신경외과학 의학박사 학위논문

수모세포종 전이와 관련된 microRNA-192의 역할

microRNA-192 suppresses leptomeningeal dissemination of medulloblastoma by modulating cell proliferation and anchoring through the regulation of dihydrofolate reductase, integrin subunits, and CD 47

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Abstract

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Introduction: The main cause of death in medulloblastoma is recurrence associated with leptomeningeal dissemination. Although the molecular basis of medulloblastoma has received considerable attention over the past decade, the role of microRNAs (miRNAs) in the acquisition of metastatic phenotype remains poorly understood. This study aimed to identify the miRNA involved in leptomeningeal dissemination and to elucidate its target mechanisms.

Materials and methods: We analyzed the miRNA expression profiles of 29 medulloblastomas according to the presence of cerebrospinal fluid (CSF) seeding.

Differentially expressed miRNAs (DEmiRNAs) were validated on 29 medulloblastoma tissues and three medulloblastoma cells. The biological function of the selected miRNA was evaluated using *in vitro* studies.

Results: A total of 12 DEmiRNAs were identified including miRNA-192 in medulloblastoma with seeding. The reduced expression of miRNA-192 was confirmed in the tumor seeding group and in the medulloblastoma cell lines. Overexpression of miRNA-192 inhibited cellular proliferation targeting dihydrofolate reductase (DHFR). miRNA-192 decreased cellular anchoring via the repression of integrin subunits (αV, β1, and β3) and CD47.

Conclusions: Medulloblastoma with seeding showed specific DEmiRNAs compared to those without. microRNA-192 suppresses leptomeningeal dissemination of medulloblastoma by modulating cell proliferation and anchoring ability.

Key words: Medulloblastoma, microRNA-192, Integrins, CD47, Dihydrofolate reductase, Seeding

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microRNAs: miRNAs

Cerebrospinal fluid: CSF

Differential expressed miRNAs: DEmiRNAs

Dihydrofolate reductase: DHFR

Sonic hedgehog: SHH

Seoul National University Children's Hospital: SNUCH

Quantitative Reverse Polymerase Chain Reaction: qRT-PCR

Negative control: NC

Extracellular matrix: ECM

Optical density: OD

Zinc finger E-box-binding proteins: ZEB

Bovine serum albumin: BSA

Standard deviation: SD

Epithelial-mesenchymal transition: EMT

Signal regulatory protein α: SIRPα

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Introduction

Medulloblastoma is one of the most common malignant brain tumors and is a leading cause of cancer-related morbidity and mortality in children. The outcome for patients with medulloblastoma is highly associated with three factors: age at diagnosis, the extent of surgical resection, and metastasis stage according to Chang and colleagues (1,2). Dissemination, a potent marker for poor prognosis, is found in up to 40% of children at diagnosis and in most children at recurrence (3). The tight correlation between metastasis and poor prognosis for medulloblastoma patients heightens the need to understand the genetic determinants of metastasis. Substantial progress has been made in recent years in the molecular understanding of medulloblastoma. Four major subgroups can be currently distinguished: WNT, sonic

hedgehog (SHH), group 3, and group 4 (4). Likewise, these molecular subgroupings are related to distinct patient demographics, histologic subtypes, genetic variations, and patient prognosis. For example, patients with group 3 tumors tend to be younger or male, have anaplastic histology, and are associated with a higher incidence of metastasis (4,5).

miRNAs are a naturally occurring class of small non-coding regulatory RNAs that modulate protein expression by binding to the 3′-untranslated region (3′-UTR) of mRNA, inhibiting mRNA translation and affecting transcription (6). Deregulation of miRNAs was discovered to play an important role in regulating the expression of various oncogenes and tumor suppressors in a wide variety of human cancers; oncogenic miRNAs are upregulated while tumor suppressor miRNAs are down-

regulated in cancers (7). Therefore, miRNAs have been proposed as novel effective targets for anticancer therapies (7). In recent studies, miRNA-21 suppression was shown to impede medulloblastoma cell migration whereas miRNA-182 promoted leptomeningeal dissemination of non-SHH-medulloblastoma (8,9). miRNA-199b-5p is described as up-regulated in non-metastatic medulloblastomas and its high expression is associated with a better overall survival (10). However, the molecular mechanisms of miRNA-mediated medulloblastoma metastasis are still largely unknown. To identify the specific roles of miRNAs, we investigated the contribution of miRNAs to tumor seeding using miRNA microarray profiling in two antithetic groups: medulloblastoma with a tumor seeding group and without a seeding group. We then performed functional studies to assess the mechanisms of the selected

miRNA in cerebrospinal fluid (CSF) seeding.

Materials and methods

1. Tissue samples

In this study, 29 frozen medulloblastoma tissues were collected from the Brain Bank of the Division of Pediatric Neurosurgery, Seoul National University Children's Hospital (SNUCH). These tumor tissues were obtained from patients that underwent microsurgery between 1998 and 2008 at the SNUCH. The samples had been snap-frozen in liquid nitrogen immediately after microsurgery. Patient selection was based on the availability of snap-frozen tissues sufficiently abundant for this study. The individual that selected the patients was blind to the patient's clinical information except diagnosis. No previous irradiation or systemic chemotherapy had been conducted on these patients prior to microsurgery. Medulloblastoma was subsequently

diagnosed based on histopathological evaluation. Detailed clinical information is listed in Table 1. To assess the molecular biological processes for tumor dissemination, 29 medulloblastoma tissues were divided into two groups: a tumor seeding group (N=9) and a tumor non-seeding group (N=20). Tumor seeding was decided by magnetic resonance imaging (MRI) findings, supplemented by lumbar CSF cytology data. We defined tumor seeding according to our previous study (11). Written consent to use tumor material for research purposes was obtained from the parents of patients according to the requirement of the Institutional Review Board of the Seoul National University Hospital and the Seoul National University College of Medicine.

2. Cells and culture conditions

The human medulloblastoma cell line, D283, was obtained from the American Type culture Collection (ATCC, Manassas, VA). MED8A and UW228 cell lines were provided by Dr. Young Shin Ra (Asan Medical Center, Seoul, Korea). D283 cells were cultured in Eagle's minimum essential medium (EMEM, ATCC), and MED8A and UW228 cells were cultured in DMEM (Welgene, Daegu, Korea). All media were supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA) and penicillin-streptomycin (Invitrogen). All medulloblastoma cells were grown at 37˚C in a humidified atmosphere of 5% CO_2.

3. miRNA microarray profiling and data analysis

Total RNA isolation and small RNA (including miRNA) enrichment procedure were

performed on medulloblastoma tissues using the mirVana^{TM} miRNA Isolation Kit (Ambion, Grand Island, NY). RNA concentration was quantified with the Nano Drop spectrophotometer and RNA integrity was evaluated using the Agilent Bioanalyzer. Agilent Human miRNA Microarray Kit (V2) was used as a miRNA microarray chip for hybridization. RNA labeling and hybridization were performed according to the manufacturer's instructions. The microarray images were scanned with the Agilent microarray scanner. The total gene signals were extracted using Agilent Feature Extraction software. A small constant, 16, was added to the scanned raw expression values to ensure that all expression values were greater than zero before $\log_2$ transformation. The $\log_2$-transformed data were normalized by the quantile normalization method using R/Bioconductor (12). To identify the differentially

expressed miRNAs (DEmiRNAs) between the tumor seeding group and the tumor non-seeding group, Bayesian moderated t-statistics were computed (13).

4. RNA extraction

Total RNAs, including miRNA, were isolated from cells using reagent (Ambion) according to the manufacturer's instructions on the indicated days after transfection. Normal human brain cerebellum total RNA was purchased from Clontech Laboratories (Mountain View, CA).

5. Real-Time quantitative Reverse Transcription Polymerase Chain Reaction (Real-Time qRT-PCR) analysis of miRNA

The DEmiRNAs identified by the microarray were validated with TaqMan probes (Applied Biosystems, Carlsbad, CA) using the Applied Biosystems 7500 Real-time PCR system. The cDNAs were prepared with the High-Capacity cDNA Synthesis kit (Applied Biosystems) using 10 ng total RNA as a template. miRNA sequence-specific RT-PCR primers for miRNA-192 and endogenous control RNU6B were purchased from Ambion. The reactions were performed under conditions specified in the ABI TaqMan Gene Quantitation assay protocol and were repeated in triplets. Signal was collected at the endpoint of every cycle. The gene expression delta cycle threshold values of miRNAs from each sample were calculated by normalizing with the internal control, RNU6B, and relative quantitation values were plotted.

6. Transfection of miRNA in medulloblastoma cells

50 nM of precursor miRNA-192 and negative control (NC) miRNA (Ambion) were transfected into the medulloblastoma cells (D283, MED8A, and UW228). Transfection was performed with Lipofectamine RNAiMAX transfection reagent (Invitrogen) according to the manufacturer's instructions. Cells were then harvested and seeded for further studies on the indicated days. Cy3-labeled NC miRNA (Ambion) was used to observe transfection efficacy by fluorescence microscope.

7. Cell viability, proliferation and cell cycle assay

Medulloblastoma cells were transfected with miRNA-192 or NC miRNA. After stabilization, cells were seeded in 96-well plates (4×10^3) and incubated for 24, 48,

and 72 hours.

For the cell viability assay, a colorimetric cell counting kit-8 (Dojindo, Kumamoto, Japan) was used. Absorbance in each well was measured at 540 nm with a micro-ELISA reader (Molecular Devices, Sunnyvale, CA). Percentage of cell viability was determined by the relative absorbance of the cells transfected with miRNA-192 versus cells transfected with NC miRNA.

For the cell proliferation assay, BrdU was added to the cells for 3 hours and the cells were treated according to the manufacture's protocol. The optical density at 450 nm was measured on an ELISA plate reader.

For the cell cycle analysis, cells were harvested and fixed in ice cold 70% ethanol. After incubation for 1 hour at –20˚C, the cells were washed with cold PBS and re-

suspended in 0.5 mg/mL RNase A (Sigma-Aldrich, St. Louis, MO). After 1 hour at 37˚C, 10 μg/ml propidium-iodine solution (Sigma-Aldrich) was added in the dark at 4˚C and the cells were observed with fluorescent microscopy. The cells were then analyzed by fluorescence activated cell sorting. All experiments were performed in triplicate and repeated at least three times.

8. Invasion assay

Invasion assay was performed using the QCM ECMatrix Cell Invasion Assay (24-well, 8 μm, colorimetric kit, Millipore, Billerica, MA) according to the manufacturer's instructions. Briefly, cells transfected with miRNA-192 or NC miRNA (5×10^4/well) in 300 μl serum-free medium were added to the upper chamber precoated with

ECMatrix™ gel. Then, 500 µl of 10 % FBS-containing medium was added to the lower chamber as a chemoattractant. Cells were incubated for 1 day at 37˚C and non-invading cells were removed completely using a cotton swab. Invasive cells were fixed, stained and rinsed in distilled water. Stained cells were soaked in ice-cold acetic acid, oscillated for 10 min and then assessed by absorbance at 570 nm wavelength using a microplate reader. All experiments were performed in triplicate.

9. Adhesion assay

Cell adhesion assay was performed using the CytoSelect 48-well cell adhesion assay kit (Cell Biolabs, San Diego, CA) according to the manufacturer's instruction. Medulloblastoma cells transfected with miRNA-192 or NC miRNA (1×10^5/well) in

300 µl serum-free medium were seeded in the 48-well plates coated with extracellular matrix (ECM) including fibronectin, collagen type I, collagen type IV, laminin type I, fibrinogen, and bovine serum albumin (BSA). After 1 hour of incubation at 37℃, unattached cells were removed by washing with PBS. Attached cells were stained and dissolved using the extraction solution. The extracted cells were transferred to a 96-well plate and were measured the optical density (OD) 560 nm in a microplate Reader. All experiments were performed in triplicate.

10. Determination of miRNA-192 targeting sequences by computational prediction

Computational prediction has already been proven to be an effective and efficient

method for predicting miRNA's targets. We performed database searches in miRNA target prediction engines, including TargetScan and microRNA.org (http://www.targetscan.org/ and http://www.microrna.org/) to predict the miRNA-192 targeting sequences. Targets predicted by both databases and related to tumor metastasis were considered relevant to our research.

11. Real-Time qRT-PCR analysis of target mRNA expression

cDNA synthesis was carried out with the EcoDry Premix-Oligo dT (Clontech Laboratories) using 1μg total RNA. Real-time qRT-PCR was performed on the 7500 Real-Time PCR System (Applied Biosystems) with Power SYBR Green PCR Master Mix (Applied Biosystems). Primer sequences used for amplifications were as follows:

dihydrofolate reductase (DHFR), -Forward: 5′-TCACCCAGGCCATCTTAAAC, Reverse: 5′-GAACACCTGGGTATTCTGGC; GAPDH, -Forward: 5′-CGTGGAAGGACTCATGAC, Reverse: 5′-CAAATTCGTTGTCATACCAG.

12. Dual luciferase miRNA target reporter assay

The 3′-UTR of DHFR was amplified by PCR using the following primers: 5′-CCG CTCGAGCTTGACATTGTCGGGCTTTT-3′ (forward) and 5′-ATAAGAAT GCGGCCGC TGCAAACACCTGAGACTTGCt -3′ (reverse). The PCR product was extracted from the gel and subsequently cloned into the pSiCHECK-2 Vector (Promega, Madison, WI). After sequencing, the construct was verified by sequencing. Medulloblastoma cells were co-transfected with 50- nM pSiCHECK-2 or

pSiCHECK-2-DHFR 3′-UTR, and 50-nM microRNA-192 or NC microRNA using Lipofectamine RNAiMAX. The 3′-UTR of integrin αV, integrin β1, integrin β3, and CD47 cloned into the pEZX vector and control vectors were purchased from GeneCopoeia (Rockville, MD). Medulloblastoma cells were co-transfected with the 100-nM pEZX or pEZX -3′-UTR of integrin αV, integrin β1, integrin β3, and CD47 and 50-nM miR-192 or NC microRNA using Lipofectamine RNAiMAX. Luciferase activities were measured by using a Dual-Luciferase Reporter Assay System (Genecopoeia) for the pEZX vector, and a Dual-Glo luciferase assay system (Promega) for the pSiCHECK vector after transfection. Relative luciferase activities were calculated by normalizing the firefly luminescence to the Renilla luminescence following the manufacturer's instructions. All experiments were performed in

triplicate.

13. Western blot analysis

Total protein was extracted using the RIPA buffer containing a protease inhibitor cocktail (Cell Signaling Technology, Danvers, MA) from selected medulloblastoma tissues and cells transfected with miRNA-192 or NC miRNA. From 29 tumor tissues available, 8 with tumor seeding and 10 with tumor non-seeding were selected. The selection of tumor tissue was based on the availability of snap-frozen tissues sufficiently abundant for this analysis. The individual that selected the patients was blind to the patient's clinical information except diagnosis. The protein concentration of the lysates was measured using a BCA Protein Assay Kit (Thermo Scientific,

Rockford, IL). Equal amount of proteins were resolved by NuPAGE Bis-Tri on 4-12% gels and transferred to polyvinylidene difluoride stacks using the iBlot® transfer system (Invitrogen). The membranes were incubated with each of the following primary antibodies: anti-DHFR (1:400, Cell Signaling Technology), anti-zinc finger E-box-binding proteins (ZEB) 2 (1:1000, Abcam, Cambridge, MA), anti-E-cadherin (1:250, Abcam), anti-vimentin (1:1500, Cell Signaling Technology), anti-integrin αV (1:1000, Cell Signaling Technology), anti-integrin β1 (1:2000, Cell Signaling Technology), anti-integrin β3 (1:2000, Cell Signaling Technology), anti-CD47 (1:2000, Abcam), and anti-β-actin (1:5000, Sigma-Aldrich, St. Louis, MO). HRP-conjugated immunoglobulins (1:5000, Jackson ImmunoResearch Laboratories, West Grove, PA) served as secondary antibodies. Signals were detected with enhanced

chemiluminescence reagent (Invitrogen) and exposed to film. The protein band intensities were normalized by the corresponding band intensities of actin from the same samples.

14. Immunofluorescence staining

Cells were seeded on 8-well chamber slides (Lab-Tech, Nunc, Naperville, IL) and transfected as described above. Subsequently, cells were fixed with 2% paraformaldehyde for 15min, permeated with 0.1% Triton X-100 in PBS for 5min and blocked with 1% bovine serum albumin (BSA) for 30min. For detection, cells were incubated with the following antibodies: anti-vimentin (1:200, Abcam), anti-integrin αV (1:1000, Cell Signaling Technology), anti-integrin β1 (1:1000, Cell Signaling

Technology), anti-integrin β3 (1:500, Millipore) and anti-CD47 (1:200, Abcam). The secondary antibodies Alexa Fluor 488-conjugated goat anti-mouse IgG or anti-rabbit IgG (1:500, Invitrogen) were applied for 1hour at room temperature. Slides were mounted with antifading solution containing 4′-6′-diamidino-2-phenyl-indole (DAPI; Vector Laboratories, Burlingame, CA). Images were taken using a confocal microscope (Zeiss, Oberkochen, Germany). All experiments were conducted in triplicate.

15. Statistical analysis

Values were presented as mean ± standard deviation (SD). Statistical significance was evaluated by Student's *t*-test and χ^2-test comparison between the two groups of

data. $P < 0.05$ was accepted as significant and indicated significant difference in the experimental groups compared with the corresponding control condition. Statistical analysis was done using GraphPad Prism software (GraphPad 4.0).

Results

1. miRNA-192 is down-regulated in the tumor seeding group and in medulloblastoma cells

From analyzing miRNA expression data using the Agilent human miRNA microarray containing probes for 723 human miRNAs between the tumor seeding group and the tumor non-seeding group, we selected 12 DEmiRNAs with minimum log2 expressions greater than 5 and range of expressions greater than 2 (all P values < 0.05, Table 2 and Figure 1A). Of these DEmiRNAs, we focused on 4 downregulated DEmiRNAs capable of functioning as tumor suppressors during medulloblastoma metastasis. Recently, a couple of studies have reported that miRNA-192 was downregulated in medulloblastomas with respect to both adult and fetal controls. The

studies also reported that miRNA-192 acted as a regulator of different steps in the proliferation-metastasis-cascade by virtue of its capacity to modulate key proliferation- and EMT-promoter genes, including ZEB 1 and 2, vimentin, and DHFR (14-17). Therefore, we hypothesized that among DEmiRNAs, miRNA-192 plays an important role in CSF seeding in medulloblastomas and focused on analyzing miRNA-192. We found that the expression level of miRNA-192 was significantly lower in the tumor seeding group (N=9) compared to the tumor non-seeding group (N=20) and/or the normal cerebellum (all P values < 0.05, Figure 1B). To evaluate the expression level of miRNA-192, we analyzed Real-Time qRT-PCR in three medulloblastoma cell lines (D283, MED8A, and UW228). We observed that the expression level of miRNA-192 was significantly lower in all of the medulloblastoma

cells than in the normal cerebellum (all P values < 0.05, Figure 1C).

2. Transfection of miRNA-192 in medulloblastoma cells

To determine the functional significance of miRNA-192, all medulloblastoma cells were transfected with miRNA-192. Transfection efficiency was monitored using Cy3-labeled pre-miRNA (Fig. 1D) and determined by Real-Time qRT-PCR (Fig. 1E). After transfection, miRNA-192 levels increased approximately 1100-fold in D283 ($P < 0.05$), 867-fold in MED8A ($P < 0.01$), and 2507-fold in UW228 ($P < 0.01$) at 48-hour and 127-fold in D283 ($P < 0.05$), 313-fold in MED8A ($P < 0.05$), and 267-fold in UW228 ($P < 0.05$) at 72-hour, compared to NC miRNA, respectively (Fig. 1E).

3. **Overexpression of miRNA-192 reduces cellular viability and proliferation and induces cell cycle arrest**

To assess the functional significance of miRNA-192, we evaluated its effect on cellular viability and proliferation using medulloblastoma cells. Overexpression of miRNA-192 suppressed cellular viability (all P values < 0.05, Figure 2A) and proliferation (all P values < 0.001, Figure 2B) at 48- and 72-hour in the medulloblastoma cells. The NC miRNA had no effect on cellular viability and proliferation, suggesting that miRNA-192-mediated inhibition of cellular viability and proliferation is specific to miRNA-192. These results indicated that miRNA-192 plays an important role in the inhibition of cell growth in medulloblastoma cells. We subsequently investigated the effects of miRNA-192 in the medulloblastoma cells on the cell cycle using flow cytometry. The proportion of cells in the G2 phase increased

after miRNA-192 transfection in the medulloblastoma cells (all P values < 0.01, Figure 2C).

4. DHFR is a downstream target of miRNA-192

DHFR is a target of methotrexate and the key enzyme responsible for intracellular folate metabolism, which is essential for DNA and RNA synthesis (18). We investigated the roles of miRNAs in regulating the expression of DHFR. Based on structural analysis of 3'-UTR in the DHFR gene and miRNA target analysis (http://www.targetscan.org/ and http://www.microrna.org/) as well as a previous study (17), we identified miRNA-192 as potentially interacting with the 3'-UTR region of DHFR mRNA (Figure 3A).

To confirm direct targeting, we cloned the DHFR 3'-UTR fragment containing this

predicted site in the pSiCHECK2 luciferase reporter. Co-transfection of pre-miRNA-192 and pSiCHECK2-DHFR significantly decreased the luciferase activity, compared with the control, in all of the medulloblastoma cells (all P values < 0.05, Figure 3B), indicating that DHFR is the direct target of miRNA-192.

We explored the mRNA and protein levels of DHFR in the medulloblastoma tissues and in three medulloblastoma cells. We analyzed the expression levels of DHFR mRNA by Real-Time qRT-PCR. DHFR mRNA was higher in the seeding group than in the normal cerebellum and the non-seeding group (all P values < 0.05, Figure 3C). We also confirmed the expression level of DHFR mRNA in the medulloblastoma cells compared to the normal cerebellum (all P values < 0.05, Figure 3C). The results showed that DHFR mRNA level was higher in all of the medulloblastoma cells than

in the normal cerebellum. The up-regulated DHFR mRNA expression was associated with down-regulation of miRNA-192 expression, most likely at the translational level.

Western blot analysis showed that overexpression of miRNA-192 significantly decreased the expression of DHFR protein (Figure 3D). We also investigated the correlation between the expression level of miRNA-192 and the expression of DHFR mRNA using previously published mRNA array data (19). The expression of miRNA-192 demonstrated an inverse correlation with the expression of DHFR (Pearson's correlation coefficient $r = -0.38$, $P = 0.044$, Figure 3E).

4. miRNA-192 does not regulate invasion and EMT in medulloblastoma cells

Given that the expression of miRNA-192 was down-regulated in the tumor seeding group, we investigated whether miRNA-192 plays an important role in

medulloblastoma cell invasion. Following miRNA-192 or NC miRNA transfection in all medulloblastoma cells, no significant differences were seen in the percentages of migrated cells (Figure 4A). There was no significant impact on the optical densities of migrated cells in the medulloblastoma cells transfected with miRNA-192 or NC miRNA (all P values > 0.05, Figure 4B). These results showed that miRNA-192 did not influence cell invasion behavior *in vitro*.

We speculated that miRNA-192 may affect EMT in medulloblastoma seeding. To confirm this hypothesis, we transfected the medulloblastoma cells with miRNA-192 and analyzed EMT-related genes and proteins such as ZEB2, E-cadherin and vimentin (Figure 4C). ZEB2 mRNA and protein expression was not affected by the overexpression of miRNA-192 in any of the medulloblastoma cells. The expression of

E-cadherin mRNA increased but protein expression did not change in the MED8A and UW228 cells. In D283 cells, E-cadherin mRNA and protein expressions increased slightly. Notably, vimentin protein was markedly diminished even though its mRNA expression was only slightly reduced in miRNA-192 overexpressed medulloblastoma cells (Figure 4D).

5. Overexpression of miRNA-192 inhibits the adhesive capability of medulloblastoma cells

To determine the functional role of miRNA-192 overexpression in the adhesion of medulloblastoma cells, we performed an adhesion assay after miRNA-192 transfection. Overexpression of miR-192 significantly inhibited adhesion to fibronectin, collagen type I, collagen type IV, laminin type I and fibrinogen in all

medulloblastoma cells compared to the NC miRNA (all P values < 0.05, Figure 5A and B). These results suggest that miRNA-192 affects cell attachment and interferes with integrin binding to the ECM.

6. miRNA-192 targets the 3′-UTR of integrin αV, integrin β1, integrin β3, and CD47and represses their expression

From the above results, we found that miRNA-192 was neither directly associated with cell invasion nor directly involved in EMT. Unlike the patterns of metastasis in other types of cancer or glioma, medulloblastoma disseminates through the CSF in the leptomeningeal space to coat the brain and spinal cord (2,20). CSF flow serves as a 'transporter' for tumor cells; therefore, cell motility itself may not be important in CSF seeding. We hypothesized that acquisition of an enhanced capacity to adhere to ECM

substrates at the target site and proliferation of tumor cells could be more important than migratory and invasive capacity for the dissemination of medulloblastoma. As integrins and integrin-related proteins are central regulators of focal adhesion dynamics, changes in their expression resulting from deregulating miRNAs in tumors represent a functionally relevant contribution to metastatic dissemination because the main function of integrin is cell attachment to the ECM (21,22). By *in silico* analyses (http://www.targetscan.org/ and http://www.microrna.org/), we found that the seed sequence of miRNA-192 was conserved in the 3′-UTR of 3 other integrin subunits (integrin αV, integrin β1, and integrin β3) and CD47 (Figure 6A). Because the binding of miRNAs to their specific seed sequences often represses gene expression, we examined the effect of miRNA-192 on the expression levels of these candidate target

integrin subunits and CD47.

We used the Firefly-Renilla dual luciferase reporter gene assay to confirm that the posttranscriptional repression of the integrin subunits (integrin αV, integrin β1, and integrin β3) and CD47 is a consequence of miRNA-192 directly binding to target seed sequences within the 3′-UTR of the transcript for the integrin subunits and for CD47. We found no differences in luciferase activity between the medulloblastoma cells transfected with control vector alone and those with control vector co-transfected with NC miRNA. In contrast, in cells transfected with the integrin subunits (integrin αV, integrin β1, and integrin β3) and CD47 3′-UTR-containing vector, overexpression of miRNA-192 resulted in a significant reduction in luciferase activity for all (all P values < 0.05, Figure 6B) but integrin β1 in D283 (P value = 0.08).

To confirm the regulation of integrin αV, integrin β1, integrin β3, and CD47 by miRNA-192, we transfected the medulloblastoma cells with miRNA-192. There was a significant reduction in the integrin αV, integrin β1, integrin β3, and CD47 protein on western blot analysis with all miRNA-192-transfected medulloblastoma cells in comparison with NC miRNA-transfected cells: integrin αV, integrin β1, integrin β3, and CD47 (all P values < 0.05, Figures 6C and D). Furthermore, we investigated the localization of integrin αV, integrin β1, integrin β3, and CD47 in the medulloblastoma cells after miRNA-192 overexpression. Consistent with our prediction, miRNA-192 decreased the protein expression of integrin αV, integrin β1, integrin β3, and CD47 and promoted nuclear accumulation of integrin αV, integrin β1, integrin β3, and CD47 (Figure 6E).

7. miRNA-192 regulates integrin αV, integrin β3, and CD47 expression in medulloblastoma tissues

To determine whether integrin subunits are related to the dissemination of medulloblastoma, the protein levels were analyzed in the seeding (N=8) and non-seeding groups (N=10). Compared with the non-seeding group, integrin αV ($P < 0.01$), integrin β3 ($P < 0.05$) and CD47 ($P < 0.01$) were significantly lower in the seeding group. Integrin β1 ($P = 1.00$) and vimentin ($P = 0.08$) were not (Figure 6F and G). We found that overexpression of miRNA-192 significantly decreased the expression of integrin αV, integrin β3, and CD47 proteins.

Discussion

We identified 12 DEmiRNAs between tumor seeding group and tumor non-seeding group in medulloblastoma. Among these, we focused on miRNA-192 because it was downregulated in medulloblastoma tumor samples and has been known to regulate EMT (14-16). Although miRNA-192 does not affect cell invasion directly, we found that miRNA-192 is associated with leptomeningeal dissemination by regulating DHFR-related cell proliferation processes and integrin-related cell anchoring. This is the first report outlining the phenocopying effects of miRNA-192 down-regulation on the leptomeningeal dissemination of medulloblastoma.

Despite the importance of miRNAs in metastasis, little has been known about the effects of miRNAs on leptomeningeal dissemination of medulloblastoma until

recently. The miRNAs may have a dual role in cancer and can act as oncogenes or tumor suppressor genes (23). miRNA-199b-5p is described as up-regulated in non-metastatic medulloblastomas and its high expression is associated with better overall survival (10). miRNA-21 regulates the expression of the metastasis suppressor programmed cell death 4 (PDCD4) protein and lack of this protein results in leptomeningeal dissemination in medulloblastoma (24). A recent report demonstrated that miRNA-182 promotes leptomeningeal spread of non-SHH medulloblastoma subgroups (25). We found that miRNA-192 expression was down-regulated in the tumor seeding group and in all medulloblastoma cells compared to the tumor non-seeding and/or the normal cerebellum group.

DHFR has important roles in the growth and proliferation of cells (17). DHFR is a

key enzyme in intracellular folate metabolism and is a target of methotrexate (MTX), which is an important chemotherapeutic agent widely used in the treatment of several malignancies including medulloblastoma (26-28). The inhibition of DHFR can limit the growth and proliferation of cells and the expression of DHFR can be regulated, at least in part, at the translational level (17,29). A previous study showed that miRNA-24 has a target site in the 3'-UTR of DHFR mRNA and that a miRNA-24-single nucleotide polymorphism results in the loss of miRNA-24-mediated inhibition of DHFR and in methotrexate resistance. miRNA-24-single nucleotide polymorphism is also associated with an increase in DHFR mRNA and protein expression (29). Other studies have shown that miRNA-192 regulates DHFR and cell proliferation through the p53-miRNA circuit and is down-regulated in metastatic cancers (17,30). However,

the role of miRNA-192 and the contribution of DHFR in medulloblastoma have not been fully explored and most of their overall biological functions remain unknown. We found that miRNA-192 significantly down-regulates DHFR expression and DHFR expression is higher in the tumor seeding group than in the non-seeding group. In addition, miRNA-192 expression inversely correlates with DHFR expression in medulloblastoma tissues and cells. Like miRNA-24, in the present study, miRNA-192 overexpression suppresses cell proliferation and restores cell cycle control by modulating DHFR expression (17). These results provide further evidence that miRNA-192 is one of the candidate miRNAs directly involved in the regulation of a key anticancer target, DHFR.

Some miRNAs control EMT by targeting either receptors that initiate signals from

EMT inducers, or multiple components of the EMT signaling pathways (31,32). In various cancers, cancer cells can undergo EMT to escape from the primary tumor, invade surrounding tissues, and eventually colonize remote sites to generate metastases (33). Initially, we hypothesized that metastatic dissemination of medulloblastoma might be associated with EMT. Although vimentin was reduced by miRNA-192 transfection in all of the medulloblastoma cells, EMT related proteins such as ZEB2 and E-cadherin proteins were not affected. miRNA-192 also did not affect cell invasion in medulloblastoma cells. Unlike other types of cancer or glioma, medulloblastoma disseminates by using the CSF as a transportation medium. Therefore, acquisition of enhanced anchoring ability of floating tumor cells to ECM substrates at the metastatic site might be more important than invasive capacity in the

dissemination of medulloblastoma. In the present study, miRNA-192 regulated cell adhesion by targeting integrin αV and integrin β. Integrin β3 is mostly associated with the ability of tumor metastasis to promote extravasation from the primary tumor, cell adhesion, intravasation, and tumor growth at the metastatic site (34,35). Vimentin interacts with integrin β3 and plectin, which together regulate the organization and distribution of vimentin in several different cell types. A possible role for integrin-β3-mediated recruitment of vimentin to the cell surface is to regulate the adhesive strength of the cells binding to the substrate (36). The cells' adhesion ability positively correlates with integrin αVβ3, indicating an increase in their metastatic potential (37,38). Based on our findings and those of others (34-38), the role of miRNA-192 in the metastatic dissemination of medulloblastoma may be more related to cell adhesion

than to EMT at the metastatic site.

When the central role of miRNAs in regulating various cell adhesion molecules is taken into consideration, it is not surprising that the altered activity of some adhesion-associated miRNAs has been found to contribute to primary tumor development and to subsequent metastatic progression (22,39). In the present study, miRNA-192 regulated cell adhesion by targeting integrin β3 and integrin αV. Integrin β3 is mostly associated with the ability of tumor metastasis to promote extravasation from the primary tumor, cell adhesion, intravasation, and tumor growth at the metastatic site (34,35). Factors that interfere with integrin β3 action abrogate the integrin αVβ3-mediated adhesion and migration of cancer cells (37,40). Moreover, the cells' abilities to adhere and migrate positively correlate with integrin αVβ3, which indicates an

increase in their metastatic potential (37,38,40). Transfection of melanoma cells with let-7a pre-miRNA down-regulates integrin β3 expression and also reduces the invasive potential of transfected cells (41). Integrin αVβ3 binds to the endothelial cell adhesion molecule L1 and this adhesive interaction may promote the metastasis of melanoma cells (42). Like the above-mentioned studies, our results showed that the overexpressed miRNA-192 reduced cell adhesion but did not affect cell invasion.

Metastatic cells have to induce angiogenesis to escape the limitations of passive diffusion of nutrients and oxygen, which hamper metastatic colonization and growth (43). Integrin β3 plays an important role in tumor-induced angiogenesis and has been described as a pro-angiogenic factor (34,37). Among all integrin subtypes, integrin αVβ3 is probably most strongly involved in the regulation of neovascularization and

tumor-induced angiogenesis (34,37,44,45). Differential expression of integrin αVβ3 is found on newly formed vessels but not on pre-existing vessels. Integrin αVβ3 is also widely expressed on blood vessels of human tumor biopsy samples but not on vessels in normal tissues (37,44,46). In addition, blocking αVβ3 integrin with monoclonal antibodies or ligand antagonists results in blunted blood vessel formation (47,48). In accordance to our results and the above-mentioned findings, miRNA-192-mediated integrin β3 (especially αVβ3) plays an important role in tumor-induced angiogenesis, which is essential for metastatic colonization and growth.

Although there were no significant differences in the protein level of integrin β1 between in the tumor seeding group and in the tumor non-seeding group in the present study, miRNA-192 contributed to the decreased expression of integrin β1 protein in

all of the miRNA-192-transfected medulloblastoma cells. Integrins are adhesion receptors involved in bidirectional signaling crucial for various cellular responses related to maintaining normal homeostasis as well as for pathologic conditions such as cancer progression and metastasis. Previous studies have demonstrated that overexpression of miRNA-124 in a glioblastoma cell line inhibits tumor migration and invasion, accompanied by decreased expression of integrin β1 and that miRNA-31-mediated inhibition of integrin β1 expression results in decreased cancer cell invasion and metastasis (39,49). On the other hand, our results showed that overexpressed miRNA-192 reduced cell adhesion but did not affect cell invasion. Integrin β1, known as a part of the focal adhesion platform, contributes to stabilizing cell attachment to ECM ligands (50). Binding of integrin β1 to collagen results in

downstream rearrangements of the actin cytoskeleton by providing a scaffold for cytoskeletal proteins and multiple signaling molecules that are involved in the regulation of cell adhesion and spreading (51). Based on our findings and those of others (50,51), miRNA-192-mediated inhibition of integrin β1 expression results in decreased cell anchoring ability during the leptomeningeal dissemination of medulloblastoma.

CD47, which has been shown to interact with and to modulate integrin αVβ3 activation, co-localizes with E-cadherin at cell-cell adhesion sites and participates in the regulation of cell-cell adhesion as well as cell migration through the reorganization of the actin cytoskeleton in epithelial cells (44,52,53). Aberrant expression of miRNAs has been implicated in expression deregulation and activity in integrin and

CD47, leading to the development and progression of primary tumors, including their acquisition of the metastatic phenotype. Blocking CD47 by neutralizing antibodies reduces migration and chemotaxis in response to collagen IV in melanoma, prostate cancer, and ovarian cancer-derived cells. These effects have been shown to be dependent on integrin αVβ3 and intracellular calcium (52). The interaction of CD47 with integrin αVβ3 is necessary for the complete inhibition of tumor cell proliferation and tumor cell adhesion to a substrate (53). In the present study, miRNA-192 regulated cell adhesion by targeting integrin β3, integrin αV, and CD47. Our results seem to be consistent with the above-mentioned findings: miRNA-192-mediated CD47 expression can regulate leptomeningeal dissemination of medulloblastoma through molecular cross talk with integrin αVβ3. Recent studies have also demonstrated

that the CD47-signal regulatory protein α (SIRPα) signaling system plays important roles in tumor immune surveillance through regulation of the phagocytic activity of macrophages. Blocking this signaling system enhances macrophage-mediated clearance of tumor cells (54,55). Although we did not determine whether miRNA-192 regulates the CD47-SIRPα immune surveillance system, this will be an area for future research.

Together with the results of other studies (30,56), our results suggest that miRNA-192 is one of the metastasis suppressor genes, whose pleiotropic functions regulate multiple steps of the cancer metastasis cascade by targeting prometastatic target genes, including integrins. This pleiotropic effect of miRNA-192 is of potential interest from a translational perspective because the modulation of a single miRNA, such as adeno-associated viral vectors and synthetic miRNA precursors (57), can affect the function

of several genes involved in cancer metastasis. However, *in vivo* studies including clinical settings are clearly warranted first. Regarding miRNA-192 down-regulation in medulloblastoma and its function as a tumor suppressor, we tentatively propose that the therapeutic approach should rely on restoring the expression of miRNA-192 using synthetic RNA duplexes that will be recognized by the RNA-induced silencing complex and mimic miRNA-192 functions (58).

In summary, this study demonstrated that loss of miRNA-192 expression has wide ranging effects on cell proliferation and cell anchoring as it modulates DHFR and molecular cross talk between integrin subunits (αV, β1, and β3) and CD47 in the leptomeningeal dissemination of medulloblastoma.

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Table 1. Characteristics of the patients with medulloblastoma (N=29)

Characteristics	CSF non-seeding group (N=20)	CSF seeding group (N=9)	P-value
Age at diagnosis (year), mean (range)	6.1 (1.1-13.0)	7.0 (0.7-15.0)	0.55
< 3	4 (20.0)	1 (11.1)	0.99
Female sex	8 (40.0)	4 (44.4)	0.99
Histopathological subtype			
Classic	12 (60.0)	8 (88.9)	0.26
Desmoplastic and nodular	3 (15.0)	0	
Anaplastic	5 (25.0)	1 (11.1)	
[a]Molecular subtypes according to Tayler *et al.* (4)			
WNT	0	0	

SHH	7	2	
Group 3	5	1	
Group 4	8	5	
Clinical follow-up period after surgery (month), mean (range)	78.1 (17.0-167.0)	65.9 (11.0-150.0)	
Final state at the last follow-up			0.26
No evidence of disease	12 (60.0)	3 (33.3)	
Progression	1 (5.0)	0	
Death	7 (35.0)	6 (66.7)	

[a]The result is based on our previous study (19).

Table 2. Differentially expressed miRNAs in the tumor seeding group compared to the tumor non-seeding group.

miRNA	Tumor seeding vs tumor non-seeding	
	$\log_2$ FC	P-value
hsa-miRNA-340-5p	− 0.595	0.008
hsa-miRNA-148a-3p	− 0.519	0.028
hsa-miRNA-101-3p	− 0.515	0.037
hsa-miRNA-192-5p	− 0.332	0.041
hsa-miRNA-34b-5p, or -3p	0.442	0.021
hsa-miRNA-32-3p	0.485	0.011

hsa-miRNA-483-3p	0.527	0.002
hsa-miRNA-574-3p	0.644	0.001
hsa-miRNA-574-5p	0.724	0.003
hsa-miRNA-630	0.725	0.031
hsa-miRNA-196a-5p	0.944	0.004
hsa-miRNA-494-3p, or -5p	1.512	0.042

FC: fold-change, hsa: Homo sapiens

Figure 1. (A), heatmap of 12 differentially expressed miRNAs between seeding and non-seeding group of medulloblastoma. Black bars at the top of the heatmap indicate the presence of seeding. (B), the expression level of miRNA-192 is significantly lower in the tumor seeding group (N=9) compared to the tumor non-seeding group (N=20) or to normal cerebellum (normal cerebellum vs. non-seeding, 1.01 ± 0.02 vs. 0.45 ± 0.12, $P < 0.001$; normal cerebellum vs. seeding, 1.01 ± 0.02 vs. 0.26 ± 0.18, $P < 0.001$; non-seeding vs. seeding, $P < 0.01$). (C), miRNA-192 expression in medulloblastoma cells (normal cerebellum vs. D283, 1.00 ± 0.02 vs. 0.19 ± 0.16, $P < 0.001$; normal cerebellum vs. MED8A, 1.00 ± 0.02 vs. 0.36 ± 0.14, $P < 0.05$; non-seeding vs. UW228, 1.00 ± 0.02 vs. 0.32 ± 0.26, $P < 0.05$). (D), Cy3 labeled miRNA visualized by fluorescent microscope to determine transfection efficiency. (E), after transfection of miRNA-192 in medulloblastoma cells, transfection efficacy was determined by RT-qPCR. The miRNA-192 was effectively overexpressed in D283 (1100-fold, $P < 0.05$ at 48-hour; 127-fold, $P < 0.05$ at 72-hour), MED8A (867-fold, $P < 0.01$ at 48-hour; 313-fold, $P < 0.05$ at 72-hour) and UW228 (2507-fold, $P < 0.01$ at 48-hour; 267-fold, $P < 0.05$ at 72-hour). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Error bars represent ± SD (standard deviation).

Figure 1A

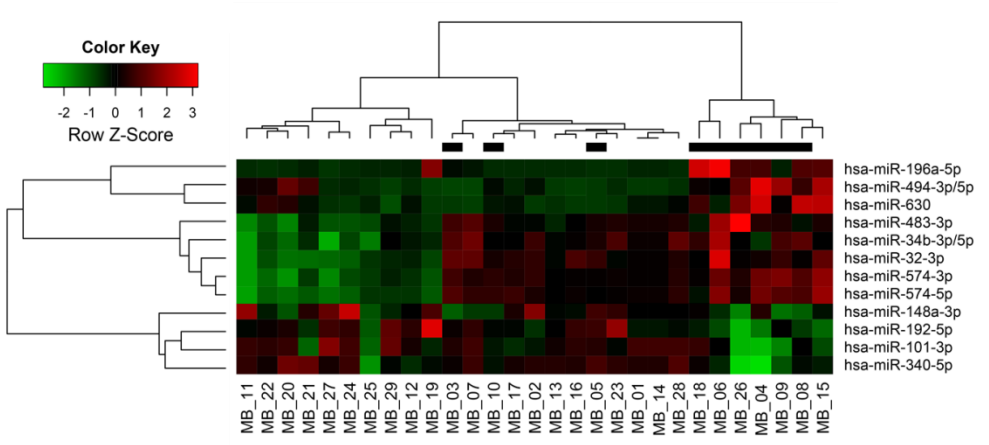


Figure 1B

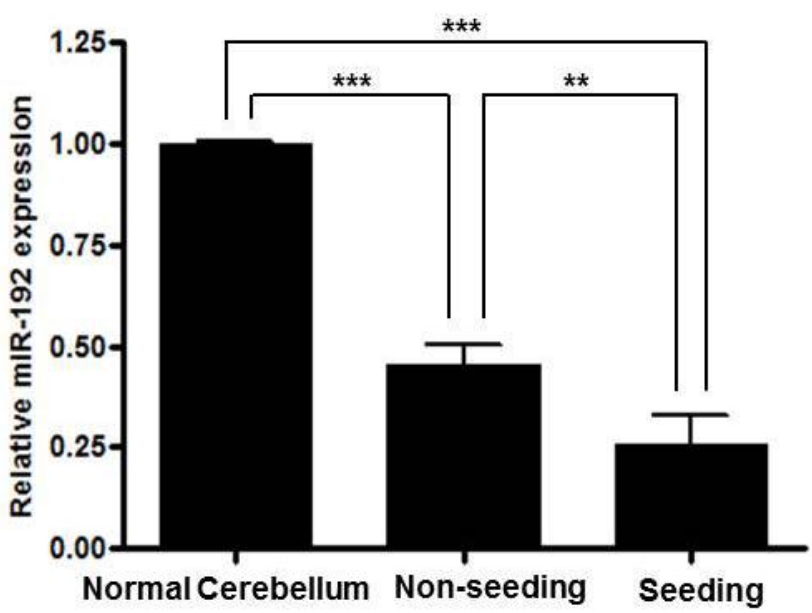


Figure 1C

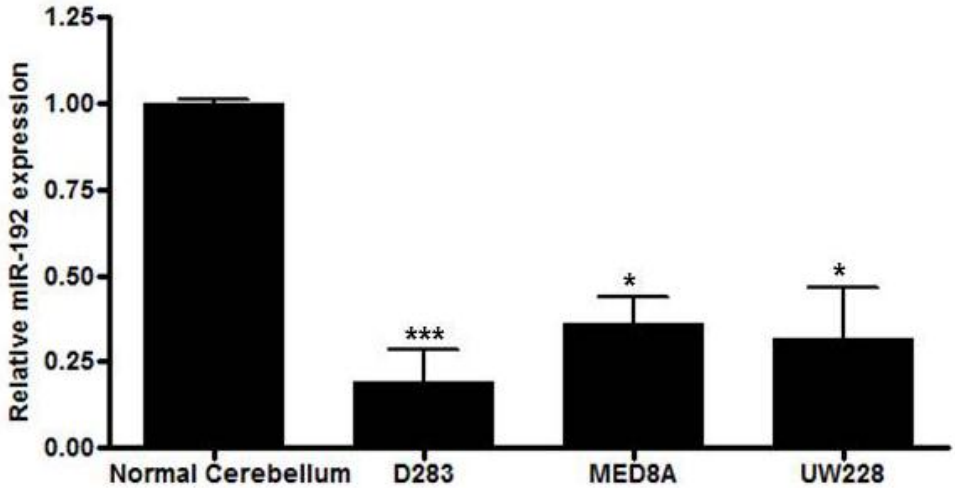


Figure 1D

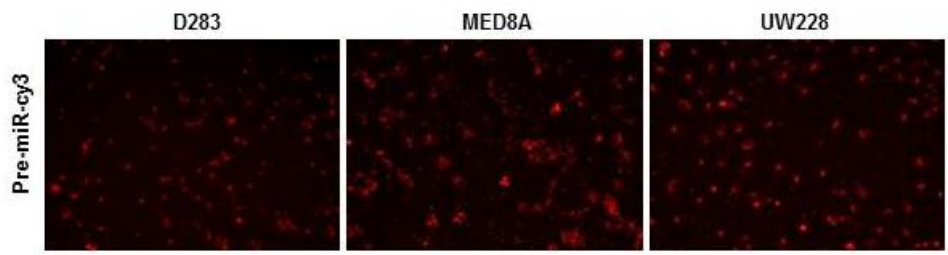


Figure 1E

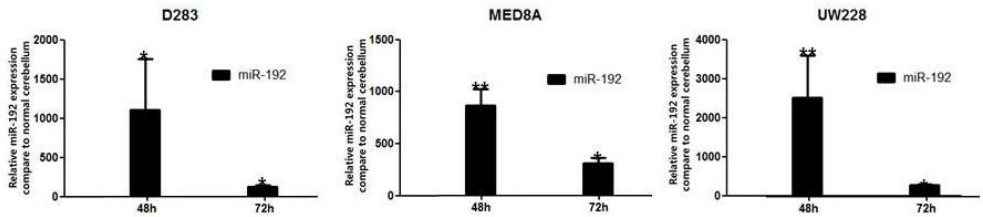

D283
MED8A
UW228
miR-192
Relative miR-192 expression compare to normal cerebellum
48h
72h

Figure 2. Functional studies of miRNA-192 transfection in medulloblastoma cells. (A), cell viability assay (normal control (NC) miRNA vs. miRNA-192: 100.2 ± 5.7 % vs. 64.2 ± 1.9 % in D283, $P < 0.05$; 100.1 ± 5.3 % vs. 61.6 ± 2.6 % in MED8A, $P < 0.001$; 100.1 ± 0.5 % vs. 65.2 ± 2.8 % in UW228, $P < 0.001$) at 48-hour. (B), BrdU cell proliferation assay. Overexpression of miRNA-192 decreases cellular proliferation (NC miRNA vs. miRNA-192: 92.8 ± 4.1 % vs. 65.2 ± 9.2 % in D283, $P < 0.001$; 139.2 ± 7.4 % vs. 88.8 ± 13.1 % in MED8A, $P < 0.001$; 111.7 ± 13.6 % vs. 72.0 ± 4.4 % in UW228, $P < 0.001$). (C), cell cycle assay. Overexpression of miRNA-192 induces cell cycle G2 arrest (NC miRNA vs. miRNA-192: 7.5 ± 0.6 % vs. 19.2 ± 0.2 % in D283, $P < 0.001$; 2.7 ± 0.3 % vs. 11.2 ± 2.0 % in MED8A, $P < 0.001$; 7.2 ± 0.1 % vs.

13.1 ± 0.5 % in UW228, $P < 0.01$). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Error bars represent ± SD.

Figure 2A

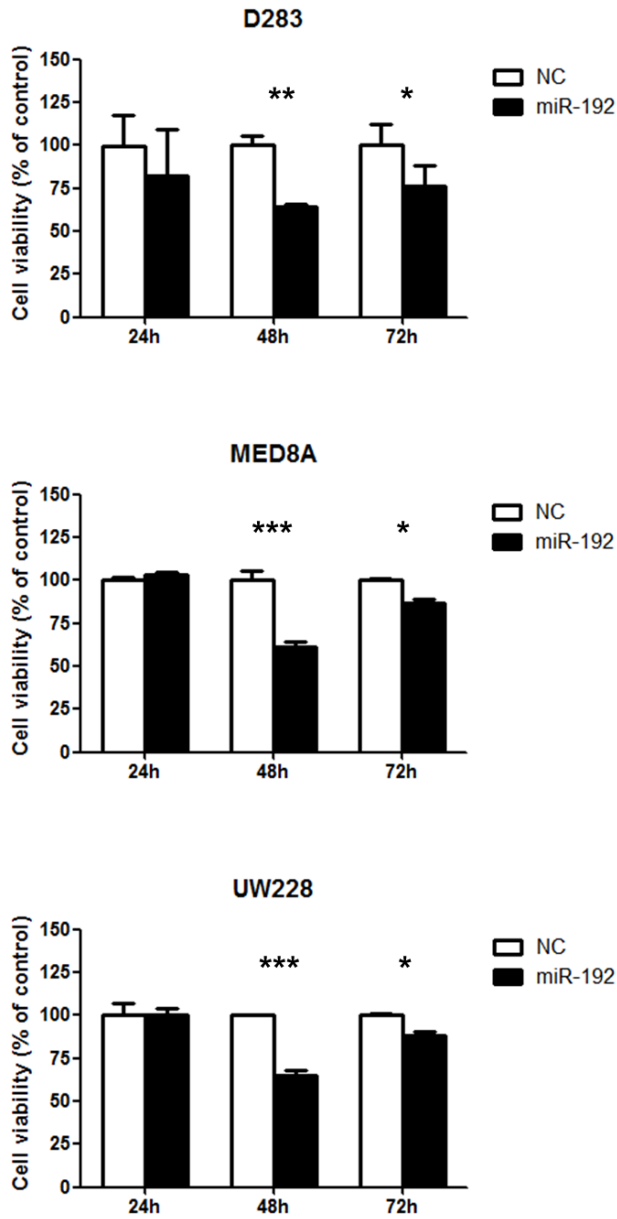

D283
Cell viability (% of control)
150
125
100
75
50
25
0
**
*
24h
48h
72h
NC
miR-192
MED8A
Cell viability (% of control)
***
*
UW228
Cell viability (% of control)
***
*

Figure 2B

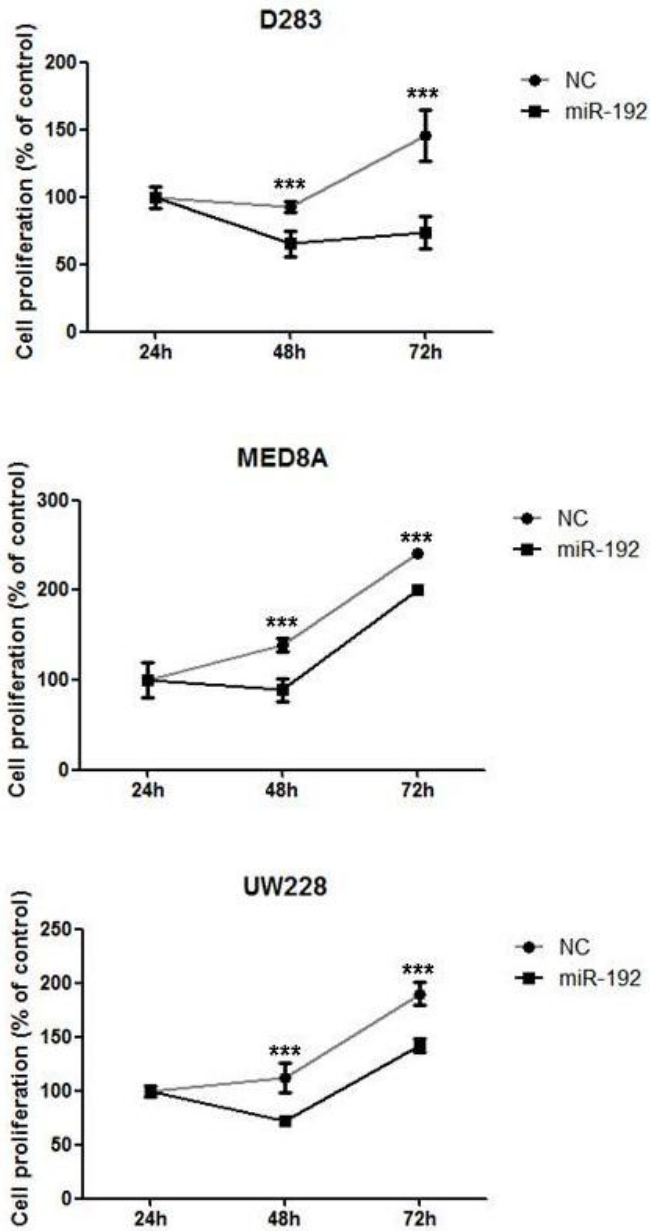

D283
Cell proliferation (% of control)
200
150
100
50
0
24h
48h
72h
***
***
NC
miR-192
MED8A
Cell proliferation (% of control)
300
200
100
0
24h
48h
72h
***
***
NC
miR-192
UW228
Cell proliferation (% of control)
250
200
150
100
50
0
24h
48h
72h
***
***
NC
miR-192

Figure 2C

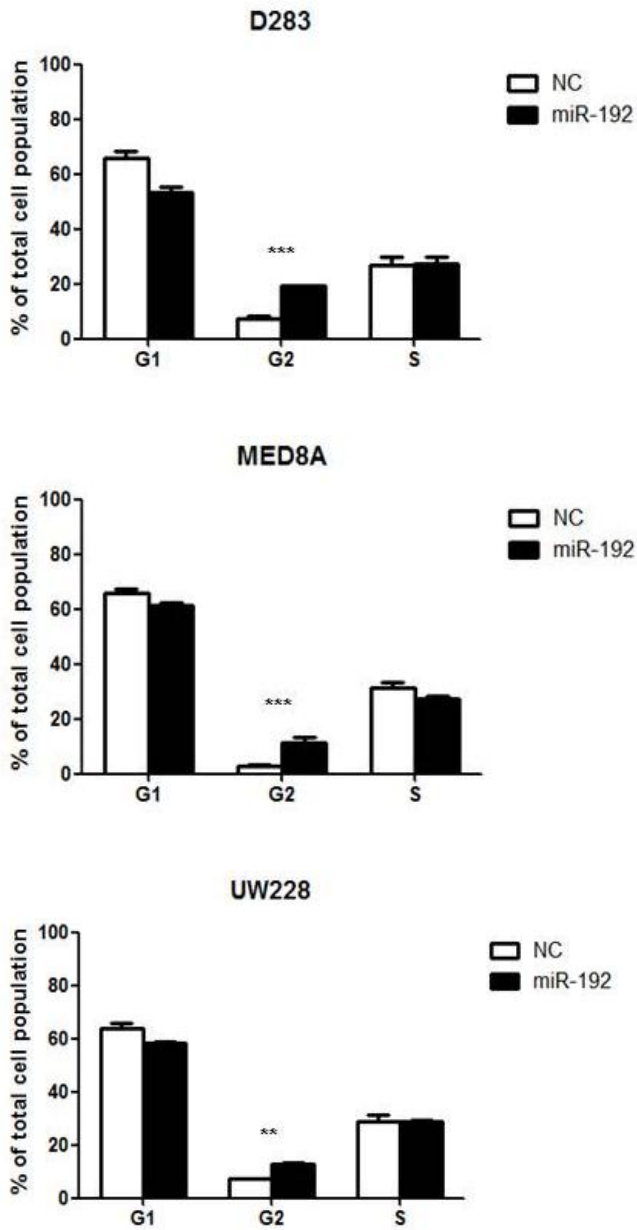

D283
% of total cell population
100
80
60
40
20
0
G1
G2
S
***
NC
miR-192
MED8A
% of total cell population
100
80
60
40
20
0
G1
G2
S
***
NC
miR-192
UW228
% of total cell population
100
80
60
40
20
0
G1
G2
S
**
NC
miR-192

Figure 3. (A), miRNA-192 binding site at the 3'-UTR of DHFR mRNA. (B), analyzing miRNA-192 targets with luciferase reporter shows that DHFR is the direct target of miRNA-192 (normal control (NC) vs. miRNA-192: 1.00 ± 0.30 vs. 0.34 ± 0.34 in D283, $P < 0.05$; 1.00 ± 0.07 vs. 0.37 ± 0.28 in MED8A, $P < 0.05$; 1.00 ± 0.11 vs. 0.51 ± 0.21 in UW228, $P < 0.05$). (C), the expression of DHFR mRNA in normal cerebellum, tumor tissues, and medulloblastoma cells (normal cerebellum vs. non-seeding, 1.00 ± 0.20 vs. 2.75 ± 1.01, $P < 0.05$; normal cerebellum vs. seeding, 1.00 ± 0.20 vs. 4.27 ± 1.43, $P < 0.01$; non-seeding vs. seeding, $P < 0.05$; normal cerebellum vs. D283, 1.00 ± 0.08 vs. 4.65 ± 0.25, $P < 0.001$; normal cerebellum vs. MED8A, 1.00 ± 0.08 vs. 3.84 ± 0.17, $P < 0.001$; normal cerebellum vs. UW228, 1.00 ± 0.08 vs. 3.42 ± 0.085, $P < 0.001$). (D), western blot analysis of the expression of DHFR protein in medulloblastoma cells transfected with miR-192. DHFR protein expression is

significantly decreased by miRNA-192 transfection in all medulloblastoma cells. (E), correlation Plot of DHFR expression against miRNA-192 expression. *P < 0.05; **P < 0.01; ***P < 0.001. Error bars represent ± SD.

Figure 3A

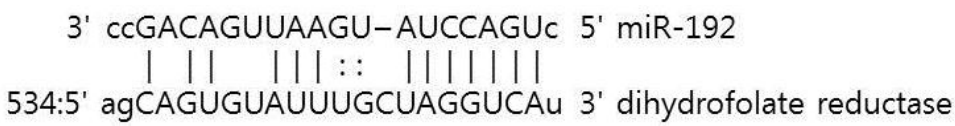


Figure 3B

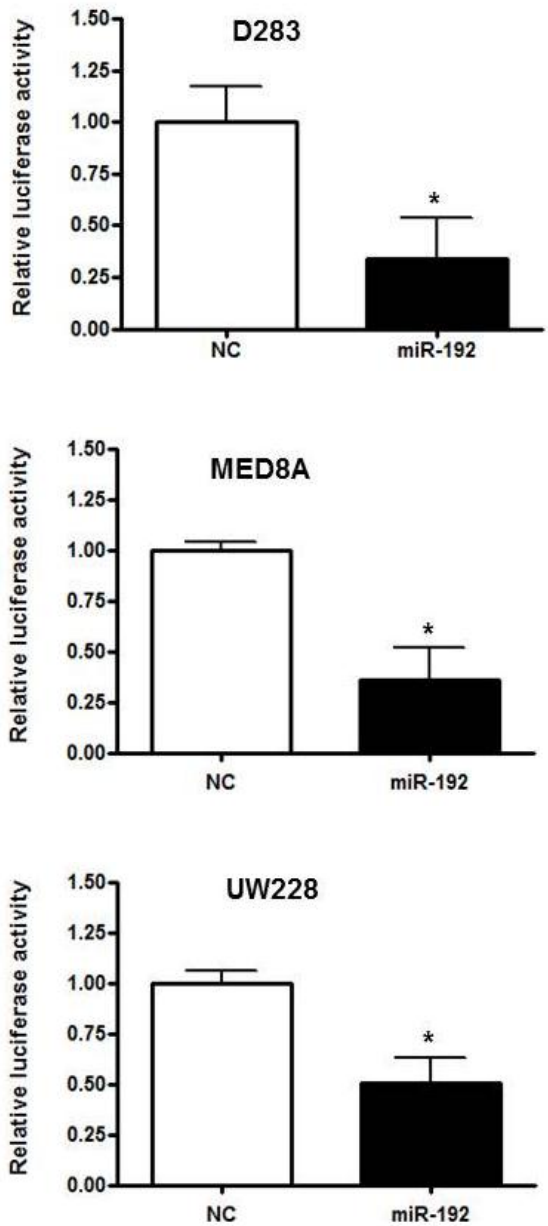

D283
Relative luciferase activity
1.50
1.25
1.00
0.75
0.50
0.25
0.00
*
NC
miR-192
MED8A
Relative luciferase activity
1.50
1.25
1.00
0.75
0.50
0.25
0.00
*
NC
miR-192
UW228
Relative luciferase activity
1.50
1.25
1.00
0.75
0.50
0.25
0.00
*
NC
miR-192

Figure 3C

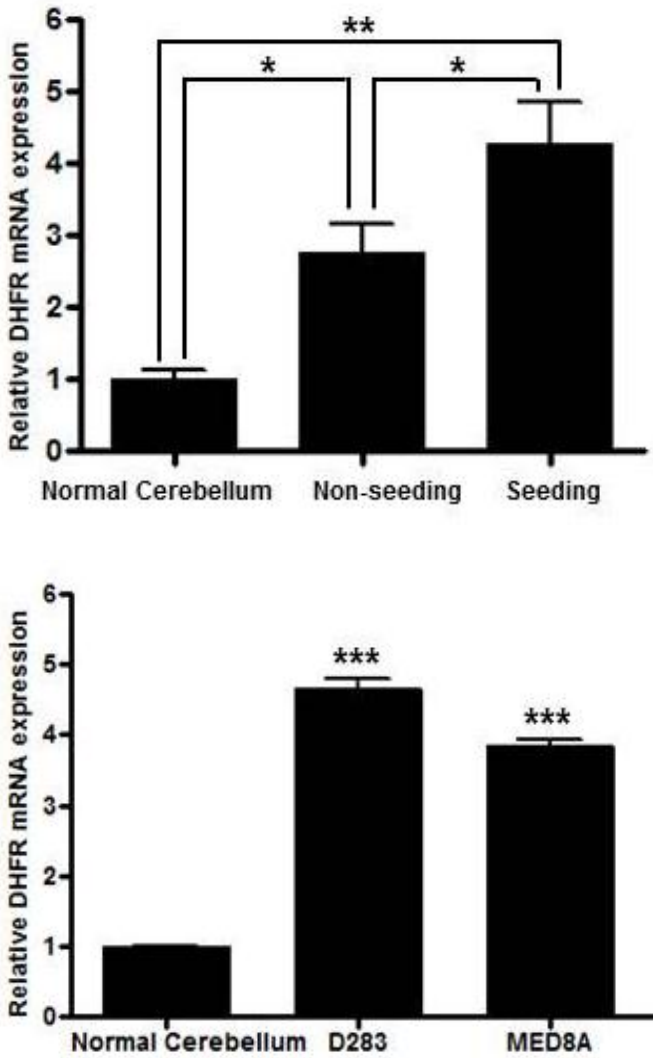

Relative DHFR mRNA expression
0
1
2
3
4
5
6
**
*
*
Normal Cerebellum
Non-seeding
Seeding
Relative DHFR mRNA expression
0
1
2
3
4
5
6
***
***
***
Normal Cerebellum
D283
MED8A
UW228

Figure 3D

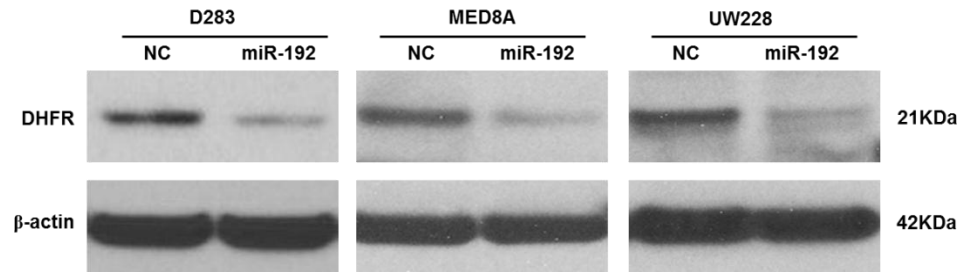


Figure 3E

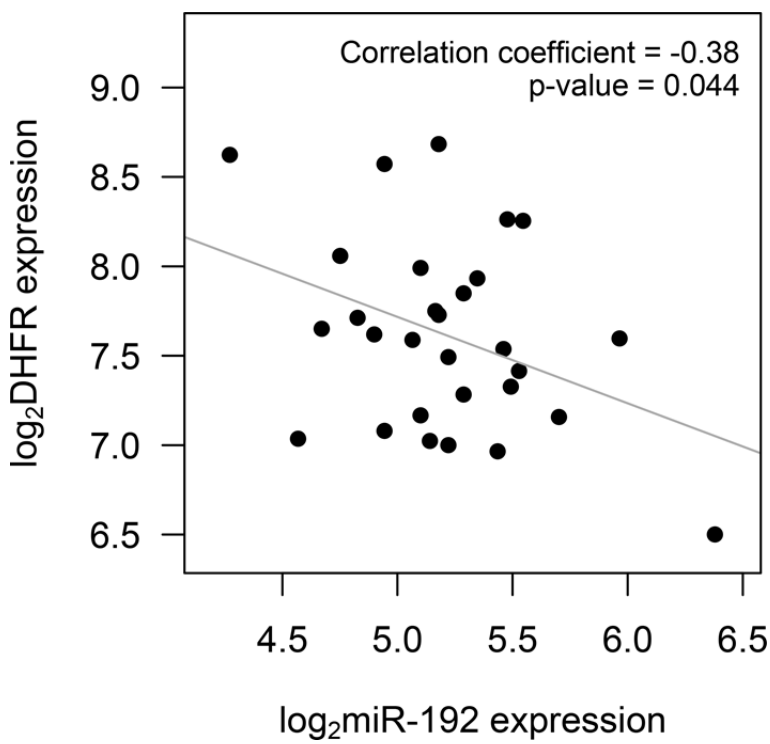


Figure 4. (A) and (B), miRNA-192 does not affect the cell invasion capability of medulloblastoma cell lines compared with normal control (NC) miRNA (NC miRNA vs. miRNA-192: 0.6 ± 0.4 vs. 0.5 ± 0.1 in D283, $P > 0.05$; 0.7 ± 0.1 vs. 0.8 ± 0.1 in MED8A, $P > 0.05$; 0.9 ± 0.4 vs. 0.8 ± 0.1 in UW228, $P > 0.05$).

EMT related mRNA and protein expression after miRNA-192 transfection. (C), the expression of ZEB2 mRNA is not affected by miRNA-192 transfection. When medulloblastoma cells are transfected with miRNA-192, increased E-cadherin mRNA expression and decreased vimentin mRNA expression are observed. (D), ZEB2 and E-cadherin protein are not affected but vimentin is reduced by miRNA-192 transfection in all of the medulloblastoma cells.

Figure 4A

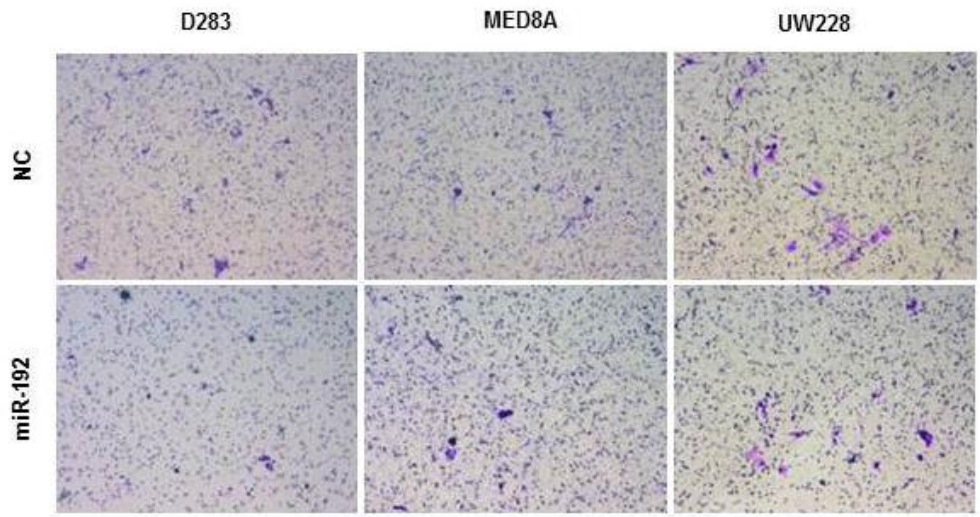


Figure 4B

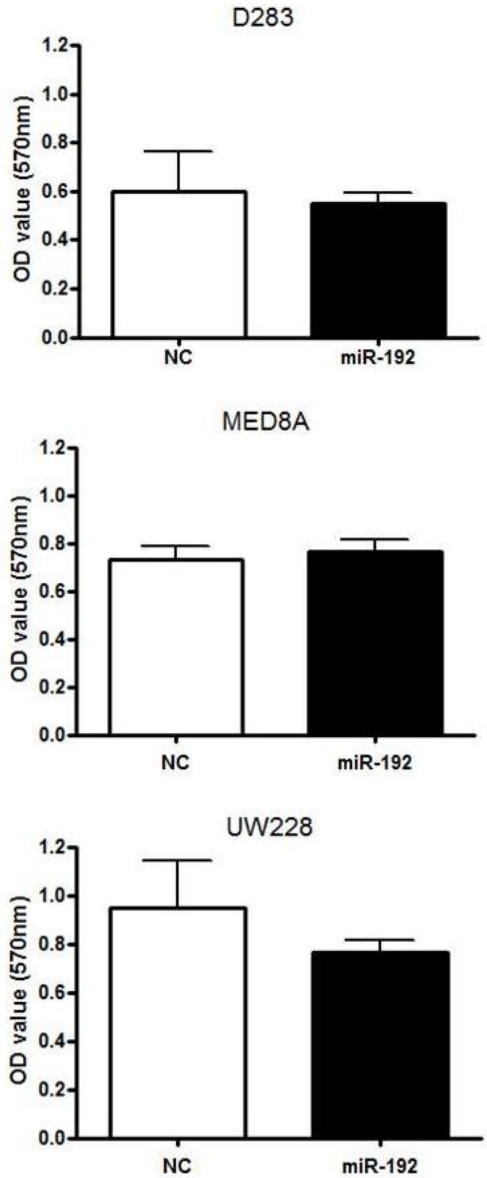

D283
OD value (570nm)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
NC
miR-192
MED8A
OD value (570nm)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
NC
miR-192
UW228
OD value (570nm)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
NC
miR-192

Figure 4C

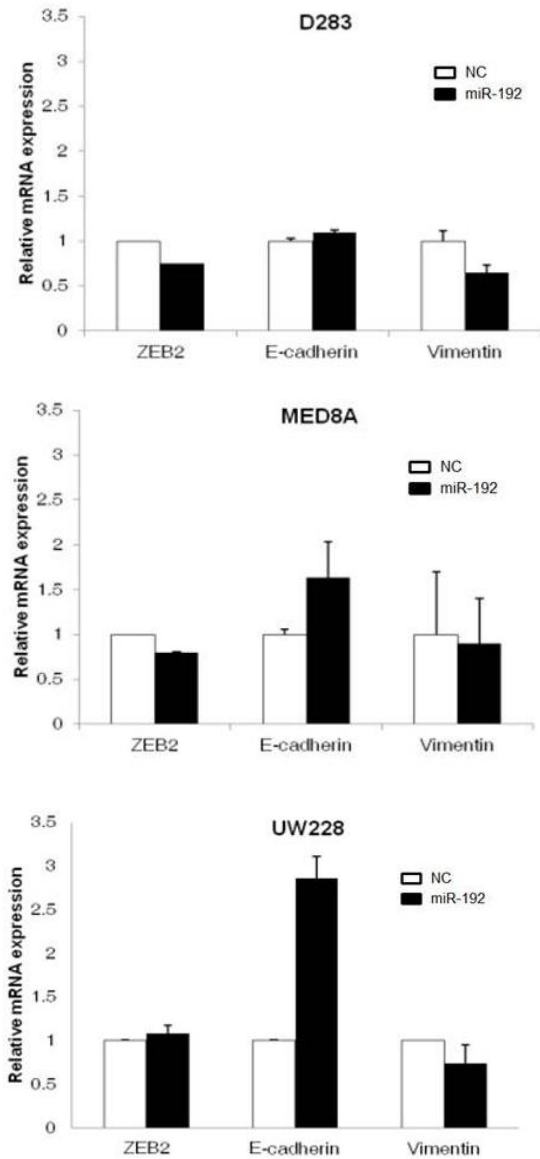

D283
NC
miR-192
Relative mRNA expression
3.5
3
2.5
2
1.5
1
0.5
0
ZEB2
E-cadherin
Vimentin
MED8A
NC
miR-192
Relative mRNA expression
3.5
3
2.5
2
1.5
1
0.5
0
ZEB2
E-cadherin
Vimentin
UW228
NC
miR-192
Relative mRNA expression
3.5
3
2.5
2
1.5
1
0.5
0
ZEB2
E-cadherin
Vimentin

Figure 4D

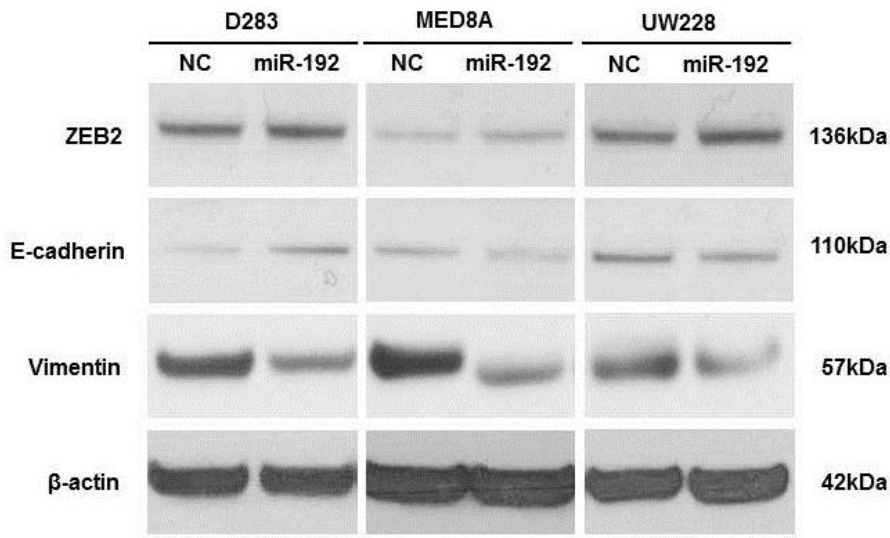

D283
MED8A
UW228
NC
miR-192
NC
miR-192
NC
miR-192
ZEB2
136kDa
E-cadherin
110kDa
Vimentin
57kDa
β-actin
42kDa

Figure 5. Cell adhesion assay. Overexpression of miRNA-192 inhibits the adhesive capability of medulloblastoma cells. Adherent cells were stained (A) and quantified at OD 560nm after extraction (B).

Figure 5A

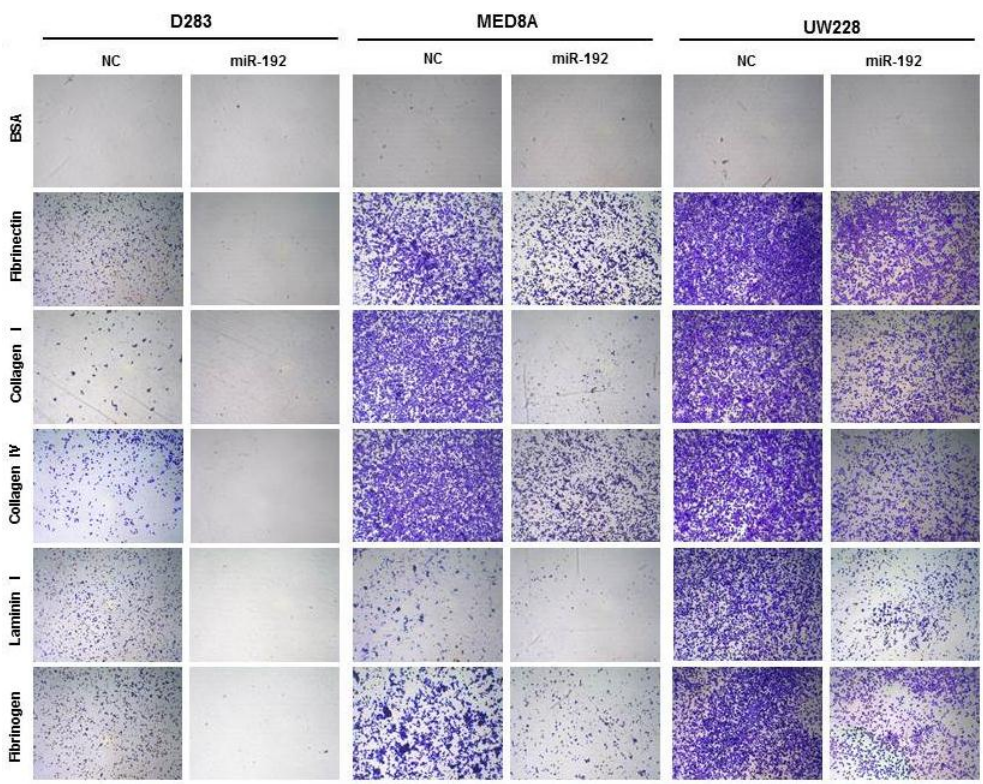


Figure 5B

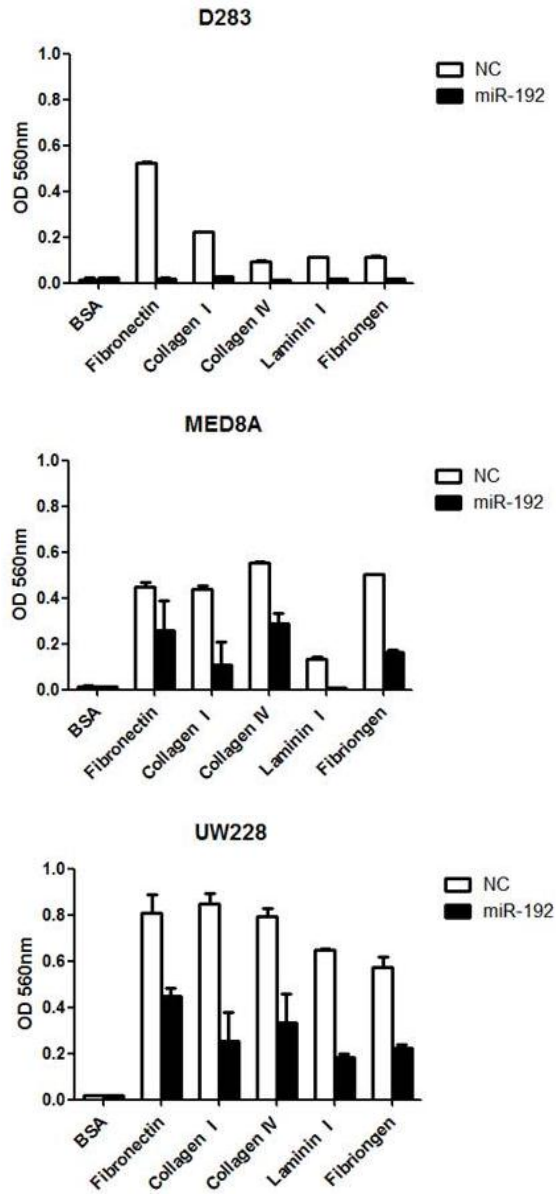

D283
NC
miR-192
OD 560nm
1.0
0.8
0.6
0.4
0.2
0.0
BSA
Fibronectin
Collagen I
Collagen IV
Laminin I
Fibriongen
MED8A
NC
miR-192
OD 560nm
1.0
0.8
0.6
0.4
0.2
0.0
BSA
Fibronectin
Collagen I
Collagen IV
Laminin I
Fibriongen
UW228
NC
miR-192
OD 560nm
1.0
0.8
0.6
0.4
0.2
0.0
BSA
Fibronectin
Collagen I
Collagen IV
Laminin I
Fibriongen

Figure 6. miRNA-192 inhibits the expression of specific α and β integrin subunits as well as CD47 *in vitro*. (A), alignment of miRNA-192 seed sequence with candidate target sequences within the 3′-UTRs of the indicated integrin subunits (αV, β1, β3, and CD47). (B), overexpression of miRNA-192 resulted in significant reduction in luciferase activity for integrin αV (normal control (NC) miRNA vs. miRNA-192: 1.00 ± 0.26 vs. 0.28 ± 0.15 in D283, $P < 0.05$; 1.00 ± 0.32 vs. 0.36 ± 0.08 in MED8A, $P < 0.05$; 1.00 ± 0.15 vs. 0.56 ± 0.15 in UW228, $P < 0.05$). (C) and (D), western blot analysis shows that miRNA-192 transfectants show a significant reduction in the expression of integrin αV, integrin β1, integrin β3, and CD47. Quantitation of western blot bands: integrin αV (NC miRNA vs. miRNA-192 in D283, 100.0 ± 38.6% vs. 51.6 ± 29.3%, $P < 0.01$; NC miRNA vs. miRNA-192: 100.0 ± 53.6 % vs. 53.8 ± 45.2 % in MED8A, $P < 0.05$; 100.1 ± 19.6 % vs. 73.9 ± 20.9 % in UW228, $P < 0.05$), integrin β1 (NC vs. miRNA-192: 100.0 ± 30.8% vs. 60.0 ± 21.2 % in D283, $P < 0.01$; 100.1 ± 6.7% vs. 41.4 ± 4.8 % in MED8A, $P < 0.01$; 100.0 ± 12.1 % vs. 23.2 ± 14.5 % in UW228, $P < 0.001$), integrin β3 (NC miRNA vs. miRNA-192: 100.1 ± 30.7 % vs. 47.9 ± 10.3 % in D283, $P < 0.05$; 100.0 ± 12.3 % vs. 47.6 ± 16.6 % in MED8A, $P < 0.001$; 100.1 ± 18.1 % vs. 34.5 ± 3.5 %

in UW228, $P < 0.01$), and CD47 (NC miRNA vs. miRNA-192: 100.0 ± 5.4 % vs. 64.6 ± 5.9 % in D283, $P < 0.05$; 100.1 ± 10.2 % vs. 64.1 ± 8.1 % in MED8A, $P < 0.01$; 100.0 ± 28.1 % vs. 17.2 ± 18.9 % in UW228, $P < 0.05$). (E), regulation of integrin subunits and related proteins by miRNA-192. The representative immunofluorescence staining shows the localization of integrin αV, integrin β1, integrin β3, CD47, and vimentin in response to miRNA-192 transfection. Scale bar indicates 50μm. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Error bars represent ± SD.

miRNA-192 inhibits the expression of integrin αV, integrin β3, CD47, and vimentin in medulloblastoma. (F), western blot analysis of integrin subunits and CD47 protein expression between seeding and non-seeding group. β-actin was used for normalization. (G), Quantitation of western blot bands. Protein expression of integrin αV ($P < 0.01$), integrin β3 ($P < 0.05$) and CD47 ($P < 0.01$) were significantly lower in the seeding group but integrin β1 ($P = 1.00$) and vimentin ($P = 0.08$) remained the same in the seeding and non-seeding groups.

Figure 6A

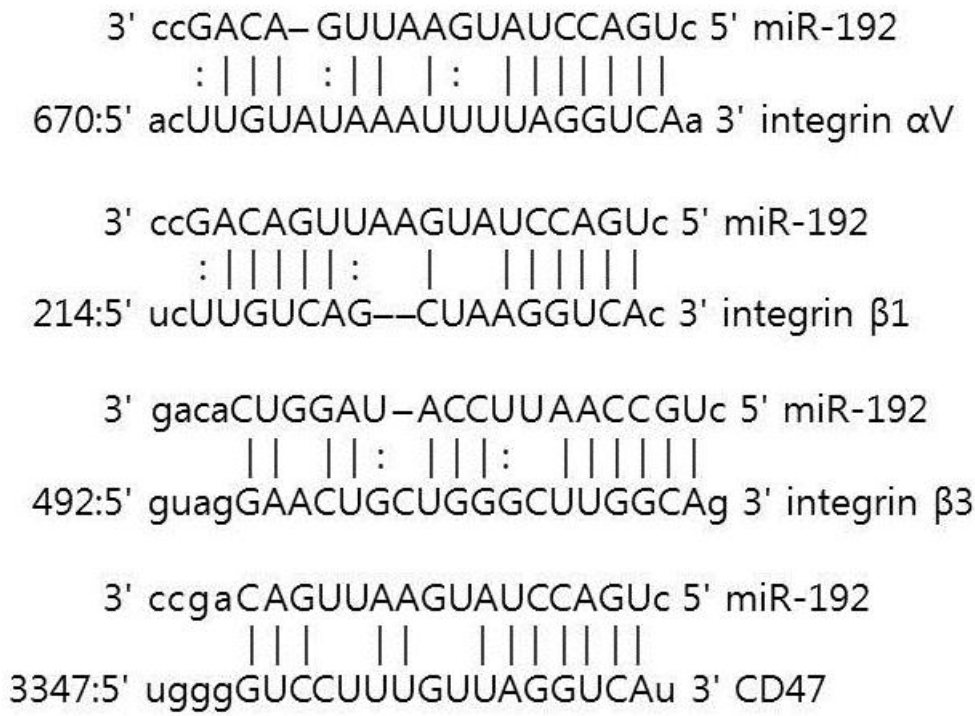

3' ccGACA– GUUAAGUAUCCAGUc 5' miR-192
670:5' acUUGUAUAAAUUUUAGGUCAa 3' integrin αV
3' ccGACAGUUAAGUAUCCAGUc 5' miR-192
214:5' ucUUGUCAG––CUAAGGUCAc 3' integrin β1
3' gacaCUGGAU–ACCUUAACCGUc 5' miR-192
492:5' guagGAACUGCUGGGCUUGGCAg 3' integrin β3
3' ccgaCAGUUAAGUAUCCAGUc 5' miR-192
3347:5' ugggGUCCUUUGUUAGGUCAu 3' CD47

Figure 6B

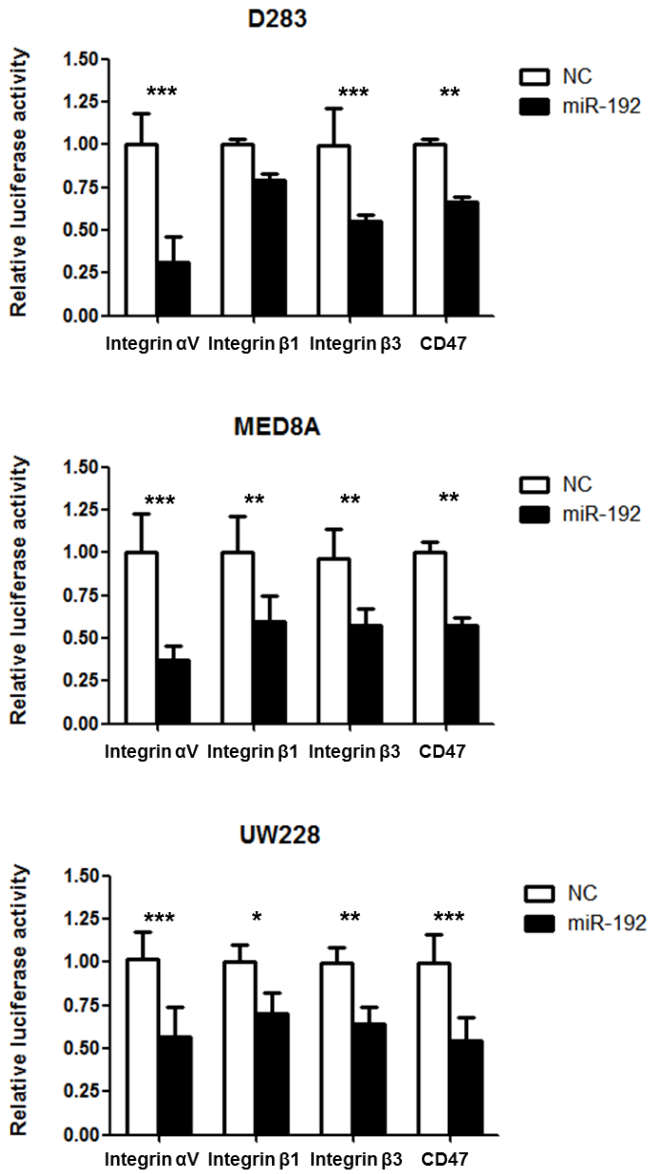

D283
Relative luciferase activity
1.50
1.25
1.00
0.75
0.50
0.25
0.00
***
***
**
NC
miR-192
Integrin αV
Integrin β1
Integrin β3
CD47
MED8A
Relative luciferase activity
***
**
**
**
NC
miR-192
Integrin αV
Integrin β1
Integrin β3
CD47
UW228
Relative luciferase activity
***
*
**
***
NC
miR-192
Integrin αV
Integrin β1
Integrin β3
CD47

Figure 6C

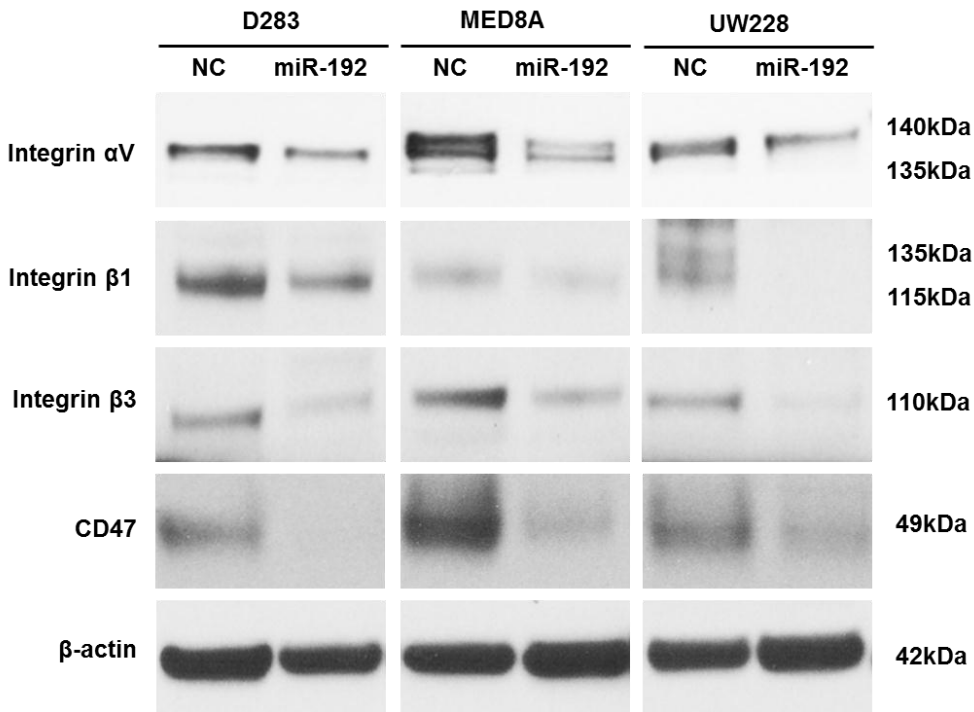

D283
MED8A
UW228
NC
miR-192
NC
miR-192
NC
miR-192
Integrin αV
140kDa
135kDa
Integrin β1
135kDa
115kDa
Integrin β3
110kDa
CD47
49kDa
β-actin
42kDa

Figure 6D

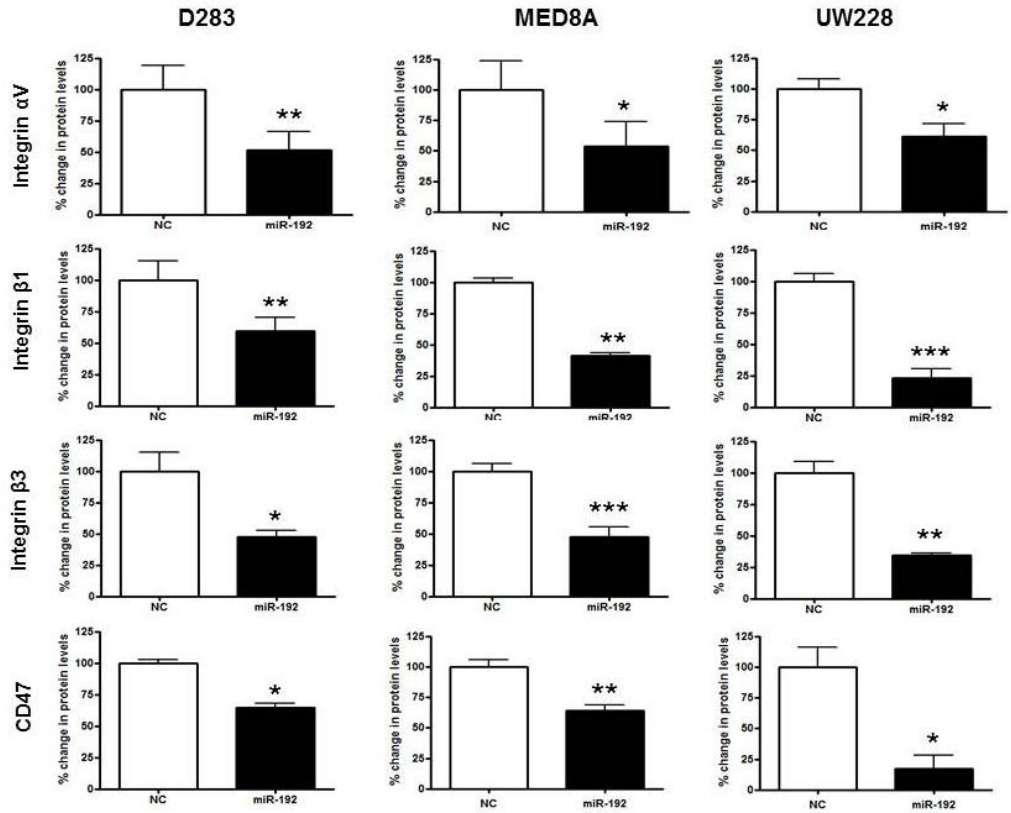

D283
MED8A
UW228
Integrin αV
Integrin β1
Integrin β3
CD47
% change in protein levels
NC
miR-192
*
**
***

Figure 6E

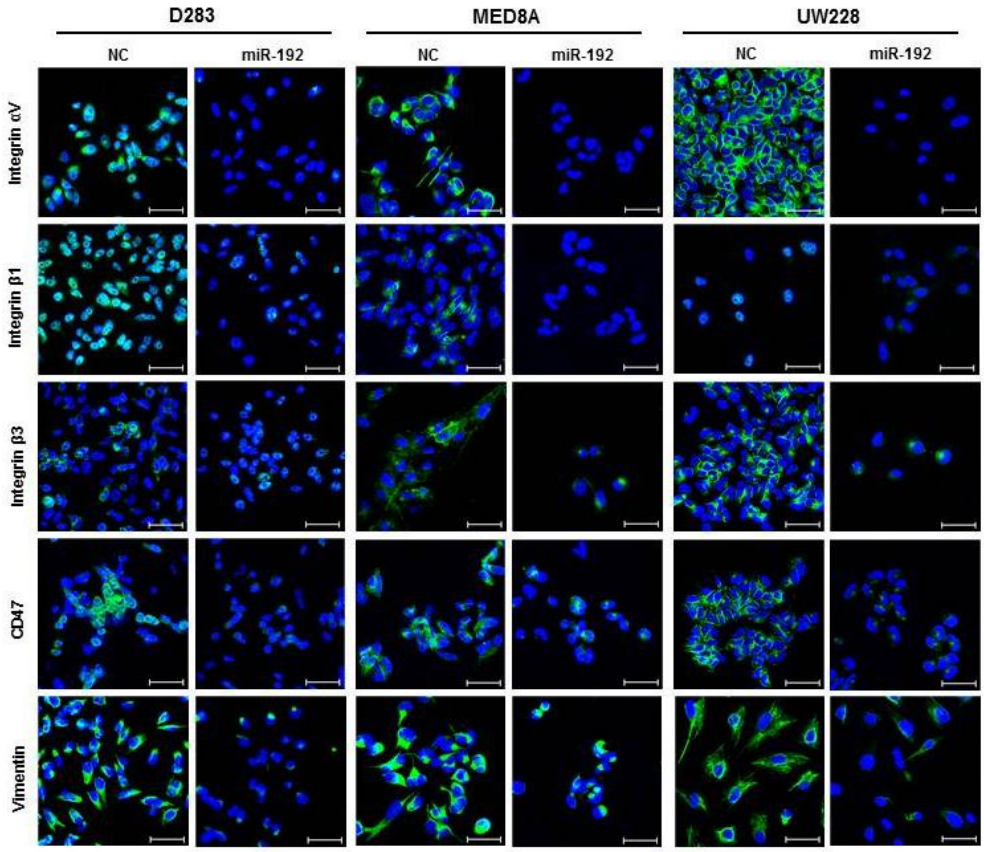

D283
MED8A
UW228
NC
miR-192
NC
miR-192
NC
miR-192
Integrin αV
Integrin β1
Integrin β3
CD47
Vimentin

Figure 6F

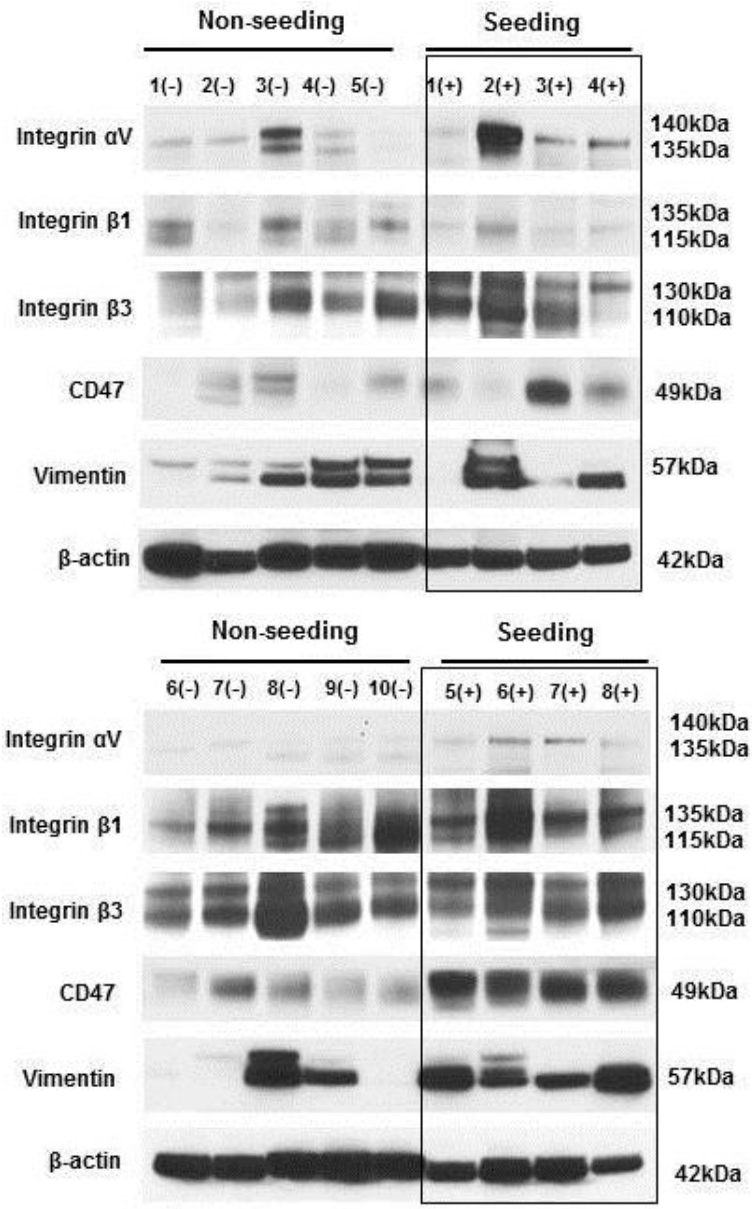
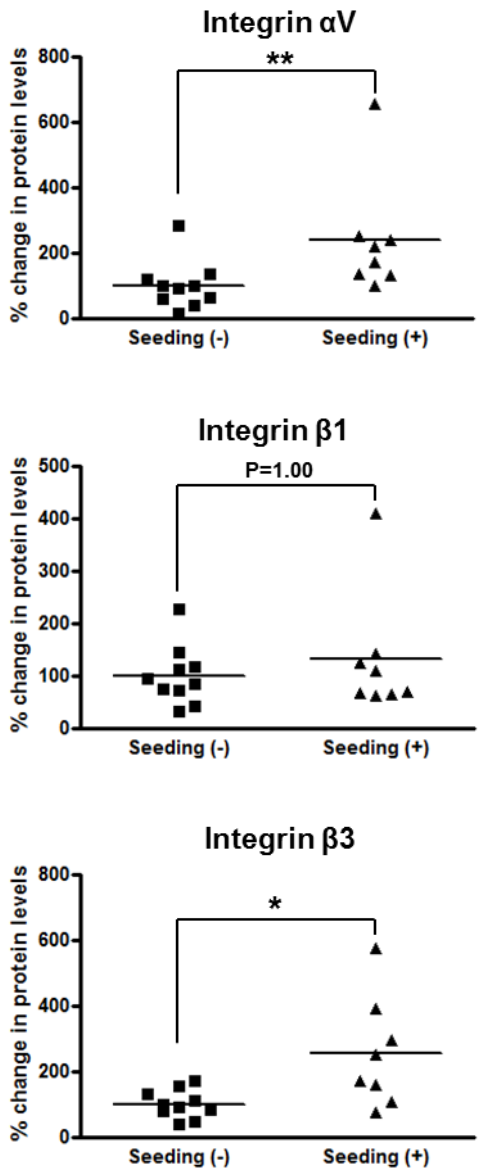
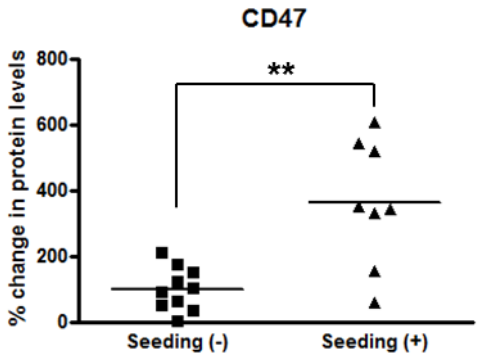
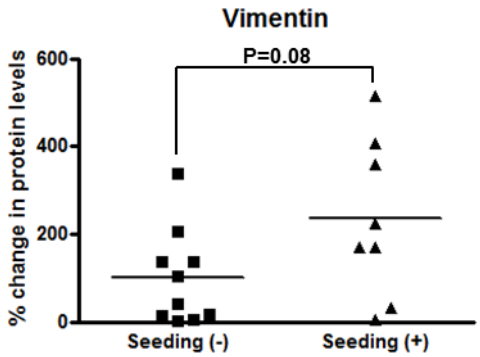

Non-seeding
Seeding
1(-) 2(-) 3(-) 4(-) 5(-)
1(+) 2(+) 3(+) 4(+)
Integrin αV
Integrin β1
Integrin β3
CD47
Vimentin
β-actin
140kDa
135kDa
135kDa
115kDa
130kDa
110kDa
49kDa
57kDa
42kDa
Non-seeding
Seeding
6(-) 7(-) 8(-) 9(-) 10(-)
5(+) 6(+) 7(+) 8(+)
Integrin αV
Integrin β1
Integrin β3
CD47
Vimentin
β-actin
140kDa
135kDa
135kDa
115kDa
130kDa
110kDa
49kDa
57kDa
42kDa

Figure 6G


Integrin αV
**
% change in protein levels
800
600
400
200
0
Seeding (-)
Seeding (+)
Integrin β1
P=1.00
% change in protein levels
500
400
300
200
100
0
Seeding (-)
Seeding (+)
Integrin β3
*
% change in protein levels
800
600
400
200
0
Seeding (-)
Seeding (+)


CD47
**
% change in protein levels
800
600
400
200
0
Seeding (-)
Seeding (+)


Vimentin
P=0.08
% change in protein levels
600
400
200
0
Seeding (-)
Seeding (+)

국문 초록

서론: 수모세포종 환자에서의 주된 사인은 연수막 전이에 의한 재발이다. 비록 수모세포종에 대한 눈부신 분자생물학적 발전이 이루어졌다고는 하지만 전이와 관련된 마이크로 리보핵산의 역할은 거의 알려진 바가 없다. 이 연구의 목적은 수모세포종에 있어서 연수막 전이와 관련된 마이크로 리보핵산을 찾아 그 기전을 알고자 한다.

방법: 우리는 뇌척수액 전이를 보였던 환자들을 포함하여 총 29 명의 환자로부터 채취한 수모세포종 조직에서 마이크로 리보핵산 발현을 분석하였다. 종양 조직과 3 종의 세포주에서 서로 다르게 발현된 마이크로 리보핵산들을 찾은 후 이들 가운데 1 종류에 대하여 생체외 실험을 진행하였다.

결과: 종양 전이를 보였던 환자군과 3 종의 세포주에서 총 12 종류의 다르게 발현된 마이크로 리보핵산들을 찾았으며 이들 가운데 발현이 감소되었던 마이크로 리보핵산-192 를 이용하여 실험을 진행하였다. 마이크로 리보핵산-192 과발현이 다이하이드로 엽산염 환원효소를 표적으로 하여 세포 증식을 억제함을 알 수 있었다. 마이크로 리보핵산-192 는 인테그린 α

V, β1, β3 와 CD47 을 억제하여 종양 세포가 조직에 부착되는 것을 감소시켰다.

결론: 수모세포종 전이에 중요한 역할을 하는 특이 마이크로 리보핵산들을 확인할 수 있었으며 이들 중에서 마이크로 리보핵산-192 는 세포 증식과 종양 세포의 조직 부착을 조절하여 연수막 전이를 억제한다는 것을 알 수 있었다.

주요어: 수모세포종, 마이크로 리보핵산-192, 인테그린, CD47, 다이하이드로 엽산염 환원효소, 전이

학 번: 2009-30526