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MicroRNA-21 suppression impedes medulloblastoma cell migration

Eveline Grunder [a,f], *Rocco D'Ambrosio* [b,f], *Giulio Fiaschetti* [a,f], *Lucia Abela* [a], *Alexandre Arcaro* [b], *Tycho Zuzak* [c], *Hiroko Ohgaki* [d], *Sheng-Qing Lv* [d,e], *Tarek Shalaby* [a], *Michael Grotzer* [a,*]

[a] *Oncology Department, University Children's Hospital of Zurich, Switzerland*
[b] *Division of Paediatric Haematology/Oncology, Department of Clinical Research, University of Bern, Switzerland*
[c] *Department of Pediatric Oncology and Hematology, University Children's Hospital Essen, Germany*
[d] *Section of Molecular Pathology, International Agency for Research on Cancer, World Health Organization, Lyon, France*
[e] *Department of Neurosurgery, Xinqiao Hospital, The Third Military Medical University, Chongqing, China*

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ABSTRACT

Medulloblastoma (MB), the most common malignant brain tumour in children, is characterised by a high risk of leptomeningeal dissemination. But little is known about the molecular mechanisms that promote cancer cell migration in MB. Aberrant expression of miR-21 is recognised to be causatively linked to metastasis in a variety of human neoplasms including brain tumours; however its function in MB is still unknown. In this study we investigated the expression level and the role of miR-21 in MB cell migration. miR-21 was found to be up-regulated, compared to normal cerebellum, in 29/29 MB primary samples and 6/6 MB-derived cell lines. Inverse correlation was observed between miR-21 expression and the metastasis suppressor PDCD4, while miR-21 repression increased the release of PDCD4 protein, suggesting negative regulation of PDCD4 by miR-21 in MB cells. Anti-miR-21 decreased protein expression of the tumour cell invasion mediators MAP4K1 and JNK, which are also known to be negatively regulated by PDCD4, and down-regulated integrin protein that is essential for MB leptomeningeal dissemination. Moreover miR-21 knockdown in MB cells increased the expression of two eminent negative modulators of cancer cell migration, E-Cadherin and TIMP2 proteins that are known to be positively regulated by PDCD4. Finally and importantly, suppression of miR-21 decreased the motility of MB cells and reduced their migration across basement membranes *in vitro*. Together, these compelling data propose miR-21 pathway as a novel mechanism impacting MB cell dissemination and raises the possibility that curability of selected MB may be improved by pharmaceutical strategies directed towards microRNA-21.

1. Introduction

MB is the most common malignant brain tumour of childhood. Evidence of leptomeningeal dissemination is the most important clinical predictor of poor clinical outcome.[1] Despite intense adjuvant therapies including craniospinal irradiation and chemotherapy, more than one-half of patients with metastatic MB die of tumour recurrence.[2] In addition, many

* *Corresponding author:* Address: University Children's Hospital, Steinwiesstrasse 75, 8032 Zurich, Switzerland. Tel.: +41 44 266 75 75; fax: +41 44 266 71 71.
E-mail address: Michael.Grotzer@kispi.uzh.ch (M. Grotzer).
[f] These authors contributed equally to this work.
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survivors exhibit long term therapy-associated late-effects. The development of novel targeted treatments will be essential to increase survival rates and reduce adverse therapy-related sequelae. Improvements in understanding the molecular mechanisms responsible for metastasis of MB will be fundamental to such advances.[3]

MicroRNAs (miRNAs) are a naturally occurring class of small non-coding RNAs that negatively regulate gene expression.[4] Deregulation of miRNAs was found to play important roles in the pathogenesis and metastasis of various malignancies and was proposed as a novel effective target for anticancer therapies.[5–8] miR-21 is one of the most commonly implicated miRNAs in cancer. Its expression is up-regulated in a variety of solid tumours, including glioblastoma, breast, lung, colon, prostate, pancreas and stomach cancers.[9] Elevated miR-21 expression has been causally linked to cell migration of several cancer cell lines.[10] Previous microarray analyses have shown that 87 miRNAs are aberrantly expressed in MB.[11] Notably, the aberrant expression of some microRNAs in MB including upregulation of miR-21, were similar to what was previously reported in glioblastoma,[11] thus supporting the general role of these miRNAs in neuronal and glial-derived tissues. However, the molecular mechanisms mediating miR-21 function in MB is not known.

Programmed Cell Death 4 (PDCD4) is a tumour suppressor gene that inhibits metastasis in human cancer cells.[12] PDCD4 has recently emerged as a major, functionally significant target that is negatively regulated by miR-21 in diverse cancers including brain tumours.[10] Studies investigating cellular functions of PDCD4 demonstrated that it suppresses the expression and or/activity of the invasion-related proteins such as AKT,[13] MAP4K1,[14] JNK/c-Jun,[15] integrin[16] and increases the release of metastasis suppressor proteins such as E-Cadherin[17] and TIMP2.[18] Hence miR-21 and its target gene PDCD4 have been proposed as potential targets for novel anti-cancer metastasis therapies.[19] Although miR-21 and its target PDCD4 have been widely explored as a cancer-related target for adult brain tumours such as glioblastoma,[20] there is no information available concerning the relevance of both genes for metastasis in childhood MB. The aims of this study are to examine miR-21 in MB patient's samples as well as in a representative panel of MB cell lines and investigate its role in MB cells migration.

2. Materials and methods

2.1. Human MB/PNET cell lines

DAOY human MB cells were purchased from American Type Culture Collection (Manassas, VA). D341, D425, UW-228-2, Med-1 human MB cells were the kind gift of Dr. Henry Friedman (Duke University, Durham). PFSK PNET cells were the kind gift of Dr. Peter Phillips (The Children's Hospital of Philadelphia, PA). All MB cells were cultured in Richter's zinc option medium (Invitrogen; Basel, Switzerland) supplemented with 10% faetal bovine serum. Non-essential amino acids were added to the medium of D341 and D425 cells to a final concentration of 1%, and G418 was added to the medium of DAOY V11 and DAOY M2 to a final concentration of 500 μg/ml. All cell cultures were maintained at 37 °C in a humidified atmosphere with 5% CO_2. Cell lines were tested for their authentication by karyotypic analysis using molecular cytogenetic techniques, such as comparative genomic hybridisation.

2.2. Patients' samples

Paraffin sections from 36 MB samples and 10 normal brain tissues surrounding MB were obtained from Neuro-Pathology of the University Hospital of Zurich, Switzerland. All tumour samples were reviewed by two independent neuropathologists and diagnosed as MB. Written informed consent for research use of biological samples was obtained from all patients, and the research project was approved by the Institutional Ethical Committee. Tumours were manually scraped from the paraffin sections, and DNA was extracted using a previously reported protocol.[21] In 29/36 MB samples with sufficient tumour material, cDNA was produced for q-RT-PCR analysis. The median age at diagnosis for these 29 MB patients was 7.5 years (range, 1.3–23 years); 21 were male and eight were female.

2.3. Genetic analyses of the miR-21 gene

To detect *miR-21* amplification/deletion, differential PCR was performed as described previously,[22] using the β-actin sequence as a reference. Primer sequences for *miR-21* were 5′-TGT TTT GCC TAC CAT CGT GA-3′ (sense) and 5′-AAG TGC CAC CAG ACA GAA GG-3′ (antisense) to produce 221 bp fragment, and those for β-actin were 5′-CTG TGG CAT CCA CGA AAC TA-3′ (sense) and 5′-AGG AAA GAC ACC CAC CTT GA-3′ (antisense) to produce a 187-bp fragment. After PCR (33 cycles), the products were separated on 8% acrylamide gels. Gels were stained with ethidium bromide. Quantitative analysis of the signal intensity was performed with Molecular Imager and the Quantity One Analysis Software (Bio-Rad, Hercules, CA). The mean ratio of *miR-21* gene to β-actin of normal DNA (10 samples of normal tissue) was about 1.0. The threshold for evidence of *miR-21* gene amplification was calculated using the formula $2 \times \text{mean} + 3 \times \text{standard deviation (SD)}$. Samples in which the ratio of *miR-21* gene to β-actin was $\leqslant 0.2$ were considered to show deletion of the *miR-21* gene.[23]

2.4. SSCP and DNA sequencing to detect miR-21 gene mutations

Pre-screening for *miR-21* mutations was carried out using single-strand conformational polymorphism (SSCP) as described previously[24] using the primers described above. Samples that showed mobility shifts on SSCP analysis were further analysed by direct DNA sequencing (ABI 3100 PRISM DNA Sequencer, Applied Biosystems, CA).

2.5. MicroRNA isolation

Total RNA was isolated using the mirVana™ miRNA Isolation Kit (Applied Biosystems; Rotkreuz, Switzerland) according to the manufacturer's instructions. RNAs were quantitated by spectrophotometer and $A_{260/280}$ ratios were calculated for quality assurance. Human cerebellum RNA was purchased from Ambion. Reverse transcription was performed each in

a total volume of 15 μl containing 0.15 μl NTP (100 mM total), 1 μl of Multiscribe™ RT Enzyme (50 U/μl), 0.19 μl of RNase Inhibitor (20 U/μl), 4.16 μl water, 5 μl RNA (a total of 10 ng) and 3 μl microRNA-21 specific stem-loop primers (Applied Biosystems; Rotkreuz, Switzerland). The reaction was carried out in a thermocycler (Mastercycler, Eppendorf) with the following cycle conditions: 30 min at 16 °C, 30 min at 42 °C and 5 min at 85 °C.

2.6. Real time quantitative polymerase chain reaction for microRNAs

First strand synthesis of mature microRNAs was followed by quantitative real-time PCR (TaqMan MicroRNA Assay) using microRNA-specific TaqMan MGB primers and probes for miR-21 ID (000397) and for control RNU6B ID (001093) and TaqMan Universal master mix for 7900HT PCR (Applied Biosystems; Rotkreuz, Switzerland). PCRs were carried out in a 20 μl volume containing 10 μl, TaqMan 2× Universal Master Mix, 7.67 μl water, 1 μl of 20× TaqMan MicroRNA Assay mix and 1.33 μl of RT product. cDNA was diluted 1:2 prior to use. Each reaction was performed in triplicate. Thermal cycling parameters included initial 10 min of Taq enzyme activation at 95 °C followed by 40 cycles of 15 s denaturation at 95 °C and 1 min of annealing/extension at 60 °C. The threshold cycle numbers (Ct) were normalised against an endogenous control (RNU6b RNA) and related to normal human cerebellum (Ambion). Real-time PCR data were analysed by relative quantification using the $2-\Delta\Delta C_T$ method.

2.7. Western blot analysis

Cells were washed twice in medium, centrifuged, and lysed in buffer [50 mmol/L Tris (pH 8), 150 mmol/L NaCl, 1% Triton X-100, 1% NP40, 0.1% SDS, 5 mmol/L EDTA] for 20 min on ice followed by centrifugation at 10,000*g* for 15 min. The crude lysates were heated for 5 min at 95 °C in the presence of 3% mercaptoethanol. Equal amounts of protein (30 μg/line) from the homogenates were separated by 10% (w/v) SDS–PAGE and transferred onto nitrocellulose membranes (Bio-Rad). Nonspecific binding sites were blocked with 10 mmol/L TBS containing 0.1% Tween 20 and 10% nonfat milk. Membranes were then incubated overnight at 4 °C with the respective first antibody, PDCD4, P-MapK Erk, P AKT, P-jun, JNK, HPK1, E-Cadherin, TMP2, Integrin, uPAR, Caspase 3, (Santa Cruz Biotechnology), β-actin (Sigma Aldrich, Switzerland). Membranes were then washed three times at room temperature, and bound immunoglobulin was detected with anti-isotype monoclonal antibody coupled to horseradish peroxidase (Santa Cruz Biotechnology). The signal was visualised by enhanced chemiluminescence (Amersham Biosciences) and autoradiography. Relative band intensities were determined using Quantity One analysis software (Bio-Rad).

2.8. Transfection with anti-miRNA-21 (transient down-regulation)

Cells (DAOY wt, PFSK and UW-228) were seeded in 6-well plates at a cell density of 3X 10^4. Transfection of anti-miRNA 21(AM 10206, Ambion Switzerland) or negative control oligonucleotide referred to as scrambled (AM 17010, Ambion Switzerland) in a final concentration of 50 nmol/l was performed 24 h after seeding using 6 μl siPort Amine transfection reagent according to the manufacturer's recommendation. Cells were harvested 48 and 72 h after transfection for subsequent experiments.

2.9. Invasion assay

The metastatic activity of MB cells was measured *in vitro* using the 24-well BD BioCoat FluoroBlok Invasion System as recommended by the manufacturer (BD Biosciences, Bedford, MA). Briefly, 3×10^4 cells were labelled for 20 min with 10 μM of the fluorescent cell probe CellTracker Green (Molecular Probes, Eugene, OR) in 10% FBS DMEM. Cells were then incubated for an additional 30 min in fresh 10% FBS DMEM. Baseline fluorescence analysis confirmed equivalent labelling of all different MB cell types. 10% FBS DMEM was then added as a chemo attractant to the lower chambers of the 24-well plates, and cells were seeded into the prehydrated upper wells of the BD BioCoat FluoroBlok chambers. Nonhydrated wells were included as controls. Cells were then cultured under standard conditions for the indicated times and invasion measured from below at excitation wavelength 485 nm and emission wavelength 530 nm using a Fusion Universal Microplate Analyzer (Perkin-Elmer Life Sciences, Boston, MA). The fluorescence of MB cells that had invaded to the undersurface of the membrane was recorded relative to that of control cells. Assays were conducted in quadruplicate.

2.10. Cell proliferation

Following transfection with anti-miRNAs, a colorimetric 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) assay (Promega; Wallisellen, Switzerland) was used to quantitate cell viability of human MB cells, as previously described.[25] Each experiment was performed in triplicate. The absorbance values of each well were measured with a microplate spectrophotometer (Molecular Devices; Sunnyvale, CA) at 490 nm. All proliferation assays were repeated as independent experiments at least twice.

2.11. Apoptosis assay

A photometric enzyme immunoassay (Cell Death Detection ELISA; Roche Diagnostics, Basel, Switzerland) was used for the quantitative determination of cytoplasmic histone-associated DNA fragments, as described previously.[49] In brief, an equal number of MB cells were seeded in a six-well plate and were either transfected with anti-miRNA-21 or control oligo. After 48 h or 72 h, cells were counted, and cell lysates of equal number of cells (5×10^4 cells/ml) were placed in a StreptAvidin-coated microtiter plate. A mixture of biotin-labelled monoclonal histone antibody and peroxidase-conjugated monoclonal DNA antibody was then added, followed by incubation for 2 h. After washing to remove unbound antibodies, the amount of mono- and oligonucleosomes was measured at 405 nm (reference wavelength 490 nm).

2.12. Statistical analysis

Data were analysed using SPSS for Windows version 14 (SPSS Inc., Chicago IL, USA). Pictures were analysed by ImageJ software. Pearson's correlation coefficient and Wilcoxon signed-rank test were used to determine statistically significant differences, with a *p*-value (always two-sided) of less than 0.05 considered to be statistically significant.

3. Results

3.1. miR-21 is up-regulated in MB cells

Increasing numbers of reports implicate miR-21 over-expression in tumour carcinogenesis and metastasis.[10] To assess the relevance of miR-21 in MB biology we first determined its expression level comparing to normal brain tissue in 29 MB patient's samples as well as in a representative panel of 6 MB cell lines using quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). Interestingly, compared with normal human cerebellum, miR-21 expression was found to be up-regulated in every MB sample and MB cell line tested, with its level between 10- to 130-fold higher in MB cell lines (Fig. 1A) and 3- to 40-fold higher in MB primary samples (Fig. 1B). The observed higher level of miR-21 expression in every MB primary sample and cell line compared to normal cerebellum suggests the involvement of miR-21 in MB biology.

3.2. Genetic alteration in miR-21 gene

Several studies have shown that genetic alterations of microRNA may be related to abnormal expression of some miRNAs.[26] To investigate whether *miR-21* is genetically altered in MB, we screened 36 MB tissue samples for amplification, deletion, or mutation of *miR-21* gene. None of the 36 MB samples analysed showed amplification of *miR-21* gene (data not shown). There was also no case with miscoding mutations or deletion in the *miR-21* gene in any of the 36 MB tissues.

3.3. miR-21 and the metastasis suppressor PDCD4 are inversely expressed in MB cell lines

PDCD4 is one of the principal miR-21 targets validated independently by several groups. It has a single highly conserved miR-21 target site within its 3′UTR and its regulation by miR-21 has been reported in a number of human cancer cells

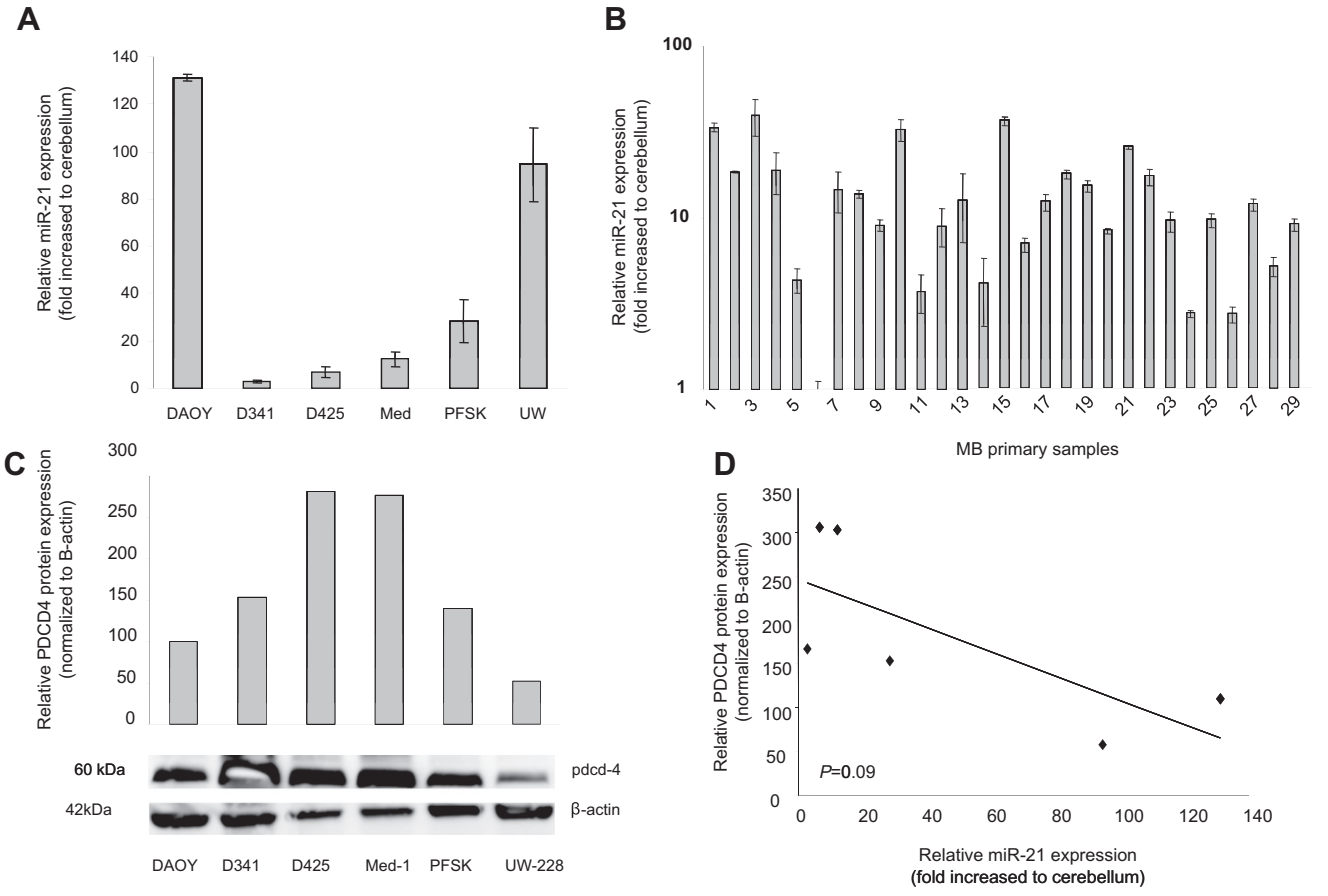

Fig. 1 – miR-21 and PDCD4 correlate inversely in MB cell lines. (A) Relative miR-21 mRNA expression in MB cell lines as determined by quantitative RT-PCR. Values represent the fold-increase of miR-21 mRNA relative to normal cerebellum = 1 (*n* = 3; ±SD). (B) Relative miR-21 mRNA expression in MB primary samples as determined by quantitative RT-PCR. Values represent the fold-increase of miR-21 mRNA relative to normal cerebellum (*n* = 3; ±SD). (C) PDCD4-protein expression as determined by Western blot and quantified by densitometry in MB cell lines. (D) Correlation between PDCD4 protein and miR-21 in MB cell lines (Pearson's correlation coefficient – 0.73; *p*-value 0.099).

including breast cancer, colorectal cancer and glioma reviewed in Ref. 9 Reduced PDCD4 expression has been reported in several types of cancer cells in which miR-21 is over-expressed.[12] To explore the relationship between miR-21 and its target gene PDCD4 in MB, we investigated the protein expression of PDCD4, in MB cell lines and compared its level to miR-21 levels. A low amount of PDCD4 protein at 60 kDa was observed on the Western blot for MB cell lines with high endogenous miR-21 (DAOY, UW-228 and PFSK) (Fig. 1A and C), whereas cell lines with low miR-21 (Med-1, D425 and D-341) showed high amounts of PDCD4 protein (Fig. 1A and C). Across all six cell lines tested, we observed an inverse correlation between miR-21 and PDCD4 protein levels (Pearson's correlation coefficient – 0.73; *P*-value 0.099) (Fig. 1D).

3.4. *miR-21 suppression mediates up-regulation of the metastasis suppressor PDCD4 protein in MB cells*

To investigate further the negative regulation of PDCD4 by miR-21 in MB, we studied the effect of miR-21 inhibition on PDCD4 protein levels. Transfection of three MB cell lines among highest miR-21 expression with anti-miR-21 for 48 h resulted in pronounced down-regulation of the endogenous miR-21 in DAOY (>90%), PFSK (83%) and UW-228 (32%) compared to control cells, as determined by qRT-PCR (Fig. 2A). Suppression of miR-21 resulted also in up-regulation of PDCD4 protein expression in DAOY (>2-fold), PFSK (>2.5-fold) and UW-228 (~1.5-fold) (Fig. 2B).

3.5. *Anti-miR-21 down-regulates PDCD4 target genes in MB cells*

To determine whether the increase in PDCD4, mediated by miR-21 inhibition, is functional and could appropriately regulate expression of known downstream targets for PDCD4, we analysed protein expression of MAP4KI and JNK that are known to be negatively controlled by PDCD4[14] (Fig. 3A). Western blot analysis showed a significant decrease in MAP4KI (80% in DAOY and 79% in PFSK) and JNK kinase (85% in DAOY and 65% in PFSK) proteins in MB cells transfected with

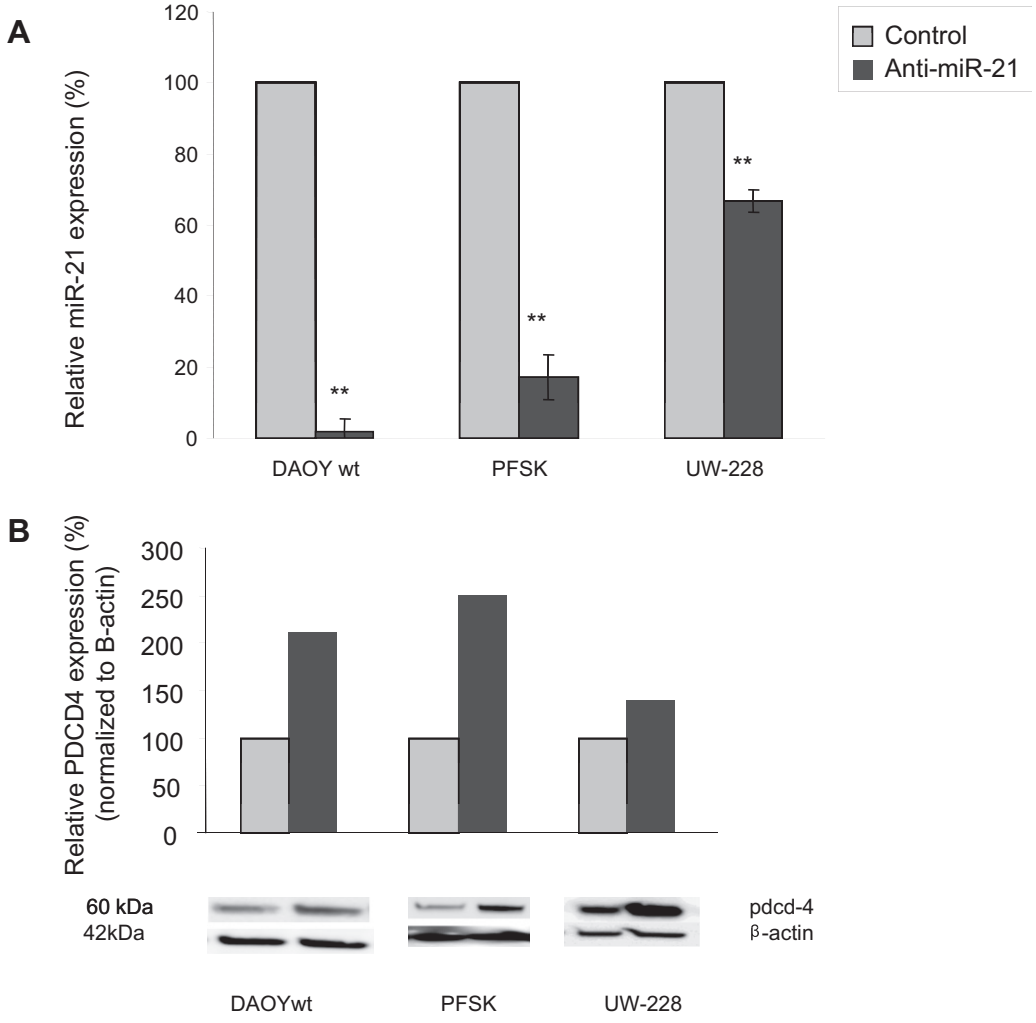

Fig. 2 – miR-21 suppression increases the expression of PDCD4 protein. (A) Relative miR-21 expression as determined by quantitative RT-PCR 48 h following transfection with either anti-miR-21 or control in indicated MB cell lines (*P < 0.005 according to Student's *t*-test). Values represent percent-decrease of miR-21 mRNA relative to control (*n* = 3; ±SD). (B) Anti-miR21 mediated up-regulation of PDCD4 protein expression as determined by Western blot and quantified by densitometric analysis in indicated MB cell lines. Values represent percent-increase of PDCD-4 protein relative to control (*n* = 3; ±SD).

anti-miR-21 compared to control (Fig. 3B). In parallel, we observed a significant decrease in the activity of c-JUN kinases, the downstream effector of JNK kinase, by 30% in DAOY and 34% in PFSK (Fig. 3B). On the other hand miR-21 suppression resulted in a decrease in the activity of ERK, that is known to be regulated by miR-21,[27] by 60% in DAOY and 40% in PFSK as well as down-regulation of AKT activity by 30% DAOY and 60% for PFSK (Fig. 3B) as measured by a decrease in kinase

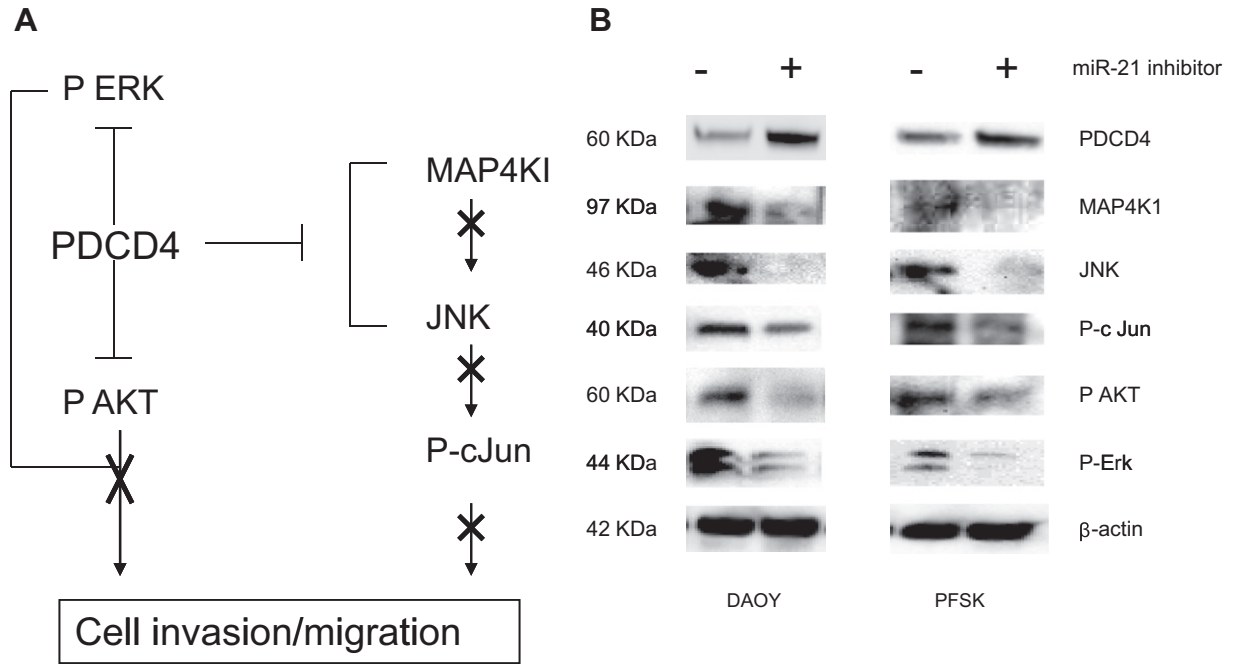

Fig. 3 – Effect of anti-miR-21 on PDCD4 target genes in MB cell lines. (A) Cartoon depicting an overview of the known functions of PDCD4. (B) Western blot showing that miR-21 inhibition increased the expression of PDCD4 protein, reduced the expression of MAP4KI, JNK proteins and reduced the phosphorylation of c-JUN, AKT and ERK in both DAOY and PFSK MB cell lines.

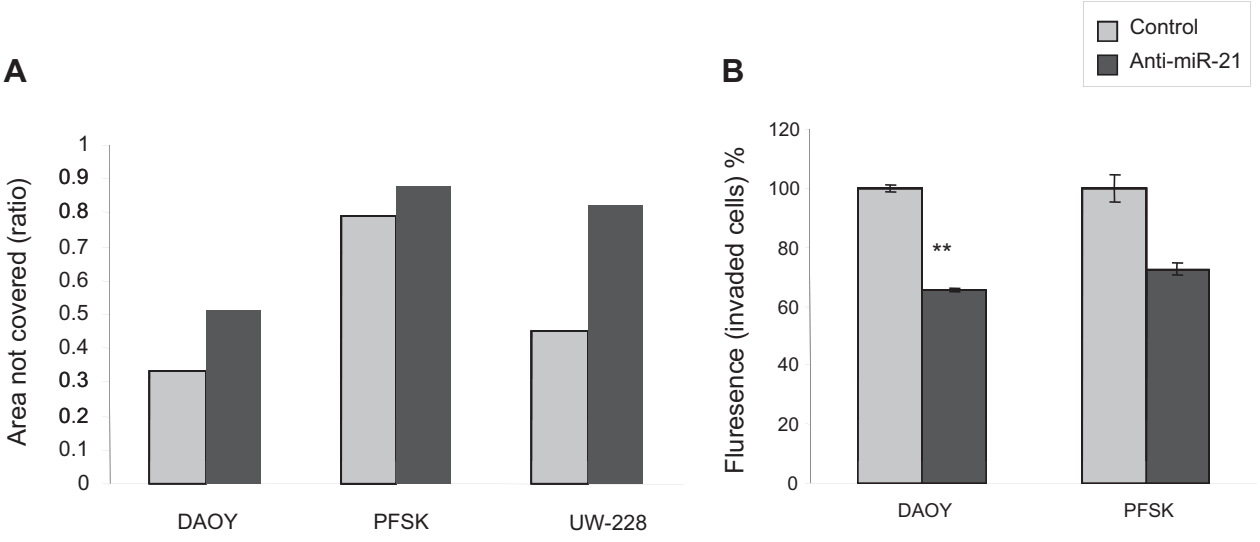

Fig. 4 – Suppression of MB cell motility and invasion by anti-miR-21. (A) Quantification of relative efficiency of wound closure assay after 48 h in three MB cell lines DAOY, PFSK and UW-228 after 48 h of transfection with anti-miR-21 compared to control transfected cells. Ratio: 1 = size of wound at 0 h (one representative experiment is shown). The extent of wound healing was defined as the remaining wound area over the original wound area. (B) Transwell invasion assay of DAOY and PFSK cells transiently transfected with either anti-miR-21 or control ($P < 0.005$ according to Student's t-test). Values represent the mean percentage of invaded cell's fluorescence compared to control transfected cells ±SD ($n = 3$; ±SD).**

phosphorylation compared to control scrambled microRNA. Collectively, these results indicate that the miR-21 suppression not only increases the expression of PDCD4 protein but also regulates the expression of known PDCD4 and miR-21 downstream targets.

3.6. *Inhibition of miR-21 reduces motility and invasiveness of MB cells*

An important component of the invasive profile of a cancer cell is its ability to be motile. High levels of miR-21 have been associated with cell motility and metastasis of different cancers including brain tumours.[10] Having this background in mind, we conducted an *in vitro* wound closure assay in order to test the effects of miR-21 on MB cell motility and invasion in DAOY, UW-228 and PFSKA MB cell lines. Cells were plated, allowed to grow to confluence, and transfected with either anti-miR-21 or control. The monolayer was then scratched to create a cleared area within the monolayer. Pictures were taken at 0 and 48 h, and the two-dimensional movement of the transfected cells was quantified by measuring the migration distance and comparing it with the front area at time zero. The results of this experiment showed that MB cells with inhibited miR-21 exhibited a limited wound closure activity while, in contrast, control transfected cells showed significant acceleration of wound closure that could be observed after 48 h (Fig. 4A). This result demonstrated that inhibition of miR-21 was able to reduce the motility of MB cells.

To measure the effect of miR-21 on MB cell migration and to confirm the results of the wound closure assay, we employed a trans-well invasion platform using the 24-well BD BioCoat FluoroBlok Invasion System. The system consists of two fluid-filled, stacked compartments separated by a porous membrane filter coated with Matrigel. Cells were grown in the upper chamber and assessed for migration through the Matrigel toward a chemo attractant (10% serum) in the lower chamber. The results of this *in vitro* experiment showed that the numbers of MB cells that migrated through the Matrigel to the lower chamber were significantly lower within MB cells that were transfected with anti-miR-21, compared to control cells (Fig. 4B). Yet again these results confirmed the

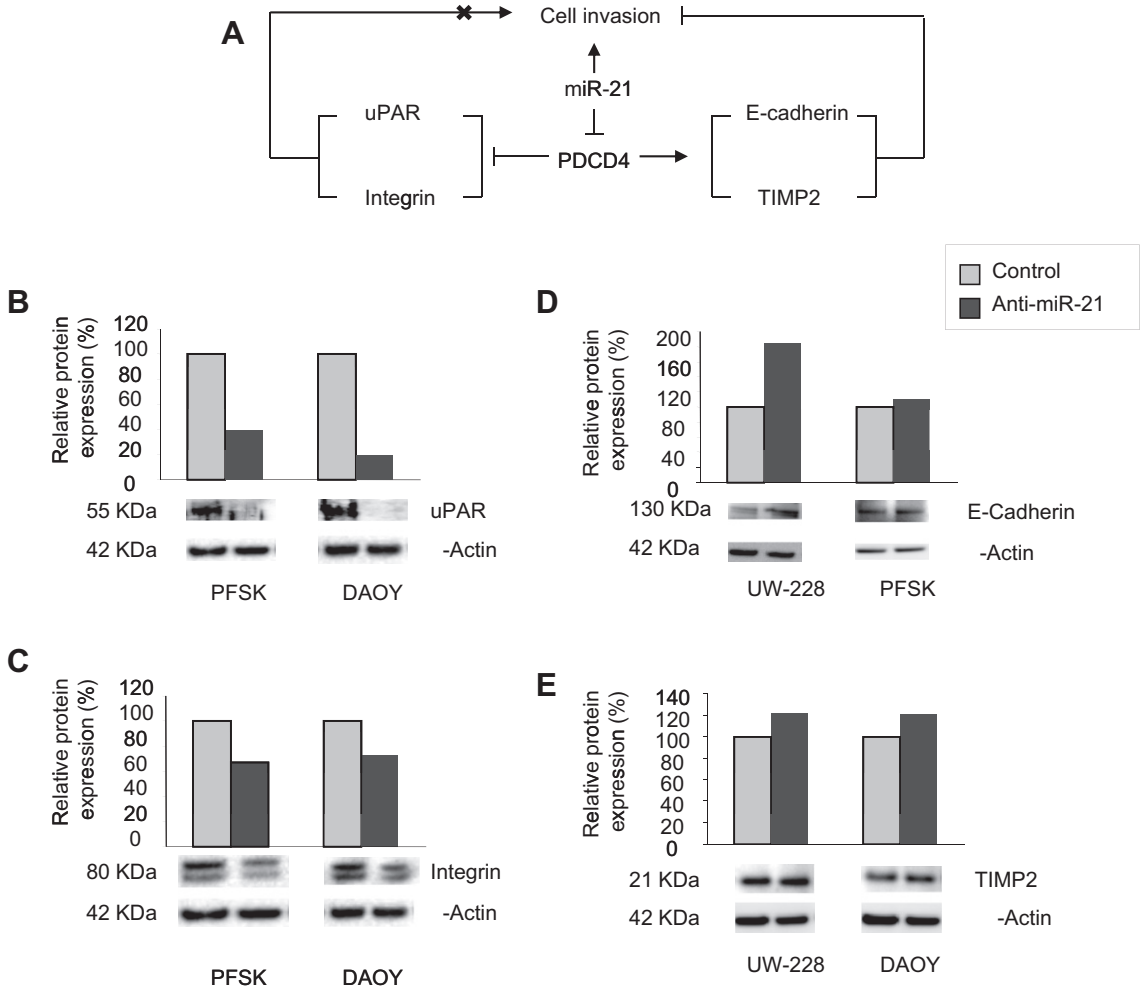

Fig. 5 – Effect of miR-21 inhibition on metastasis associated genes in MB cell lines. (A) Cartoon depicting known regulatory mechanisms for PDCD4 to genes involved in cell migration. Western blots show that miR-21 inhibition result in reduction of proteins associated with metastasis such as uPAR (B) and Integrin (C) while increased E-Cadherin (D) and up-regulated TIMP2 (E) proteins that negatively modulate cell migrations in the indicated MB cell lines.

involvement of miR-21 in the regulation of MB cell motility and suggested a biological role for miR-21 in controlling MB cell ability for migration and invasion.

3.7. *miR-21 regulates genes associated with invasion and metastasis in MB cell lines*

To investigate the mechanisms by which miR-21 suppression inhibits MB invasion, we measured the effect of miR-21 down-regulation on the protein expression levels of four fundamental metastasis-related genes in solid cancers TIMP-2,[28] uPAR,[29] E-Cadherin,[30] and Integrin[31] (Fig. 5A). Western blot analysis in (Fig. 5B) shows that miR-21 suppression in MB cell lines DAOY and PFSK has resulted in a decrease in the expression of uPAR protein, that is known to be negatively regulated by PDCD4[32] and reduction of integrin protein (Fig. 5C) in the two cell lines tested. Both genes are known to be essential genes for cancer cell invasion.[33] miR-21 inhibition has also resulted in the release of E-Cadherin protein in both UW-228 and PFSK (Fig. 5D) and increased expression of TIMP-2 in UW-228 and DAOY (Fig. 5E). Both proteins are positively regulated by PDCD4 and known to negatively modulate cell migrations.[17] These results demonstrate that miR-21 controls multiple genes in MB known to be associated with cancer cell migration and accordingly provide evidence that miR-21 is a potential pro-metastatic miRNA in MB.

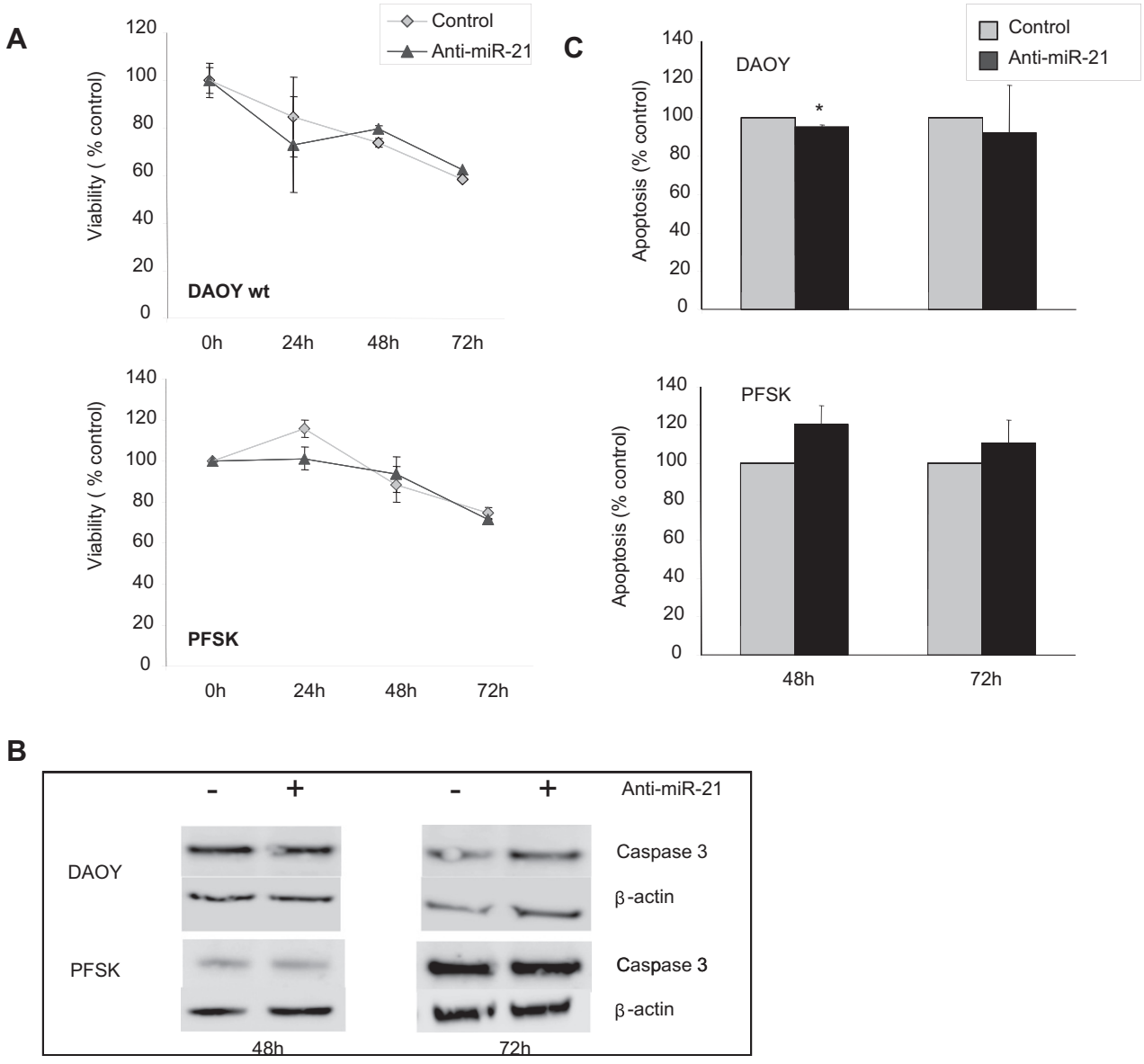

Fig. 6 – miR-21 inhibition has no effect on MB cell proliferation or apoptosis. (A) DAOY and PFSK cells were transfected with anti-miR-21 or a negative control and cell viability was measured at the indicated time points by MTS assay. Values represent the mean percentage of viability compared to control anti-miR transfected cells ±SD ($n = 3$; ±SD). (B) Expression level of caspase-3 protein measured by Western blot. (C) Apoptotic cell death measured by the mean absorbance of cytoplasmatic histone-associated DNA fragments. Values represent the mean absorbance of cytoplasmatic histone-associated DNA fragments compared to control anti-miR transfected cells ±SD ($n = 3$; ±SD). (*$P < 0.05$ according to Student's t-test.)

3.8. *Inhibition of miR-21 in MB cell lines does not affect cell proliferation or apoptosis*

Several studies have demonstrated that miR-21 is an oncomir with proliferative and antiapoptotic functions.[34] Through a loss of function approach, we investigated the effect of miR-21 knockdown using anti-miR-21 on MB cell proliferation as well as apoptosis. Surprisingly, the impairment of miR-21 failed to affect the proliferation of either of the MB cell lines DAOY or PFSK *in vitro* at all time points tested (Fig. 6A). Furthermore, miR-21 knockdown did not trigger apoptosis as revealed by the lack of substantial caspase-3 activation (Fig. 6B) as well as by the absence of the cytoplasmic histone-associated DNA fragments as measured by Cell Death Detection ELISA (Fig. 6C). These data suggest that miR-21 does not appear to regulate cell growth or apoptosis in MB cells.

4. Discussion

Metastasis is a major cause of MB-related death. Elevated miR-21 expression has been causally linked to cell metastasis in brain tumours including glioblastoma.[9] In the present study, we found increased expression of miR-21 in 100% (29/29) of MB patient's samples and in (6/6) MB cell lines analysed as compared to normal cerebellum. Little is known about the mechanisms by which microRNAs are altered in cancer cells, but all the usual genomic regulatory systems are likely to operate (mutations, gene deletion, and amplification).[26] To investigate whether miR-21 up-regulation in MB is caused by alterations in *miR-21* gene we screened tissue samples from 36 cases of MB for amplification of *miR-21*. Interestingly none of the 36 MBs analysed showed amplification of the *miR-21* gene. These findings propose that over-expression of miR-21 in MB is not caused by *miR-21* amplification, suggesting the involvement of alternative mechanisms such as transcriptional dysregulation or altered processing and editing mechanisms.[35] Fujita et al. performed extensive miR-21 gene expression analyses and showed that miR-21 expression is driven by its own promoter that contains binding site for various transcription factors such as protein one (AP-1) and (PU.1),[36] and Stat3.[37] Other studies demonstrated that female sex hormone oestrogen (E2) affects miR-21 expression in breast cancer cell line MCF7.[38] Moreover, steroid hormone receptor, androgen receptor (AR), also directly interacted with miR-21 promoter and up-regulated miR-21 expression in AR-positive prostate cancer cell lines C4-2 and CWR22Rv1.[39]

The link between miR-21 and cell metastasis in several cancer types including glioblastoma,[20] suggests the presence of metastatic pathways that are common to different cancers and raised the question regarding the mechanisms of how miR-21 may impact the metastatic potential of MB cells. PDCD4 was identified by several independent laboratories as metastasis-related target of miR-21 in various cancers.[10,18] Our results showed an inverse correlation between miR-21 and the PDCD4-protein expression in MB cells. In addition, miR-21 knockdown in MB cells resulted in a significant increase in PDCD4 protein and decrease in the expression of its downstream target MAP4K1 and JNK proteins which are recognised to be negatively regulated by PDCD4.[12] Taken together, these findings suggest that the metastasis suppressor PDCD4 is regulated by miR-21 in MB cells. This is in consistence with recent reports that showed a negative regulation of PDCD4 by miR-21 in breast cancer,[40] bladder carcinoma,[41] cholangio-carcinoma,[42] oesophageal carcinoma,[43] and glioblastoma[20] reviewed in Ref. 12.

c-Jun is frequently over-expressed in diverse tumour types and has been implicated in promoting cellular metastasis[44] while in addition disruption of c-Jun activity reduces cellular invasion.[45] Studies investigating cellular functions of PDCD4 have shown that its ability to suppress cell invasion involves the down-regulation of c-Jun kinase activity.[13] Yang et al. showed that PDCD4 is able to down-regulate c-Jun-activation by inhibiting the expression of MAP4K1, which is upstream of JNK[13] and downstream component of the PDCD4 pathway. Moreover over-expression of a dominant negative MAP4K1 mutant in colon cancer cells inhibited both c-Jun-activation and Matrigel invasion.[12] Given this background, we measured the phosphorylation level of c-Jun kinase following miR-21 inhibition in MB cell lines. Interestingly, our own results showed a significant decrease in the activity of c-Jun kinase in parallel to the increase of PDCD4 and the decrease in both MAP4K1 and JNK proteins expression. Taken together, these results suggest that miR-21 controls the activity of c-Jun, a key component of the cancer cellular invasion pathway in MB.

To further characterise the effect of miR-21 on MB, we examined the protein expression of some other metastasis associated target genes of PDCD4 including p-AKT, AKT, p-ERK and ERK that is known to regulate cancer cell migration.[16,46,47] Previous works in glioblastoma multiforme have implicated miR-21 as one of the essential molecules required for regulation of phosphorylated AKT (p-AKT) expression in brain tumour cells[48] as well as P-ERK activity.[49] The present work illustrates that miR-21 inhibition decreases both p-AKT and p-ERK expression in MB cells. However, no major alteration in total AKT or ERK levels was observed (data not shown). Yet these data add more evidence suggesting miR-21 to be a regulator of another two important invasion-related genes AKT and ERK in MB cells.

Several reports suggest that miR-21 is involved in a number of positive and negative feedback loops, that are part of the complex regulatory network operating in cancer cell metastasis, by impacting more than one gene that plays a crucial role in metastasis-related genes such as E-Cadherin,[50] TIMP2,[51] and uPAR.[32] Loss of E-Cadherin promotes cell migration by enabling the first step of the metastatic cascade – the dissociation of carcinoma cells from one another.[52] Matrix metalloproteinases (MMPs) belong to a larger family of proteases that play a major role on cell migration by degrading extracellular matrix proteins. The significance of E-Cadherin loss for metastasis has been demonstrated in a variety of *in vitro* and *in vivo* models.[51] There is abundant evidence suggesting that PDCD4 suppresses cancer cells invasion by increasing the expression of MMP inhibitors, such as TIMP2[18] and/or by increasing E-Cadherin expression.[13] Our results show that miR-21 knockdown in MB cell lines increased TIMP2 and E-Cadherin protein expression. Although the alteration in protein levels of some individual miR-21 targets appears modest in MB cells with miR-21 suppression, collectively these results suggest a possible negative regulation of

both of the metastasis suppressor proteins by miR-21 in MB cells probably via PDCD4. In support of our observations we can refer to recent studies that identified TIMP as a functional target of miR-21 in cell invasion and metastasis in glioma and cholangiocarcinoma.[53]

Another important membrane protein that mediates degradation of extracellular matrix components and is regulated by the expression of PDCD4 is uPAR.[32] Expression of uPAR protein is up-regulated in several metastatic cancers including MB[54] while its inhibition reduces cancer cell invasion.[55] Our results showed that miR-21 inhibition that increased expression of PDCD4 and decreased MB cell migration has resulted in the down-regulation of uPAR in MB cells suggesting that miR-21 and PDCD4 signalling may perform a critical function in the regulation of MB cell metastasis.

MB cells have an additional way to invade neighbouring CNS tissues through the leptomeningeal dissemination. One of the key unresolved questions in MB biology is the identification of genes that regulate this process. In leptomeningeal dissemination MB tumour cells migrate from the primary site to the surface of the brain and spinal cord, and importantly need to adhere to these secondary sites, and establish new colonies.[56] Integrin is the main neural adhesion molecule that was reported recently to be expressed in the leptomeningeal disseminated MBs[57] while antisense against integrin reduced MB cell spreading.[58] Integrin, has also been associated with the adhesion of several other cancers that are known to disseminate by surface spread rather than by infiltration, such as ovarian cancer and mesothelioma.[59,60] Based on these observations, we investigated whether miR-21 repression had any effect on integrin expression in MB cells that might contribute to MB tumour cell invasion. Interestingly the results of our Western blot analysis showed that integrin was significantly down-regulated after the repression of miR-21 in MB cell lines. Yet additional evidence, and rather a new discovery, is that miR-21 regulates the expression of this migration promoting protein in MB.

Taken together the biological effects of miR-21 on MB cell invasion are probably due to the simultaneous repression of migration suppressive proteins such as PDCD4 and/or activation of other different invasion-mediating genes. Certainly, additional mechanisms are likely to contribute to miR-21-induced tumour cell invasion. It is also worth noting that miR-21 inhibition leads to reduced expression of other critical oncogenes associated with MB including MYC.[9] Therefore, it appears that multiple critical proteins associated with the MB invasion, rather than a single signalling pathway, are regulated by miR-21.

In some tumour models including glioblastoma, the over-expression of miR-21 and consequently the down-regulation of its target gene PDCD4, have been associated with proliferative potential and anti-apoptotic activity of cancer cells.[9] However, there are conflicting data concerning their role in proliferation and/or cell death. Folini et al.[61] reported recently that miR-21 knockdown in prostate cancer cells is not sufficient *per se* to affect proliferation or apoptosis. It has also been reported that miR-21 deficiency did not affect colon cancer cell's apoptosis or cell proliferation[62] while over-expression of PDCD4 does not alter cell cycle or induce apoptosis in colon carcinoma cells.[14] In agreement with these reports, Lankat-Buttgereit et al.[63] found no correlation between PDCD4 levels and expression of proteins associated with cell cycle and/or apoptosis in different cell lines and concluded that a role for PDCD4 in apoptosis might be limited to certain cell types. The results of our current study showed that despite miR-21 is upregulated in MBs, impairment of miR-21 failed to either down regulate proliferation or increase apoptosis in any of the MB cell line tested. Our data suggest that miR-21 is not a central player in the MB growth as its single knock down is not enough to counter the viability of MB cells. This is consistent with the notion that the oncogenic properties of miR-21 and its canonical target gene PDCD4 could be cell and tissue dependent. Given the fact that the function of a miRNA strictly depends on the cell phenotype and, therefore, on the presence of specific signal transduction pathways in the tissue, we wondered whether the absence of antiproliferative or apoptotic effect upon miR-21 knockdown could be due to the lack of its key downstream targets or in a reduced ability to suppress them in MB cells.

In summary, the results of this study revealed the aberrant expression of miR-21 in MB cells and showed that miR-21 regulates the expression of multiple target proteins that are associated with tumour dissemination, many of which are implicated in MB biology. Importantly, repression of microRNA-21 inhibited MB cellular migration *in vitro*, possibly due to down-regulation of the tumour suppressor PDCD4. Together these compelling data provide preliminary evidence that miR-21 is a pro-metastatic miRNA in MB and raise the possibility that curability of selected MB may be improved by pharmaceutical strategies directed towards microRNA-21.

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Conflict of interest statement

None declared.

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