIRPα expression is induced. Therefore, we achieved forced SIRPα expression in MB cells by transduction of an inducible, human SIRPα expression construct. Upon tetracycline treatment, a strong induction of both SIRPα mRNA (Fig. 6A) and protein (Fig. 6B) expression was observed, whereas in the negative controls,
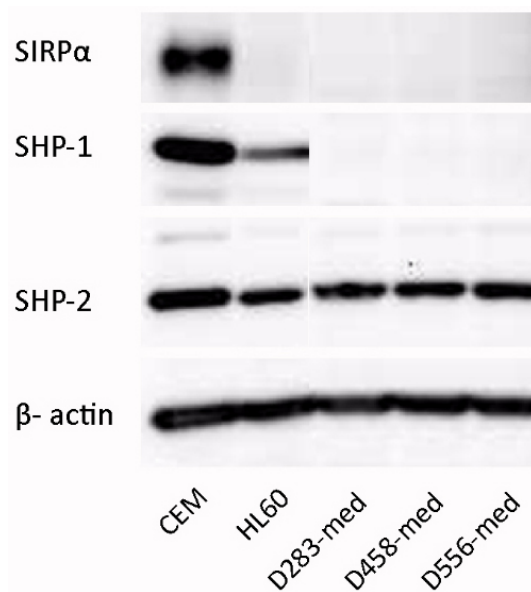

Fig. 3. Immunoblot of baseline SIRPα (75-110 kD), SHP-1 (57 kD), SHP-2 (80 kD) and β-actin (42 kD) in MB cell lines and control cell lines (CEM and HL60 cells respectively).
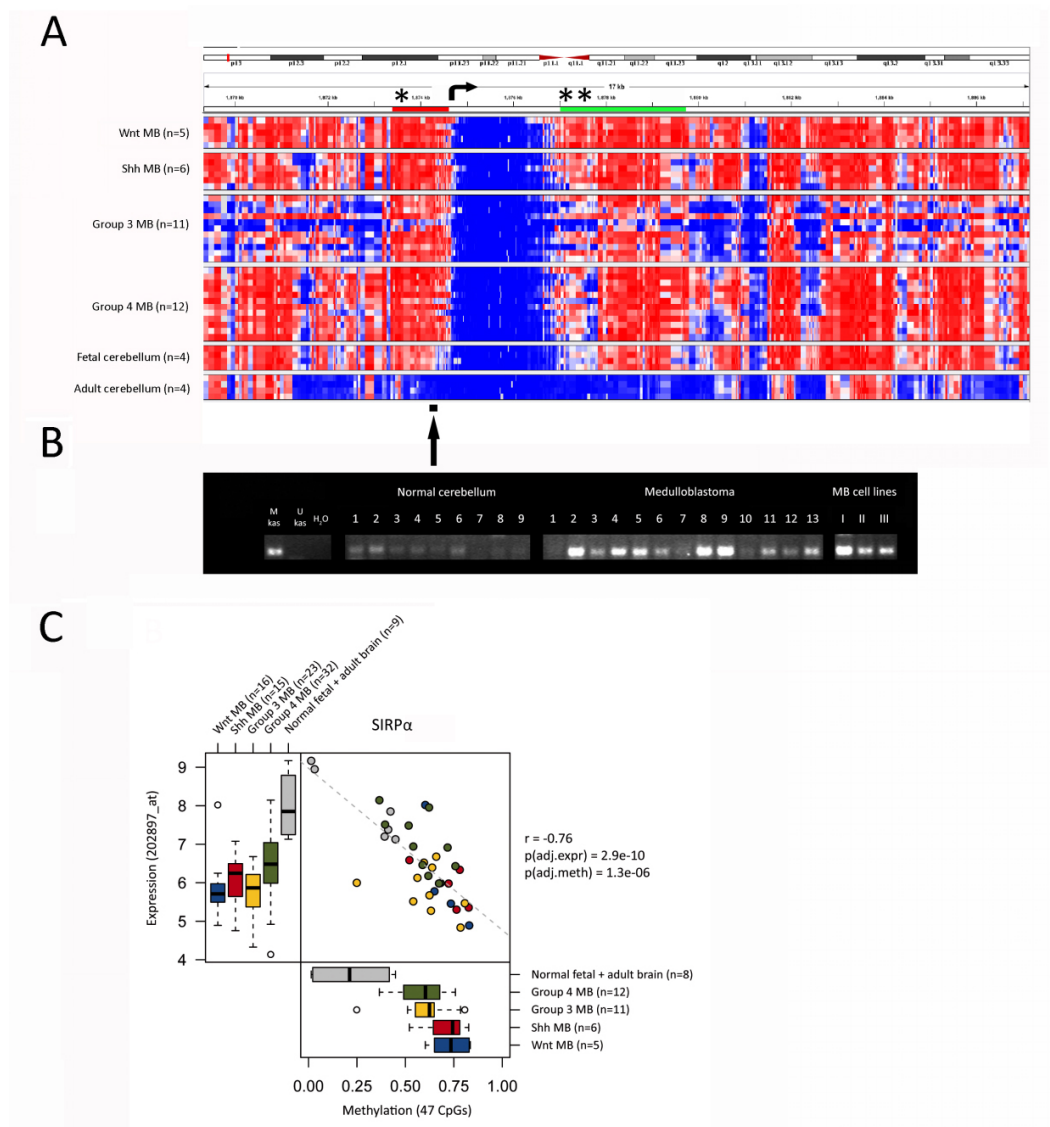
Fig. 4. Epigenetic silencing of SIRPα in MB. (A) SIRPα hyper- (red), and hypomethylation (blue) in MB, normal fetal cerebellum, and normal adult cerebellum. Depicted are the CpG island promoter (red bar*), and the region downstream of the transcription start site (green bar**). (B) Strong negative correlation of SIRPα CpG island methylation with SIRPα mRNA expression in normal adult and fetal brain and MB subgroups (r = −0.76). (C) Methylation-specific PCR (MSP) on two regions (R1/R2) in SIRPα promoter CpG island on an independent set of normal cerebellum tissues, MB and MB cell lines, showing methylation in 7/13 MB tissues, versus 2/10 normal cerebellum tissues in R1 and methylation in 5/13 MBs and in 0/10 normal cerebellar tissues in R2. Hypermethylation of both regions was detected in all three MB cell lines.

D458-med cells expressing an empty TET-repressor, no induction of SIRPα was observed. However, no differences in tumor cell viability between SIRPα expressing and non-expressing D458-med cells were observed (Fig. 6C). In addition, restoration of SIRPα expression did not influence protein expression of its downstream ligand phosphatase, SHP-2 (Fig. 6B).

3.6. *Expression of Oncomir cluster miR-17-92 negatively correlates with SIRPα mRNA expression*

As SIRPα expression was recently described to be post-transcriptionally regulated in macrophages by microRNA cluster miR-17-92 [20], which is overexpressed in MB [21–23], we investigated the correlation of microRNA cluster miR-17-92, and its paralo-gous clusters miR-106a-363 and miR-106b-25 in normal cerebellum and MB tumor specimens; the results are depicted in Fig. 5. A trend to 3.4-fold upregulation of miR-17 ($p = 0.079$) and miR-20a (2.9-fold, $p = 0.058$) and a significant 4.0-fold overexpression of miR-106a ($p = 0.038$) was observed in MB specimens, when comparing normal cerebellum with MB. Significant differences ($p < 0.0001$) were observed when comparing normal cerebellum with MB tumor cell lines D283-med, D458-med and D556-med, with 11-, 10- and 18-fold upregulation of miR-17, miR-20a and miR-106a, respectively (Fig. 7A). No differences between normal cerebellum and MB specimens or MB tumor cell lines were observed for MiR-106b and miR-20b. MiR-363 was significantly, 5.4-fold, down-regulated in MB, compared to normal cerebellum ($p = 0.01$).
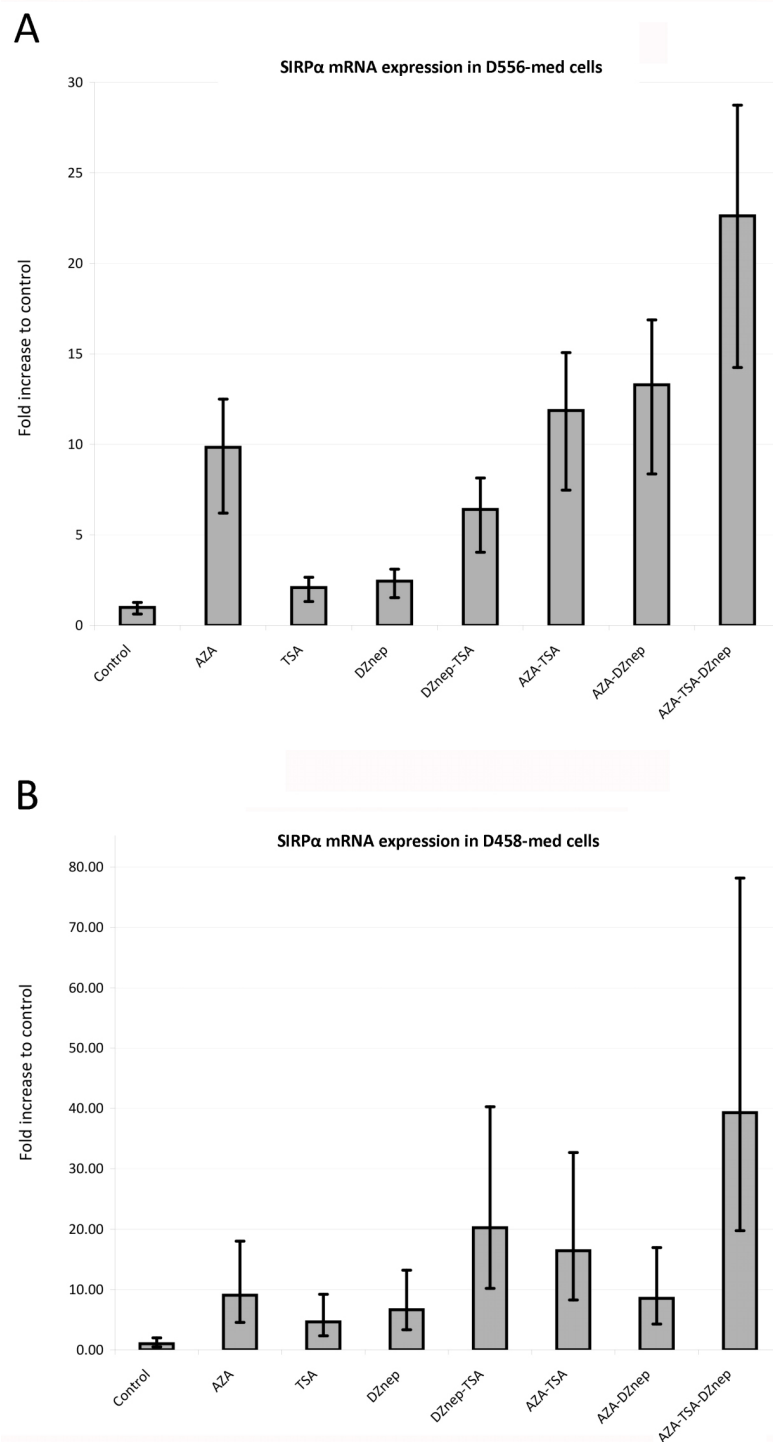
Fig. 5. Restoration of SIRPα mRNA expression upon epigenetic modulation. Seventy two hours of treatment of the MB cell lines D458-med and D556-med with 5-aza-cytidine (5-Aza), 3-deazaneplanocin A (DZNep) and Trichostatin A (TSA) increased SIRPα expression up to 20-40-old with the combination of the drugs.

Using sample 9 as a reference, SIRPα mRNA expression was plotted against miR-17, miR-20a, miR-106a, miR-363, miR106b and miR20b (Fig. 7B), and Spearman's rank correlation coefficients (ρ)were calculated for each micro-RNA. For miR-17, miR-20a and miR-106a, a significant correlation was found with SIRPα mRNA, with ρ-values of −0.68, −0.66 and −0.68 respectively, ($p < 0.005$). MiR-106b and MiR-20b expression was not significantly correlated with SIRPα expression ($\rho = -0.21$, $p = 0.37$ and $\rho = 0.40$, $p = 0.08$ respectively).
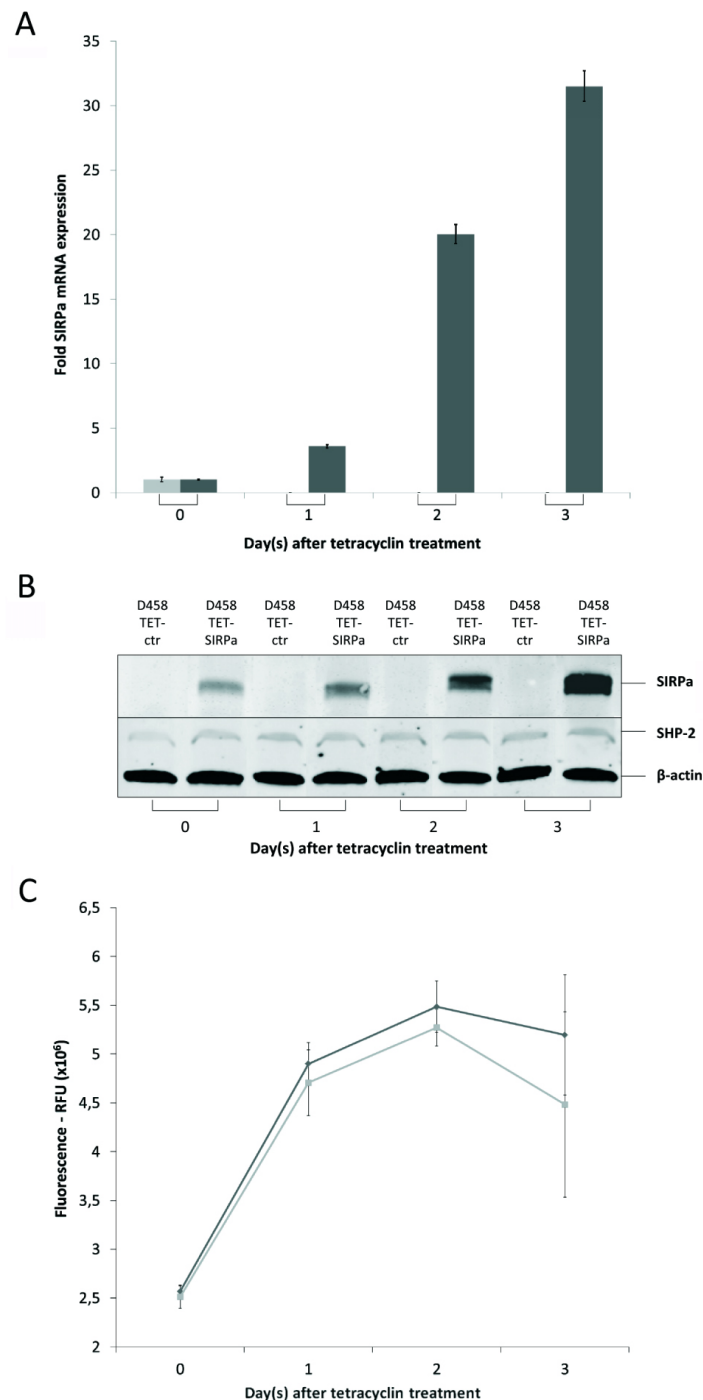
Fig. 6. Overexpression of SIRPα overexpression in MB cell line D458-med by using an inducible lentiviral construct. Strong induction of (A) SIRPα mRNA and (B) protein was observed in D458-med cells with TET-on inducible SIRPα overexpression construct (black bars), versus no induction of expression in control cells (grey bars). (C) Cell viability in D458-med cells is independent of the expression of SIRPα. No difference in viability between D458-TET-SIRPα cells (black line) versus D458-TET-control cells (grey line).

4. Discussion

Based on its established role in other cancers, we investigated the neuronal synaptic protein SIRPα in MB. We found that SIRPα was not expressed in MB, in striking contrast with the normal cerebellum, in which strong expression was observed. In MB tumor cell lines D283-med, D458-med and D556-med, SIRPα was also not detectable. Remarkably, these cells did express SHP-2, which is a phosphatase known to elicit, mostly inhibitory, signaling by SIRPα. This raised the question of how SIRPα re-expression would affect the behavior of MB tumor cells. In elucidating the mecha-
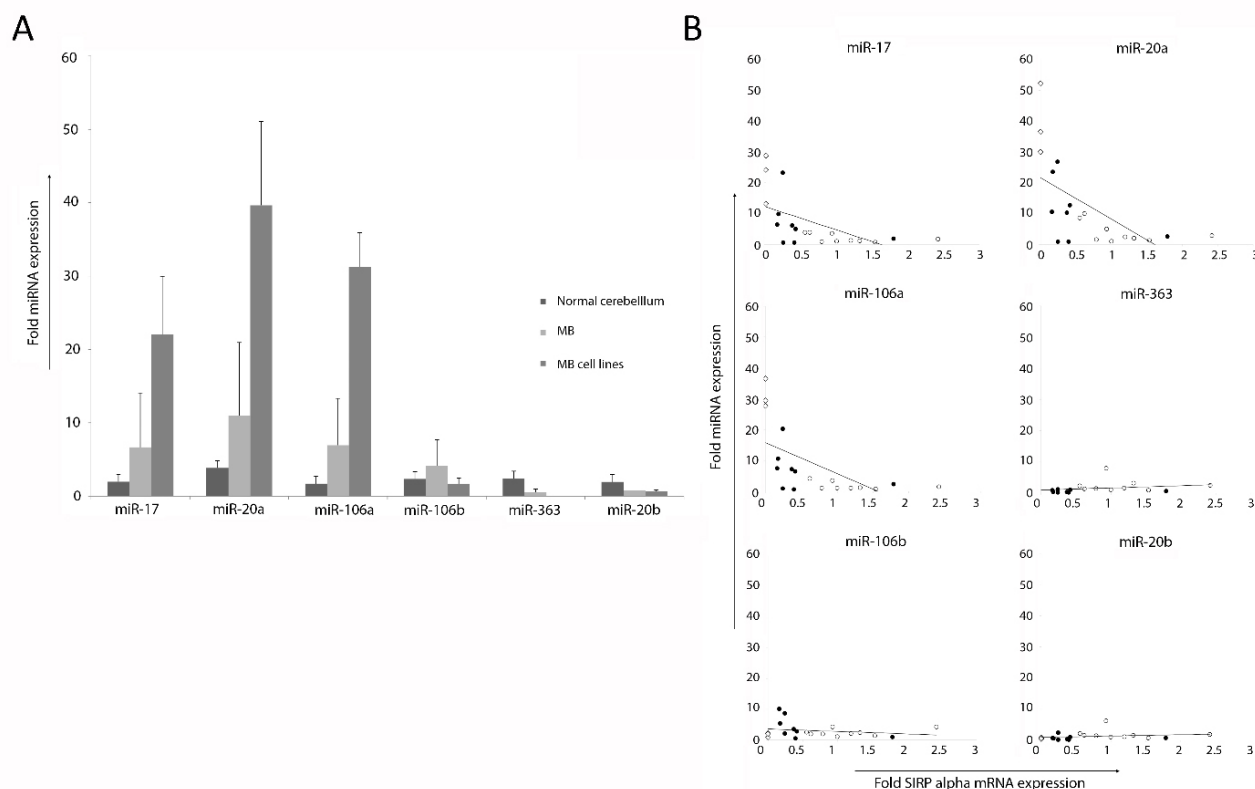
Fig. 7. Expression of oncomiR cluster 17-92 correlates with SIRPα mRNA expression. (A) As compared to normal cerebellum, miR-106a is significantly overexpressed in MB ($p = 0.038$), while miR-17 and miR-20a show a trend to upregulation ($p = 0.076$ and $p = 0.058$ respectively). miR-17, miR-20a and miR-106a are overexpressed in MB cell lines D283-med, D458-med and D556-med ($p < 0.0001$). miR-363 is significantly downregulated in MB ($p = 0.01$). (B) miR-17, miR-20a and miR-106a Are negatively correlated with SIRPα mRNA expression (Spearman's ρ $= -0.68$, -0.66 and -0.68 respectively). miR-106b, miR-363 and MiR20b do not significantly correlate.

nisms underlying loss of SIRPα expression, we found SIRPα to be transcriptionally downregulated in MB patient specimens and MB tumor cell lines by epigenetic repression via CpG island promoter hypermethylation. This hypermethylation was also observed in fetal cerebellum, whereas in adult cerebellum it was absent. This supports the idea that MB is a result of impairment of normal cerebellar precursor cell development. Epigenetic mechanisms seem to play an important role in gene expression in MB. In the latter brain tumor, multiple developmental pathways are epigenetically silenced both through CpG island promoter hypermethylation [24–27] as well as methylation of histones [28]. In recent years, comparative genomic hybridization (CGH) and gene expression profiling studies have made a revolutionary increase in the insight in MB biology and revealed that MB actually consists of four distinct disease entities, with sonic-hedgehog (Shh), wingless (Wnt), group 3 and group 4 tumors, each with its own unique molecular background, prognosis and potential therapeutic targets. Interestingly, these four MB subgroups were recently more robustly defined by 450k DNA methylation analysis, which re-emphasized that MB is actually a group of brain cancers characterized by epigenetic dysregulation of normal developmental processes [29–35]. Notably, with *in silico* analysis, non-promoter CpG island hypermethylation was also observed in a CpG island downstream from the SIRPα gene transcription start site. Non-promoter CpG island hypermethylation, has been described to both positively and negatively influence gene expression in other cancers [36, 37].

We employed epigenetic modulators in MB cell lines D283-med and D458-med, aiming at re-expression of SIRPα, and subsequent activation of SHP-2, to decrease cell viability, as postulated from the tumor-suppressive effects seen in other cancers. We therefore treated these MB tumor cells with a combination of DNA methyltransferase inhibitor 5-Aza-Cytidine (AZA), HDAC inhibitor trichostatin A (TSA) and histone methyltransferase EZH2 inhibitor 3-deazaneplanocin A (DZnep) leading to restored expression of SIRPα (Fig. 5), which concurrently resulted in a strong reduction in cell viability. To ascertain whether the strong effects of epigenetic therapy on MB cell survival are attributable to SIRPα upregulation or other 'un-silenced' genes, we transduced D458-med cells with a TET-repressed, inducible SIRPα overexpression construct. Induction with tetracycline resulted in a 40-fold upregulation of SIRPα mRNA in these cells, after 72 hr of treatment. Furthermore, strong induction of SIRPα protein expression was observed. However, no differences in cell viability were observed when compared to control D458-med TET-repressor cells. This finding is in sharp contrast with other tumors, in which signaling via SHP-2, negatively influences tumor cell survival [3, 38]. The absence of an effect on cell viability could not be explained by differences in expression of downstream effector phosphatase SHP-2, since this was indifferently expressed, irrespective of epigenetic therapy (Fig. 6B). The observed SIRPα hypermethylation and silencing in MB tissues and in fetal brain, together with

an absence of effect upon SIRPα overexpression in MB cells, might suggest that SIRPα silencing is not a tumor specific event, but rather reflects a developmental state of cerebellar precursor cells that give rise to MB. Other factors such as SIRPα protein mislocalization, or absent ligand (CD47, thrombospondins) or downstream effector expression might explain that no effect of SIRPα expression in MB was seen. These surprising findings require further investigation. In this respect, epigenetic cancer therapeutics are of increasing interest in MB [39]. Short exposures to lower doses of these drugs were also able to induce SIRPα in MB cells (Supplementary Figure S1), albeit to a lesser extent. More research is therefore needed to optimize the dose and schedules when combining these epigenetic modulator agents, since these are likely to influence their demethylating and/or cytotoxic properties [40].

Besides epigenetic changes, increasing evidence suggests that microRNAs [28, 41, 42] post-translationally regulate gene expression in MB. MicroRNA clusters miR-17/92 (OncomiR) and paralogous clusters miR-106a/25 and miR-106b/363 were previously described to be upregulated during neuronal lineage differentiation in stem cells, and in MB, mainly in Shh group, which are MYC/MYCN-driven tumors [21, 28, 43]. These microRNAs have been described to target and silence SIRPα mRNA [20] in myeloid cells. Interestingly, we found miR-17, miR-20a and miR-106a expression to be negatively correlated with SIRPα mRNA expression (Spearman's rank correlation coefficients (ρ) of -0.68, -0.66 and -0.68, respectively, $p < 0.005$), in samples of MB tumors and normal cerebellum from infants, older children and adults, suggestive of post-translational silencing of target gene SIRPα by these miRNAs. For miR-363, and, as expected, for miR-20b, no significant correlation was found. Recently, miR-520d-5p and miR-520d-3p have been found to be associated with SIRPα downregulation in astrocytes [44] and might be interesting to investigate further in MB.

In conclusion, we found that SIRPα is transcriptionally and, possibly, post-transcriptionally silenced in MB as well as during human fetal brain development. Histone modification and inhibition of DNA methylation resulted in markedly increased SIRPα gene expression. This epigenetic modulation strongly reduced viability in MB cells, but in a SIRPα-independent manner, since inducible SIRPα overexpression did not influence cell viability. This suggests that SIRPα hypermethylation in MB reflects the precursor cell state of these cells, rather than being a tumor-specific event. Epigenetic therapies and, possibly future micro-RNA targeted therapies could be of therapeutic benefit in MB, warranting further research to elucidate which genes are involved in these observed strong anti-tumor effects.

Acknowledgements

Conflict of Interest

The authors declare no conflict of interest related to the work described in this article.

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