MicroRNA-206 inhibits the viability and migration of medulloblastoma cells by targeting LIM and SH3 protein 1

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Abstract. MicroRNA (miR)-206 has been found to be deregulated in various types of human cancer, including medulloblastoma. However, the regulatory mechanism of miR-206 in medulloblastoma growth and metastasis remains largely unclear. In the present study, reverse transcription-quantitative polymerase chain reaction data indicated that miR-206 was significantly downregulated in medulloblastoma tissues compared with adjacent non-tumor tissues (P<0.01). Furthermore, low expression of miR-206 was significantly associated with seeding at presentation and anaplastic histology (P<0.01), but not with sex, age, or residual tumors. Overexpression of miR-206 significantly reduced the viability and migration of medulloblastoma D341 cells (P<0.01). LIM and SH3 protein 1 (LASP1) was further identified as a novel target of miR-206 in D341 cells. mRNA levels of LASP1 were significantly higher in medulloblastoma tissues compared to adjacent non-tumor tissues (P<0.01), with an inverse correlation to the miR-206 levels in medulloblastoma tissues. In addition, protein expression levels of LASP1 ere negatively regulated by miR-206 in D341 cells. Further investigation showed that overexpression of LASP1 significantly eliminated the inhibitory effects of miR-206 on the migration and invasion of D341 cells (P<0.01). In conclusion, our study demonstrates that miR-206 has a suppressive role in medulloblastoma cell viability and invasion, partly at least, via the targeting of LASP1. Our study highlights the importance of the miR-206/LASP1 in medulloblastoma.

Introduction

Medulloblastoma is the most common malignant pediatric brain tumor and is one of the leading causes of cancer-related mortality in children (1,2). Although great advances in the diagnosis and treatment of medulloblastoma have been made, the prognosis for patients with advanced medulloblastoma remains poor (3,4). Various oncogenes and tumor suppressors including LIM and SH3 protein 1 (LASP1) have been found to be deregulated in medulloblastoma (5,6). LASP1 was reported to be significantly upregulated in medulloblastoma, and knockdown of LASP1 reduced cell proliferation and migration, and increased cell adhesion in medulloblastoma (6). However, the molecular mechanism underlying the regulation of LASP1 expression in medulloblastoma still remains largely unknown.

MicroRNAs (miRs), which is a type of small non-coding RNAs, is able to negatively regulate gene expression by directly binding to the 3'-untranslational region (UTR) of target mRNA, resulting in either translation inhibition or mRNA degradation (7,8). Through mediation of their target genes, miRs have been found to be involved in various cellular biological processes, including cell proliferation, differentiation, apoptosis, cell cycle progression, migration, and tumorigenesis (9). Deregulations of various miRs have been observed in medulloblastoma, which may contribute to the development and malignant progression of this disease (10,11). For instance, miR-192 suppresses leptomeningeal dissemination of medulloblastoma by inhibiting cell proliferation and anchoring through the repression of DHFR, integrins, and CD47 (12).

miR-206 has been demonstrated to be involved in various physiological and pathological processes. For instance, expression of miR-206 is reduced in breast cancer and may be associated with advanced disease progression and poor patient prognosis (13). miR-206 was also found to control the transition from growth to differentiation in rhabdomyosarcoma cells by integrating multiple components of differentiation pathways (14). In addition, miR-206 is downregulated in ERα-positive endometrioid adenocarcinoma, and has suppressive effects on tumor cell proliferation and invasion (15). Recently, miR-206 was reported to act as a tumor suppressor in medulloblastoma (16). miR-206 levels were significantly downregulated in medulloblastoma tissues and cell lines, and overexpression of miR-206 effectively inhibited the proliferation of medulloblastoma cells, likely via the inhibition of OTX2 protein expression, an oncogenic miR-206 target, which was overexpressed in medulloblastoma (16). As one miR has many targets and may function through different targets in

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different cell types (7), whether other targets of miR-206 exist in medulloblastoma still remains to be studied. The present study aimed to investigate the clinical significance and regulatory mechanism of miR-206 in medulloblastoma.

Materials and methods

Tissue samples. The study protocol was approved by the Ethics Committee of People's Hospital of Binzhou City (Binzhou, China). A total of 63 cases of medulloblastoma tissues, as well as their matched adjacent non-tumor tissues, were collected at the People's Hospital of Binzhou City (Binzhou, China). Written informed consent was obtained from all participants. Clinical characteristics of medulloblastoma patients re summarized in Table I. All tissues were immediately snap-frozen in liquid nitrogen after surgical removal and stored at -80˚C before use.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's instructions. miR-206 expression was detected by qPCR using a mirVana™ RT-PCR microRNA detection kit (Thermo Fisher Scientific, Inc.), in accordance with the manufacturer's instructions. U6 was used as an internal reference (catalogue no. HmiRQP9001; Fulgene, Guangzhou, China). mRNA expression was detected using a SYBR-Green RT-PCR kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. GAPDH was used as an internal reference. Primer sequences were as follows: LASP1 forward, 5'-TGCGGCAAGATCGTGTATCC-3' and reverse, 5'-GCA GTAGGGCTTCTTCTCGTAG-3'; and GAPDH forward, 5'-GGAGCGAGATCCCTCCAAAAT-3' and reverse, 5'-GGC TGTTGTCATACTTCTCATGG-3'. Relative expression levels were determined according to the $2^{-\Delta\Delta Cq}$ method (17).

Cell culture. Human medulloblastoma cell line D341 was purchased from Cell Bank of Chinese Academic Institute, (Shanghai, China). All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Thermo Fisher Scientific, Inc.) in a humidified incubator at 37˚C with 5% CO_2.

Cell transfection. Cell transfection was conducted using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. D341 cells, at a density of $5x10^6$ were transfected with scramble miR mimic (miR-NC), miR-206 mimic, NC inhibitor, miR-206 inhibitor, or co-transfected with LASP1 siRNA and miR-206 inhibitor, or pcDNA3.1-LASP1 plasmid and miR-206 mimic, respectively.

MTT assay. To determine cell viability, D341 cell suspension was plated in a 96-well plate, and cultured at 37˚C in a humidified incubator with 5% CO_2 for 0, 24, 48 or 72 h. Subsequently, MTT (10 μl; 5 mg/ml) was added into each well and incubated at 37˚C in a humidified incubator with 5% CO_2 for 4 h. The supernatant was removed and 100 μl DMSO was added into each well. Optical density (OD) at 570 nm was measured using an ELx800 Absorbance Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA).

Table I. Association between miR-206 expression and clinicopathological characteristics in medulloblastoma.

Variables	N	Low miR-206 (n=34)	High miR-206 (n=31)	P-value
Sex				0.622
Male	36	20	16	
Female	29	14	15	
Age at diagnosis				0.779
>3 years	48	26	22	
<3 years	17	8	9	
Residual tumor				0.430
>1.5 cm^2	7	5	2	
<1.5 cm^2	58	29	29	
Seeding at presentation				0.045[a]
Yes	28	19	9	
No	37	15	22	
Anaplastic histology				0.007[b]
Yes	11	10	1	
No	54	24	30	

[a]P<0.05; [b]P<0.01. miR, microRNA.

Wound healing assay. D341 cells, at a density of $1x10^5$ were cultured to confluence. Wounds of 1-mm width were created with a plastic scriber, and cells were washed and incubated in serum-free DMEM. After wounding for 24 h, the medium was replaced with DMEM supplemented with 10% FBS. Cultures at 0 and 48 h were fixed and observed under a light microscope.

Bioinformatics analysis and dual luciferase reporter assay. TargetScan 7.0 software (targetscan.org) was used to predict the putative target genes of miR-206. Wild-type (WT) or mutant type (WT) 3'-UTR of LASP1 were constructed and inserted into the multiple clone sites (MCS) in the psiCHECK2 luciferase reporter vector (Promega Corp., Madison, WA, USA), respectively. For luciferase reporter assay, D341 cells, at a density of $5x10^6$ were co-transfected with WT-LASP1-3'UTR or MT-LASP1-3'UTR reporter plasmids with miR-206 mimic or miR-NC, respectively, using Lipofectamine 2000. Luciferase activities were determined after transfection for 48 h using a dual-luciferase reporter assay system (Promega Corp.). Renilla luciferase activity was normalized to firefly luciferase activity.

Western blot analysis. Cells were lysed in cold radioimmunoprecipitation lysis buffer. Protein concentration was examined using a BCA Protein Assay kit (Thermo Fisher Scientific, Inc.). A total of 50 μg of protein was separated by 10% SDS-PAGE, transferred to a polyvinylidene difluoride membrane (Thermo Fisher Scientific, Inc.), and incubated in 5% nonfat dried milk in PBS (Thermo Fisher Scientific, Inc.) at 4˚C overnight. Following this, the membrane was incubated with monoclonal rabbit LASP1 primary antibody (1:50; ab156872; Abcam,

Cambridge, MA, USA) and polyclonal rabbit GAPDH primary antibody (1:100; ab9485; Abcam) at room temperature for 3 h. Following washing with PBS three times, the membrane was incubated with HRP-conjugated goat anti-rabbit secondary antibody (1:10,000; ab6271; Abcam) for 40 min at room temperature. The membrane was washed again with PBS three times and an enhanced chemiluminescence western blotting kit (Thermo Fisher Scientific, Inc.) was used to detect the immune complexes. Image-Pro Plus software (version 6.0; Media Cybernetics, Inc., Rockville, MD, USA) was used to analyze the relative protein expression levels, as represented as the density ratio vs. GAPDH.

Statistical analysis. Data were expressed as mean ± standard deviation. SPSS 17.0 (SPSS, Inc., Chicago, IL, USA) was used to conduct statistical analysis. Comparison of quantitative data was analyzed using an unpaired t-test. Comparison of qualitative data was analyzed by the Chi-square test. Correlation was determined by Pearson correlation analysis. P<0.05 was considered to indicate a statistically significant difference.

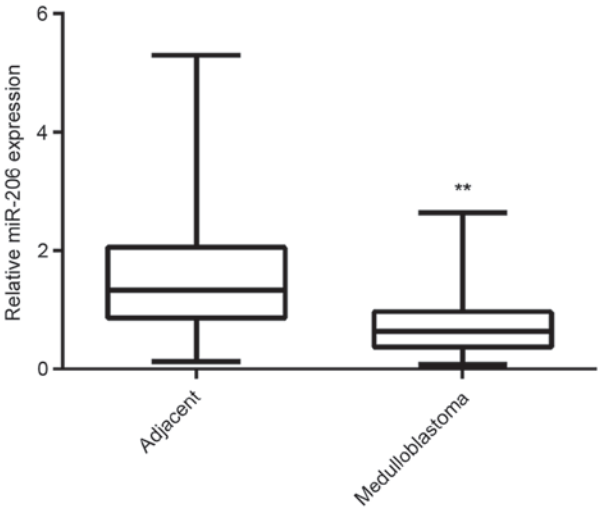

Figure 1. Reverse transcription-quantitative polymerase chain reaction was conducted to examine the miR-206 levels in a total of 65 cases of medulloblastoma tissues compared to adjacent non-tumor tissues. **P<0.01 vs. adjacent.

Results

miR-206 is downregulated in medulloblastoma. RT-qPCR was conducted to determine the miR-206 levels in 63 paired primary medulloblastoma tissues and adjacent non-tumor tissues. Our data showed that miR-206 levels were significantly reduced in medulloblastoma tissues when compared with adjacent non-tumor tissues (P<0.01; Fig. 1).

Clinical significance of miR-206 expression in medulloblastoma was further investigated. According to the mean expression of miR-206 in medulloblastoma tissues, we divided these patients into high miR-206 expression and low miR-206 expression subgroups. As indicated in Table I, low expression of miR-206 was significantly associated with seeding at presentation and anaplastic histology (P<0.05 and P<0.01, respectively), but not with sex, age, or residual tumor. These findings suggest that the reduced expression of miR-206 may contribute to the malignant progression of medulloblastoma.

miR-206 has suppressive effects on the viability and migration of medulloblastoma cells. To further elucidate the exact role of miR-206 in medulloblastoma, miR-206 mimic was to transfect medulloblastoma D341 cells. Transfection with miR-NC was used as a control. As shown in Fig. 2A, miR-206 levels were significantly higher in the miR-206 group compared with the miR-NC group (P<0.01). MTT assay and wound healing assay were conducted to examine cell viability and migration. As indicated in Fig. 2B and C, overexpression of miR-206 caused a significant decrease in D341 cell viability and invasion, when compared to the miR-NC group, respectively (P<0.01). According to these data, we suggest that miR-206 may have suppressive effects on medulloblastoma growth and metastasis.

LASP1 is a target gene of miR-206 in D341 cells. Potential target genes of miR-206 were further investigated using bioinformatics analysis. TargetScan software predicated that LASP1 was a putative target of miR-206 (Fig. 3A). To clarify this association, MT or WT 3'-UTRs of LASP1 was constructed and inserted into the MCS in the psiCHECK2 luciferase reporter vector, respectively (Fig. 3B and C). Luciferase reporter assay data showed that the luciferase activity was significantly reduced in D341 cells co-transfected with miR-206 mimic and WT-LASP1-3'UTR reporter plasmid, but remained unchanged in the other groups, when compared to the control group (P<0.01; Fig. 3D). These data indicate that LASP1 is a direct target of miR-206 in medulloblastoma cells.

LASP1 is upregulated in medulloblastoma and inversely correlated to miR-206 expression. To further clarify the relationship between miR-206 and LASP1 in medulloblastoma, we further examined the expression of LASP1 in medulloblastoma tissues and adjacent tissues. RT-qPCR data indicated that the mRNA levels of LASP1 were significantly increased in medulloblastoma tissues, when compared with those in adjacent non-tumor tissues (P<0.01; Fig. 4A). We observed an inverse correlation between the LASP1 and miR-206 levels in medulloblastoma tissues (r=0.532; Fig. 4B). These findings suggest that the upregulation of LASP1 may be due to the downregulation of miR-206 in medulloblastoma.

Protein expression of LASP1 is negatively regulated by miR-206 in D341 cells. As miRs generally inhibit the expression of their target genes at the post-transcriptional level, we examined the protein expression of LASP1 using western blotting. LASP1 protein expression levels were markedly reduced in miR-206-overexpressing D341 cells compared with the control group (Fig. 5A). To further confirm these findings, D341 cells were transfected with miR-206 inhibitor and or NC inhibitor as the control. Following transfection, miR-206 levels were significantly reduced in the miR-206 inhibitor group when compared with the NC inhibitor group (P<0.01; Fig. 5B). Further investigation showed that knockdown of miR-206 induced a significant increase in protein levels of LASP1 (Fig. 5C). Accordingly, miR-206 negatively regulates the protein expression of LASP1 in D341 cells.

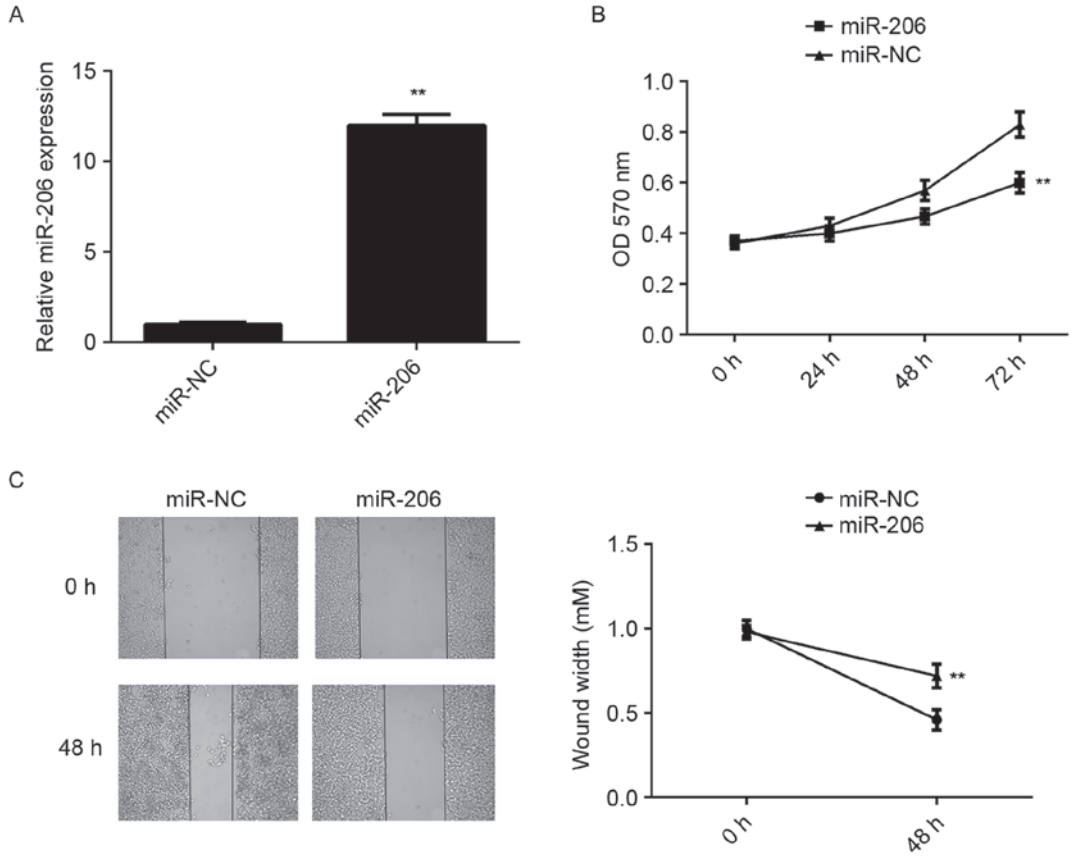

Figure 2. CD341 cells were transfected with miR-206 mimic or scramble miR mimic (miR-NC) as the control group. (A) Reverse transcription-quantitative polymerase chain reaction was conducted to examine the miR-206 expression. (B) MTT assay and (C) wound healing assay were used to examine the cell proliferation and migration. **P<0.01 vs. miR-NC.

A

Conserved

	Predicted consequential pairing of target region (top) and miRNA (bottom)	Site type	Context++ score	Context++ score percentile	Weighted context++ score	Conserved branch length	P_{CT}
Position 353-360 of LASP1 3' UTR	5' ...UCCUCACAUACCCACACAUUCCA...	8 mer	-0.24	93	-0.24	3.657	0.66
hsa-miR-206	3' GGUGUGUGAAGGAAUGUAAGGU						

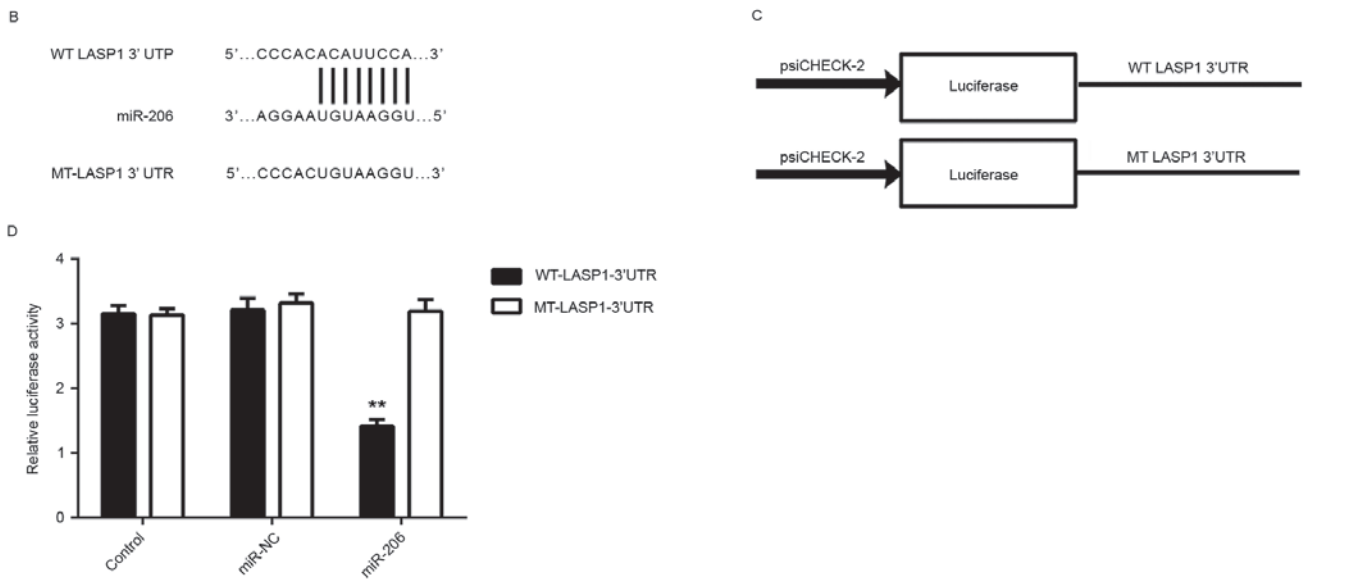

Figure 3. (A) TargetScan software indicated that LASP1 was a target gene of miR-206. (B and C) Luciferase reporter vectors containing WT or MT of LASP1 3'-UTR were generated. (D) Luciferase activity was significantly reduced in CD341 cells co-transfected with the WT-LASP1-3'UTR luciferase reporter vector and miR-206 mimics, when compared to the control group. However, these effects were eliminated by transfection with MT-LASP1-3'UTR luciferase reporter vector. **P<0.01 vs. control. LASP1, LIM and SH3 protein 1; WT, wild-type; MT, mutant; UTR, untranslated region.

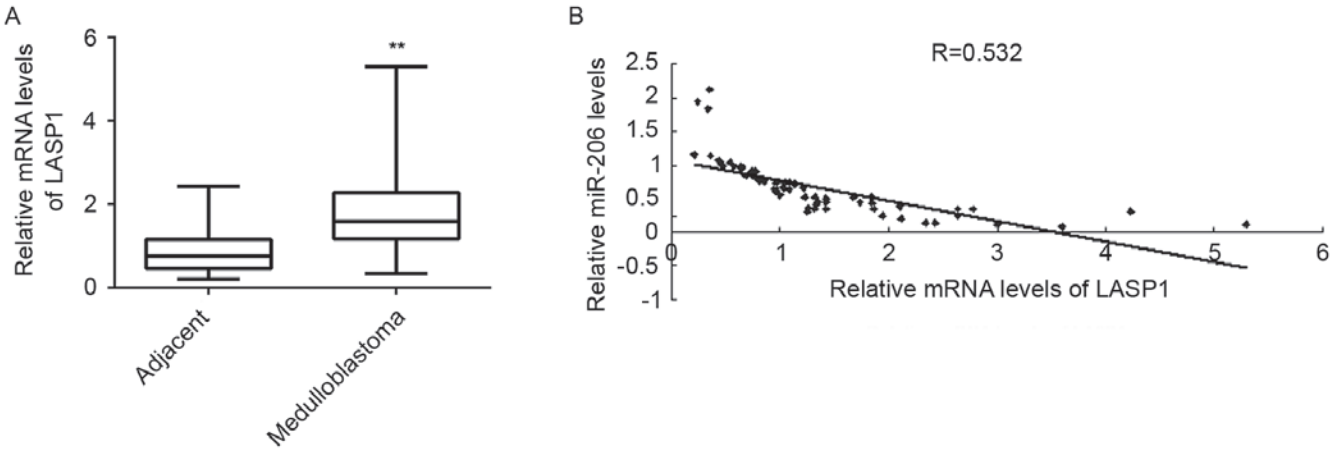

Figure 4. (A) Reverse transcription-quantitative polymerase chain reaction was used to examine the mRNA levels of LASP1 in a total of 65 cases of medulloblastoma tissues compared to adjacent non-tumor tissues. **P<0.01 vs. adjacent. (B) An inverse correlation was observed between the miR-206 and LASP1 mRNA levels in medulloblastoma tissues (r=0.532). LASP1, LIM and SH3 protein 1.

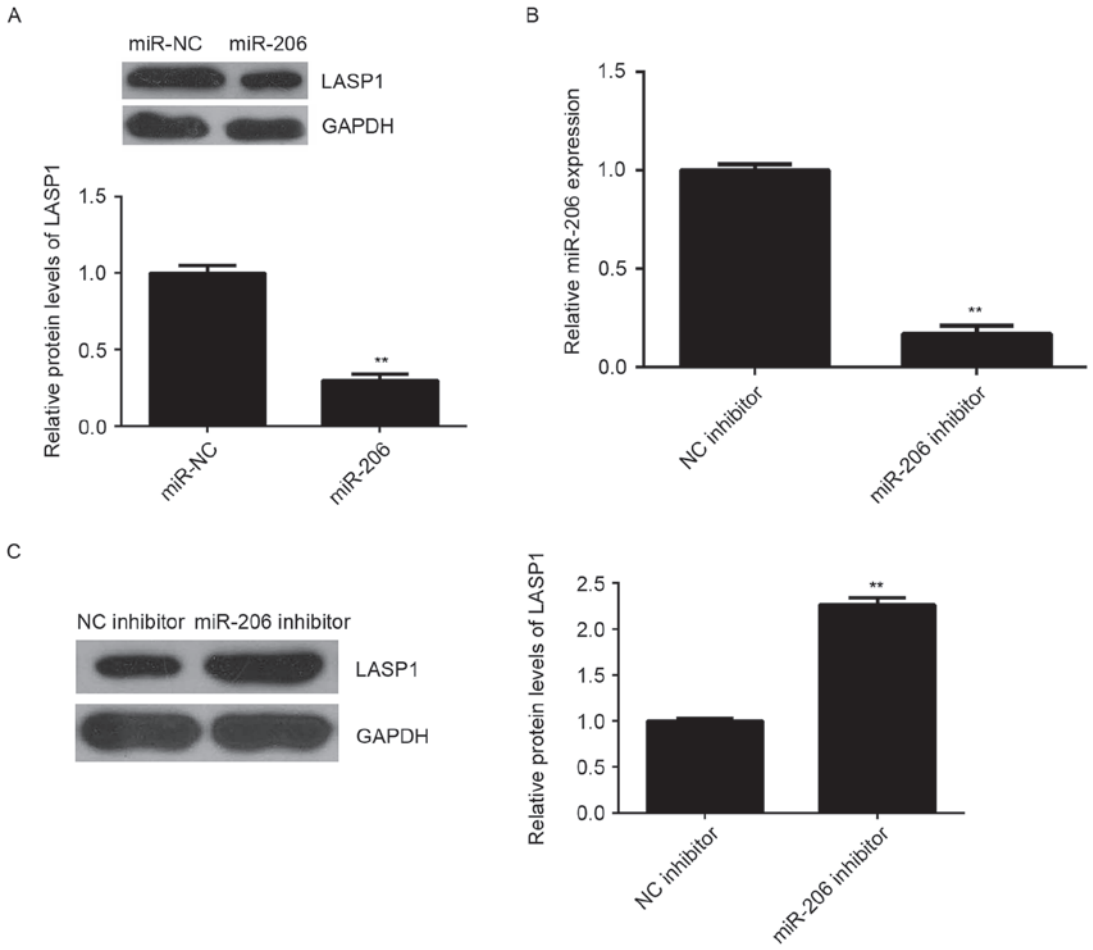

Figure 5. (A) Western blot analysis was used to examine the protein expression of LASP1 in CD341 cells transfected with miR-206 mimic or scramble miR mimic (miR-NC). **P<0.01 vs. miR-NC. (B) Reverse transcription-quantitative polymerase chain reaction was used to examine the miR-206 levels in CD341 cells transfected with miR-206 inhibitor or NC. (C) Western blot was used to examine the protein expression of LASP1. **P<0.01 vs. NC inhibitor. LASP1, LIM and SH3 protein 1; NC, negative control.

miR-206 inhibits medulloblastoma cell migration via targeting LASP1. We investigated whether LASP1 acted as a downstream effector in the miR-206-mediated viability and migration of medulloblastoma cells. miR-206-overexpressing D341 cells were transfected with pcDNA3.1-LASP1 expression plasmid. As shown in Fig. 6A, protein expression levels of LASP1 were significantly increased in the miR-206 + LASP1 group, when compared with the miR-206 group (P<0.01). MTT assay and wound healing assay data further indicated that the viability and migration of D341 cells were significantly increased in the

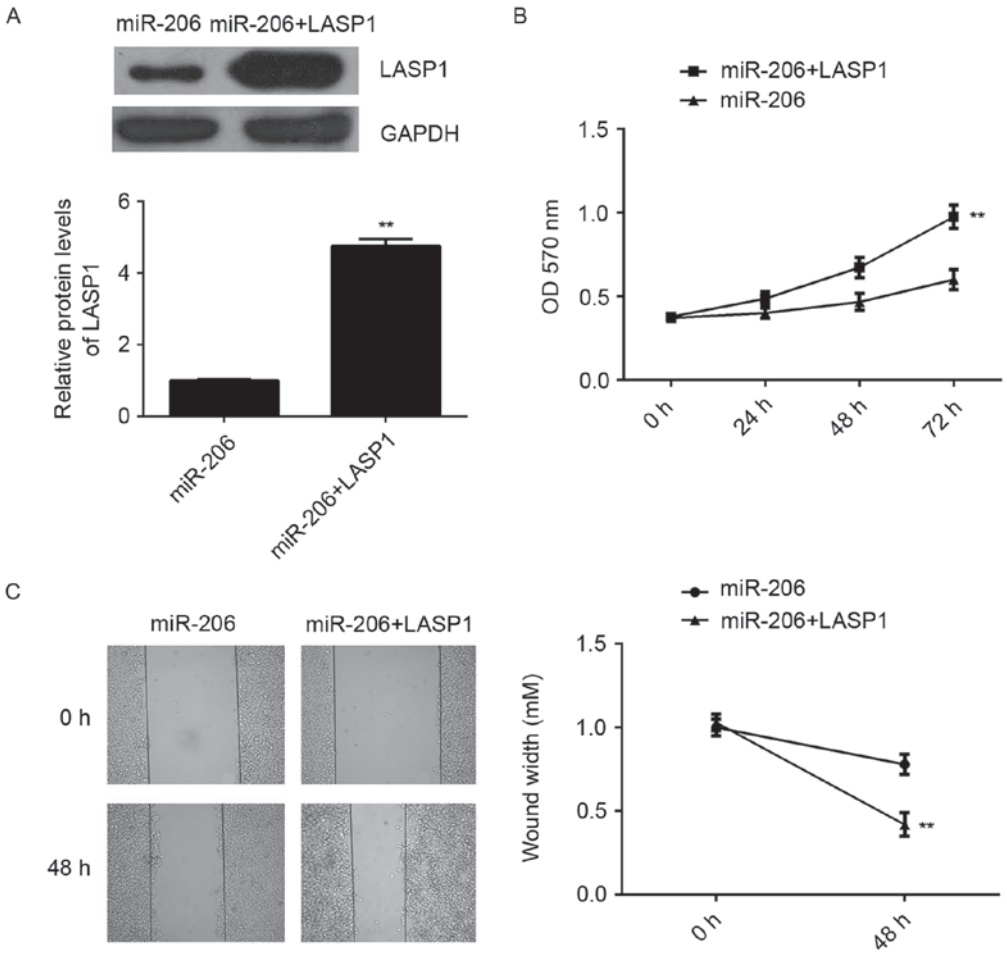

Figure 6. CD341 cells were transfected with miR-206 mimic, or co-transfected with miR-206 mimic and LASP1 expression plasmid, respectively. (A) Western blot analysis was conducted to examine the protein expression levels of LASP1. (B) MTT assay and (C) wound healing assay were used to examine cell viability and migration, respectively. **P<0.01 vs. miR-206. LASP1, LIM and SH3 protein 1.

miR-206 + LASP1 group compared with the miR-206 group (P<0.05), indicating that overexpression of LASP1 attenuated the inhibitory effects of miR-206 on D341 cells viability and migration (Fig. 6B and C). Taken together, our findings suggest that miR-206 inhibits the viability and migration of medulloblastoma cells, partly at least, via directly targeting LASP1.

Discussion

miR-206 has been demonstrated to have a tumor suppressive role in medulloblastoma (16). However, the underlying mechanism of miR-206 in medulloblastoma growth and metastasis remains unclear. The present study demonstrated that miR-206 was significantly downregulated in medulloblastoma tissues, as compared with adjacent non-tumor tissues, and low expression of miR-206 was significantly associated with seeding at presentation and anaplastic histology, but not with sex, age, or residual tumor. Overexpression of miR-206 significantly reduced the viability and migration of medulloblastoma D341 cells. LASP1, which was upregulated in medulloblastoma, was further identified as a target gene of miR-206, and its protein expression was negatively regulated by miR-206 in D341 cells. Further investigation indicated that overexpression of LASP1 significantly eliminated the inhibitory effects of miR-206 on the migration and invasion of D341 cells.

Many miRs have been found to be involved in medulloblastoma development and malignant progression, acting as oncogenes or tumor suppressors. For instance, microRNA-10b was reported to be significantly upregulated in medulloblastoma tissues, and to have a promoting role in tumor cell proliferation and survival (18). miR-367 was found to promote proliferation, invasion, and stem-like traits in medulloblastoma cells (19). On the contrary, miR-148a was able to inhibit the invasion and tumorigenic potential of medulloblastoma cells by targeting Neuropilin 1 (20). miR-31 was found to suppress the proliferation of medulloblastoma cells by inhibiting DNA replication via minichromosome maintenance 2 (21). Lucon *et al* (22) performed microarray analysis and found 64 miRs were downregulated, whereas 20 miRs were upregulated in medulloblastoma. The majority of downregulated miRs, including miR-206, were at the 14q32 locus, suggesting that this miR locus is regulated as a module in medulloblastoma. However, no previous study has focused on the clinical significance of miR-206 expression in this disease. In this study, we found that miR-206 was significantly downregulated in medulloblastoma tissues compared to adjacent non-tumor tissues. Furthermore, we observed that low expression of miR-206 was significantly associated with seeding at presentation and anaplastic histology, but not with sex, age, or residual tumor. These data suggest that the decreased expression of miR-206 may contribute to medulloblastoma progression. We

further conducted *in vitro* experiments and found that miR-206 had suppressive effects on the viability and migration of medulloblastoma D341 cells. Panwalkar *et al* (16) also reported that overexpression of miR-206 could inhibit the proliferation of medulloblastoma cells.

LASP1, which is a LIM protein and a member of the nebulin family of actin-binding proteins, is a cAMP and cGMP dependent signaling protein that binds to the actin cytoskeleton (23). LASP1 has been demonstrated to have an oncogenic role in different cancer types, including colorectal cancer, prostate cancer, breast cancer, gastric cancer, and esophageal squamous cell carcinoma (24-28). Traenka *et al* (6) reported that LASP1 may have a role in medulloblastoma as LASP1 was upregulated in medulloblastoma (6); this is consistent with our findings. Furthermore, high protein levels of LASP1 were found to be strongly correlated with metastatic dissemination and poor prognosis of patients with medulloblastoma. This previous study also showed that knockdown of LASP1 inhibited cell proliferation and migration, and increased cell adhesion in medulloblastoma (6). However, the molecular mechanism underlying LASP1 expression in medulloblastoma has never been studied, to our knowledge. In the present study, LASP1 was identified as a novel target gene of miR-206 using bioinformatic analysis and luciferase reporter assay, and the protein expression of LASP1 was demonstrated to be negatively regulated by miR-206 in medulloblastoma cells. According to our findings and the previous studies outlined, we hypothesize that LASP1 may act as a downstream effector in the miR-206-mediated viability and migration of medulloblastoma cells. To clarify this speculation, miR-206-overexpressing D341 cells were transfected with pcDNA3.1-LASP1 expression plasmid to upregulate the expression levels of LASP1, and it was demonstrated that LASP1 overexpression significantly eliminated the suppressive effects of miR-206 on the viability and migration of D341 cells. These findings suggest that miR-206 inhibits the viability and migration of medulloblastoma cells by directly targeting LASP1.

In conclusion, to the best of our knowledge, the present study is the first to demonstrate the regulatory mechanism of the miR-206/LASP1 axis in medulloblastoma growth and metastasis, suggesting that it may become a potential therapeutic target for medulloblastoma. Future investigations should aim to further reveal the regulatory role of the miR-206/LASP1 axis in medulloblastoma *in vivo*.

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