Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;48:947-958
DOI: 10.1159/000491963
Published online: July 23, 2018
Accepted: April 10, 2018







**Original Paper**

# Kaiso (ZBTB33) Downregulation by Mirna-181a Inhibits Cell Proliferation, Invasion, and the Epithelial–Mesenchymal Transition in Glioma Cells

Ligang Wang[a] Jichao Ma[b] Xiaoxiong Wang[a] Fei Peng[a] Xin Chen[a]
Bingjie Zheng[a] Chunlei Wang[a] Zhibo Dai[a] Jing Ai[b] Shiguang Zhao[a]

[a]Department of Neurosurgery, First Affiliated Hospital, Harbin Medical University, Harbin, [b]Department of Pharmacology, The State-Province Key Laboratories of Biomedicine-Pharmaceutics of China, College of Pharmacy of Harbin Medical University, Harbin, China

**Key Words**
Glioma • MiRNA-181a • Kaiso • Proliferation • Invasion • EMT

**Abstract**
***Background/Aims:*** Kaiso (ZBTB33) expression is closely associated with the progression of many cancers and microRNA (miRNA) processing. MiR-181a plays critical roles in multiple cancers; however, its precise mechanisms in glioma have not been well clarified. The goal of this study was to evaluate the interaction between Kaiso and miR-181a in glioma. ***Methods:*** Quantitative real-time PCR (qRT-PCR) was performed to detect the levels of Kaiso and miR-181a in glioma tissues and cell lines. Cell proliferation, invasion, and the epithelial–mesenchymal transition (EMT) were evaluated to analyze the biological functions of miR-181a and Kaiso in glioma cells. The mRNA and protein levels of Kaiso were measured by qRT-PCR and western blotting, respectively. Meanwhile, luciferase assays were performed to validate Kaiso as a miR-181a target in glioma cells. ***Results:*** We found that the level of miR-181a was the lowest among miR-181a–d in glioma tissues and cell lines, and the low level of miR-181a was closely associated with the increased expression of Kaiso in glioma tissues. Moreover, transfection of miR-181a significantly inhibited the proliferation, invasion, and EMT of glioma cells, whereas knockdown of miR-181a had the opposite effect. Bioinformatics analysis predicted that Kaiso was a potential target gene of miR-181a, and the luciferase reporter assay demonstrated that miR-181a could directly target Kaiso. In addition, Kaiso silencing had similar effects as miR-181a overexpression in glioma cells, whereas overexpression of Kaiso in glioma cells partially reversed the inhibitory effects of the miR-181a mimic. ***Conclusionss:*** miR-181a inhibited the proliferation, invasion, and EMT of glioma cells by directly targeting and downregulating Kaiso expression.



Shiguang Zhao
and Jing Ai

Department of Neurosurgery, First Affiliated Hospital, Harbin Medical University
No. 23 Youzheng Street, Nangang District, Harbin, Heilongjiang 150001 (China)
Tel: 86 451-85552799, E-Mail zhaoshiguangharbin@yahoo.com, azhrbmu@126.com

## Introduction

The most common cerebral neoplasms are gliomas, which comprise about 80% of primary malignant tumors of the central nervous system [1]. Based on histopathological analysis, diffuse gliomas are divided into three subtypes: astrocytic, oligodendroglial, and oligoastrocytic tumors, and are graded according to World Health Organization (WHO) classification as low-grade astrocytomas (grade II), anaplastic astrocytomas (grade III), and glioblastoma (GBM, grade IV) [2, 3]. Despite advancements in chemotherapy and radiotherapy, the prognosis of patients with glioma is still poor, with only a 12-month median survival after aggressive multimodal therapy [4]. Thus, there is an urgent need to identify novel approaches for the diagnosis, prognostic evaluation, and treatment of glioma by understanding the precise mechanisms underlying this disease.

Kaiso (also called ZBTB33) is a member of the BTB/POZ family, and its target genes have been linked to tumor onset, invasion, and metastasis [5–7]. The clinical importance of Kaiso has attracted an increasing amount of attention, and several studies have reported that it is involved in many cancers, including colorectal cancer, prostate cancer, pancreatic ductal adenocarcinoma, non-small cell lung cancer, and triple-negative breast cancer [8–12], with poor prognostic features and advanced disease status. However, the relationship between Kaiso and microRNA (miRNA) in tumor progression remains unknown.

Many studies have reported that miRNAs regulate one-third of human genes [13], and dysregulated miRNA profiles modulate tumor phenotypes via suppression of their target genes [14, 15]. Therefore, dysregulated miRNAs might induce the downregulation of Kaiso in glioma by binding to the 3′ untranslated region (3′UTR) of target genes. The miR-181 family constitutes four members, namely, miR-181a, miR-181b, miR-181c, and miR-181d. It has been shown that miR-181 family members inhibit the proliferation, invasion, migration, and epithelial–mesenchymal transition (EMT) of glioma by targeting several genes [16–20]. However, which of these four miRNAs is most important in glioma remains unclear.

In this study, we found that miR-181a had the lowest expression of these four miRNAs. However, the precise mechanism of miR-181a on the proliferation, invasion, and EMT of glioma cells has yet to be clarified. We also confirmed the significant downregulation of miR-181a in glioma tissues and cells. Overexpression of miR-181a inhibited the proliferation, invasion, and EMT of glioma cells. Moreover, Kaiso was found to be the direct target of miR-181a in glioma. Restoration of Kaiso reversed the inhibitory effects of miR-181a. These results demonstrate the critical roles of miR-181a in the pathogenesis of glioma, thereby suggesting its possible application in cancer treatment.

## Materials and Methods

*Human tissue samples*

A total of 10 normal brain tissues (NBTs), 20 low-grade glioma (LGGs; WHO grade II) and 35 high-grade glioma (HGGs; WHO grades III and IV) samples were collected at the First Affiliated Hospital of Harbin Medical University (Heilongjiang, China) between 2013 and 2017. Both NBTs and glioma specimens were histologically confirmed. All samples were immediately frozen in liquid nitrogen for subsequent quantitative real-time polymerase chain reaction (qRT-PCR) analysis. The Research Ethics Committee of Harbin Medical University approved our study. Written informed consent was obtained from each patient before participation.

*Cell culture*

Human glioma cell lines, namely, U87, T98, LN229, H4, U118, U251, and A172, were obtained from the Chinese Academy of Sciences Cell Bank (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, GIBCO, New York, NY, USA), 1% penicillin, and 1% streptomycin (GIBCO). Normal human astrocytes (NHAs) were obtained from Lonza (Basel,

Switzerland) and cultured in the provided astrocyte growth media supplemented with recombinant human epidermal growth factor, insulin, ascorbic acid, GA-1000, L-glutamine (GIBCO), and 5% FBS. All cells were cultured at 37°C in a humidified atmosphere of 5% on 0.1% gelatin-coated culture flasks.

*Transient transfection*

MiR-181a mimic, miR-181a inhibitor (2′-*O*-methyl-modified antisense oligonucleotides specifically targeting mature miR-181a), negative control (NC), small interfering RNA (siRNA) against Kaiso (si-Kaiso), and si-NC were synthesized and purified by GenePharma (Shanghai, China). The Kaiso overexpression plasmid was generated by inserting Kaiso cDNA into the pcDNA3.1 vector, and sequencing was confirmed by GenePharma. The miR-181a mimic (50 nM), miR-181a inhibitor (100 nM), si-Kaiso (100 nM), and Kaiso overexpression plasmid (100 nM) were transfected using Lipofectamine 3000 Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocols. Total RNA and protein were collected 48 h after transfection.

*RNA isolation and qRT-PCR*

Total RNA was isolated using TRIzol according to the manufacturer's instructions, and analysis of mRNA levels was performed on the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with SYBR Green-Based real-time PCR for all genes. For miRNA quantification, cDNA extraction was performed using a miRNA Extraction Kit (Tiangen, Beijing, China) and TaqMan miRNA assays (Life Technologies, San Francisco, CA, USA), and reverse-transcription real-time PCR was performed for miRNA quantifications according to the manufacturer's instructions. The primer sequences used for Kaiso, proliferating cell nuclear antigen (PCNA), p21, p27, cyclin-dependent kinase 2 (CDK2), CDK4, cyclin D1, cyclin E1, matrix metalloproteinase 2 (MMP-2), MMP-9, N-cadherin, E-cadherin, vimentin, Twist, Slug, zinc finger E-box-binding homeobox 1 (ZEB1), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) detection are shown in Table 1.

*Cell proliferation assay*

To study the effect of miR-181a on the proliferation of glioma cells, U251 cells were seeded in a 96-well plate and allowed to grow overnight in complete DMEM medium. Then, the medium was removed, and the cells were transfected with miR-181a mimic or inhibitor for 48 h. The Cell Proliferation ELISA-BrdU (colorimetric) Kit (Roche Diagnostics, Indianapolis, IN, USA) was used to detect cell proliferation according to the manufacturer's protocols [21].

*In vitro invasion assay*

According to a previous study [22], the Transwell invasion assay was conducted by adding 100 μL Matrigel (BD Bioscience, Franklin Lakes, NJ, USA) to the upper chamber of the Transwell and by placing cells onto the Matrigel. Next, non-invasive cells above the surface of the membrane were removed. The invasive

**Table 1.** Sequence of primers for qRT-PCR

| Gene | Primer Sequence |
|---|---|
| Kaiso | F: 5′-TGCTGAACTCCTTGAATGAGC-3′ |
| | R: 5′-CGGAATTTTCGGTCTTCCACAA-3′ |
| PCNA | F: 5′-CCTGCTGGGATATTAGCTCCA-3′ |
| | R: 5′-CAGCGGTAGGTGTCGAAGC-3′ |
| CDK2 | F: 5′-TGTTTAACGACTTTGGACCGC-3′ |
| | R: 5′-CCATCTCCTCTATGACTGACAGC-3′ |
| CDK4 | F: 5′-GGGGACCTAGAGCAACTTACT-3′ |
| | R: 5′-CAGCGCAGTCCTTCCAAAT-3′ |
| cyclin D1 | F: 5′-GCTGCGAAGTGGAAACCATC-3′ |
| | R: 5′-CCTCCTTCTGCACACATTTGAA-3′ |
| cyclin E1 | F: 5′-AAGGAGCGGGACACCATGA-3′ |
| | R: 5′-ACGGTCACGTTTGCCTTCC-3′ |
| p21 | F: 5′-TGTCCGTCAGAACCCATGC-3′ |
| | R: 5′-AAAGTCGAAGTTCCATCGCTC-3′ |
| p27 | F: 5′-AACGTGCGAGTGTCTAACGG-3′ |
| | R: 5′-CCCTCTAGGGGTTTGTGATTCT-3′ |
| E-cadherin | F: 5′-TACACTGCCCAGGAGCCAGA-3′ |
| | R: 5′-TGGCACCAGTGTCCGGATTA-3′ |
| N-cadherin | F: 5′- TCAGGCGTCTGTAGAGGCTT-3′ |
| | R: 5′- ATGCACATCCTTCGATAAGACTG-3′ |
| Vimentin | F: 5′-GACGCCATCAACACCGAGTT-3′ |
| | R: 5′-CTTTGTCGTTGGTTAGCTGGT-3′ |
| Twist | F: 5′- GTCCGCAGTCTTACGAGGAG-3′ |
| | R: 5′- GCTTGAGGGTCTGAATCTTGCT-3′ |
| Slug | F: 5′-CGAACTGGACACACATACAGTG-3′ |
| | R: 5′-CTGAGGATCTCTGGTTGTGGT-3′ |
| ZEB1 | F: 5′-GATGATGAATGCGAGTCAGATGC-3′ |
| | F: 5′-ACAGCAGTGTCTTGTTGTTGT-3′ |
| U6 | F: 5′-CTCGCTTCGGCAGCACA-3′ |
| | F: 5′-AACGCTTCACGAATTTGCGT-3′ |
| GAPDH | F: 5′-GAGTCAACGGATTTGGTCGTATTG-3′ |
| | R: 5′-CCTGGAAGATGGTGATGGGATT-3′ |

cells were fixed in methanol for 15 min, and then stained with 0.1% crystal violet for 20 min. Cells were imaged from at least five grids per field. Then the membranes were rinsed with 30% glacial acetic acid. Finally, the wash solution was examined at 540 nm to count the number of glioma cells. All assays were independently repeated three times.

*Protein extraction and western blot analysis*

The proteins used for western blotting were lysed with RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) containing a protease inhibitor cocktail (Roche, Basel, Switzerland). The proteins were quantified using the BCA Protein Assay Kit (Beyotime Biotechnology). The western blot system was established using the Bio-Rad Bis-Tris Gel system according to the manufacturer's instructions. Primary antibodies against Kaiso (ab124777), MMP-2 (ab37150), MMP-9 (ab76003), Twist (ab175430), Slug (ab106077; Abcam, Cambridge, UK) and ZEB1 (#3396), E-cadherin (#5296), N-cadherin (#3195), vimentin (#3390; Cell Signaling Technology Inc., Danvers, MA, USA) were prepared in 5% blocking buffer at a dilution of 1:1000. Primary antibodies were incubated with the membrane at 4°C overnight, followed by washing and incubation with secondary antibody (1:5000; Abcam, Boston, MA, USA) marked by horseradish peroxidase for 1 h at room temperature. After rinsing, the polyvinylidene difluoride membrane was transferred to the Bio-Rad ChemiDoc™ XRS system, and then 200 μL Immobilon Western Chemiluminescent HRP Substrate (Millipore, Burlington, MA, USA) was added to cover the membrane surface. The signals were captured and the intensity of the bands was quantified using Image Lab™ Software (Bio-Rad, Shanghai, China).

*Measurement of MMP-2 and MMP-9 levels by enzyme-linked immunosorbent assay*

According to a previous study [23], the supernatants of U251 cells were collected after treatment, and the concentrations of MMP-2 and MMP-9 were measured using a sandwich enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions. Briefly, primary antibody was coated onto ELISA plates and incubated for 2 h at room temperature. Samples and standards were added to the wells and incubated for 1 h. Then, the wells were washed and biotinylated antibody was added for 1 h. The plates were washed again, and streptavidin conjugated to horseradish peroxidase was added for 10 min. After washing, tetramethylbenzidine was added for color development and the reaction was terminated with 1 M sulfuric acid. Absorbance was measured at 490 nm. Values were expressed as ng/mL.

*Luciferase reporter assay*

The 3′UTR target site was generated by PCR and luciferase reporter constructs with the Kaiso 3′UTR harboring a putative miR-181a-binding site in the pGL3 (Promega, Fitchburg, WI, USA) luciferase report vector were amplified by PCR. Cells were co-transfected with the reporter construct, control vector, and miR-181a mimic or corresponding controls using Lipofectamine 3000 (Invitrogen). After transfection for 48 h, firefly and renilla luciferase activities were measured using the dual-luciferase assay system (Promega) according to the manufacturer's instructions. All experiments were performed in triplicate.

*Statistical analysis*

The data are expressed as the mean ± standard error of the mean. The number of independent experiments is represented by "n". The relationship between miR-181a and clinicopathological characteristics was evaluated by the chi-square test. Correlations between miR-181a and Kaiso mRNA levels were analyzed using Pearson's correlation coefficient. Multiple comparisons were performed using one-way analysis of variance followed by Tukey's multiple-comparison test. Other comparisons were analyzed using two-tailed Student's *t*-test. P values less than 0.05 were considered statistically significant.

## Results

*High expression of Kaiso correlates with low miR-181a expression in glioma specimens*

Kaiso expression is closely associated with many kinds of cancers [5–12]. In this study, we detected the mRNA and protein levels of Kaiso in glioma tissues for the first time. Our results showed that the mRNA level of Kaiso was significantly increased in glioma tissues compared with that in adjacent tissues (Fig. 1A). Furthermore, we also observed that the

expression of Kaiso was more enhanced in HGGs than in LGGs (Fig. 1A). To further study Kaiso expression regulated by miRNA, we detected the levels of miR-181a–d in glioma tissues, as predicted by the TargetScan 6.2 online database. We found that the levels of miR-181a–d were dramatically reduced in glioma tissues compared with adjacent tissues (Fig. 1B), which was consistent with previous studies [16–20]. Furthermore, the level of miR-181a was the lowest among the four miRNAs in the miR-181 family (Fig. 1B). Meanwhile, our results revealed that miR-181a expression was significantly downregulated in glioma tissues, especially in HGG (Fig. 1C). To determine whether the expression of Kaiso was associated with miR-181a in glioma, Pearson's correlation analysis revealed a significant negative correlation between Kaiso and miR-181a expression in HGG (Fig. 1D). To investigate the functional roles of Kaiso and miR-181a in glioma, several glioma cell lines were evaluated. Subsequently, we determined the levels of Kaiso and miR-181a in several glioma cell lines, namely, U87, T98, LN229, H4, U118, U251, A172, and NHAs. Compared with NHAs, the level of Kaiso in U251 cells was higher than that in other glioma cell lines (Fig. 1E), and the level of miR-181a was lowest in U251 cells (Fig. 1F). Therefore, U251 cells were used for subsequent experiments.

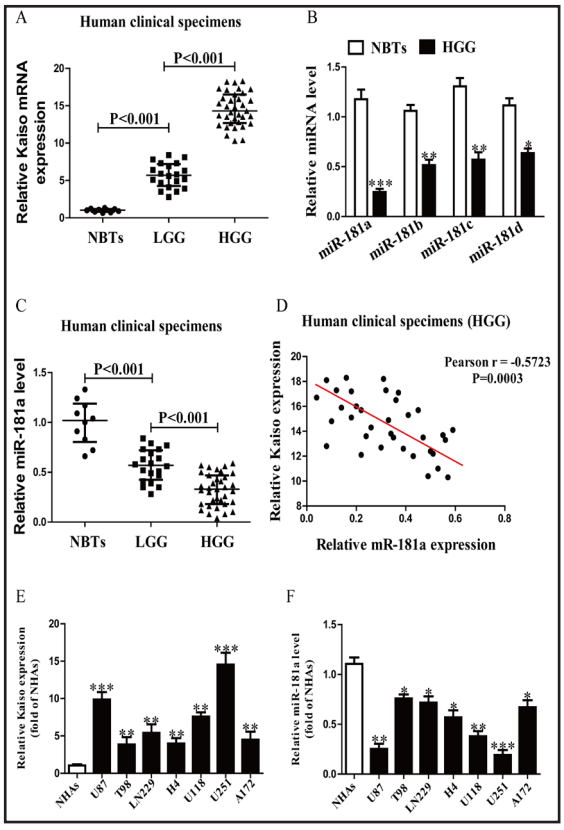



**Fig. 1.** Expression of Kaiso and miR-181a in glioma tissues and cell lines. (A) qRT-PCR analysis of Kaiso expression in 10 NBTs, 20 LGGs, and 35 HGGs. Transcript levels were normalized by GAPDH expression. (B) The levels of miR-181a–d in NBTs and HGG were detected by qRT-PCR (n = 6). (C) qRT-PCR analysis of miR-181a level in 10 NBTs, 20 LGGs, and 35 HGGs. Transcript levels were normalized by U6 level. (D) Pearson's correlation analysis of the relative expression levels of miR-181a and relative Kaiso mRNA levels in HGG. (E) Relative Kaiso expression analyzed by qRT-PCR in seven glioma cell lines was normalized by GAPDH expression (n = 6). (F) Relative miR-181a level analyzed by qRT-PCR in seven glioma cell lines was normalized with U6 (n = 6). All data are presented as the mean ± SEM. *P<0.05, **P<0.01, ***P<0.001 vs. NBT or NHA.

*miR-181a inhibited proliferation and induced G1-phase arrest in glioma cells*

After transfection with a miR-181a mimic or inhibitor, qRT-PCR analysis showed that the level of miR-181a was significantly upregulated or downregulated in the miR-181a mimic or miR-181 inhibitor group compared to thr NC group (Fig. 2A). To investigate the effect of miR-181a on glioma cell proliferation, the results from Brdu-ELISA assay indicated that introduction of miR-181a markedly inhibited the proliferation of U251 cells (Fig. 2B). Next, flow cytometry revealed that miR-181a could induce cell cycle arrest at the G0/G1 phases in U251 cells (Fig. 2C). However, cell proliferation and the cell cycle were induced in U251 cells transfected with a miR-181a inhibitor compared with the NC group (Fig. 2B, C). To confirm these results, we evaluated the effects of miR-181a on several proliferation- and cell cycle-related genes. As shown in Fig. 2D, the overexpression of miR-181a decreased the mRNA levels of PCNA, CDK2, CDK4, cyclin D1, and cyclin E1 and increased the mRNA levels of p21 and p27 in glioma cells. Knockdown

of miR-181a led to the opposite effects (Fig. 2D).

*Effects of miR-181a on the invasion and EMT in glioma cells*

To determine the effects of miR-181a on the invasion of glioma cells, we transfected a miR-181a mimic or inhibitor into U251 cells, and the invasive ability of glioma cells was assessed by the Transwell assay. The number of invading glioma cells in the miR-181a mimic group was significantly lower than that in the NC group (Fig. 3A). However, cell invasion ability in the miR-181a inhibitor group was stronger than that in the NC group (Fig. 3A). Next, the expression of MMP-2 and MMP-9 was determined. Western blot analysis and ELISA assays demonstrated that MMP-2 and MMP-9 protein expression and secretion in the culture supernatants were markedly decreased by overexpression of miR-181a in U251 cells (Fig. 3B, C), whereas knockdown of miR-181a had the reverse effect on expression (Fig. 3B, C). Then, we examined the effect of miR-181a on the mRNA and protein expression of EMT markers in glioma cells. Overexpression of miR-181a dramatically enhanced the expression of epithelial marker E-cadherin, and reduced the expression of mesenchymal markers N-cadherin and vimentin in U251 cells

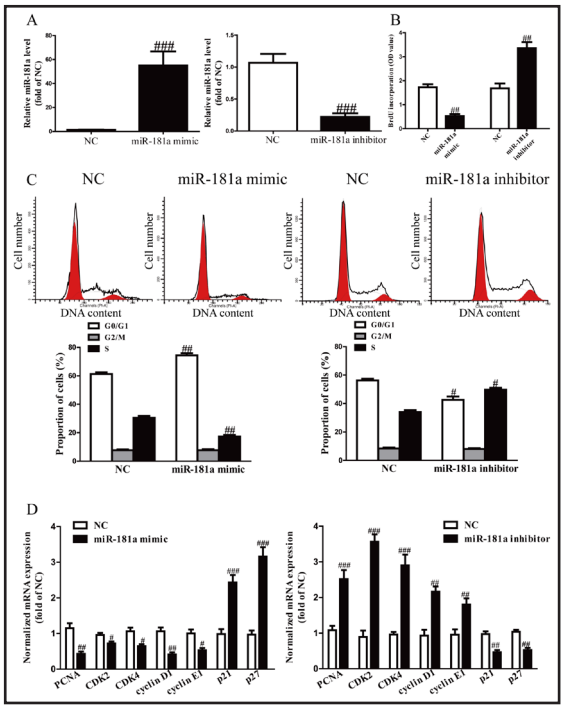
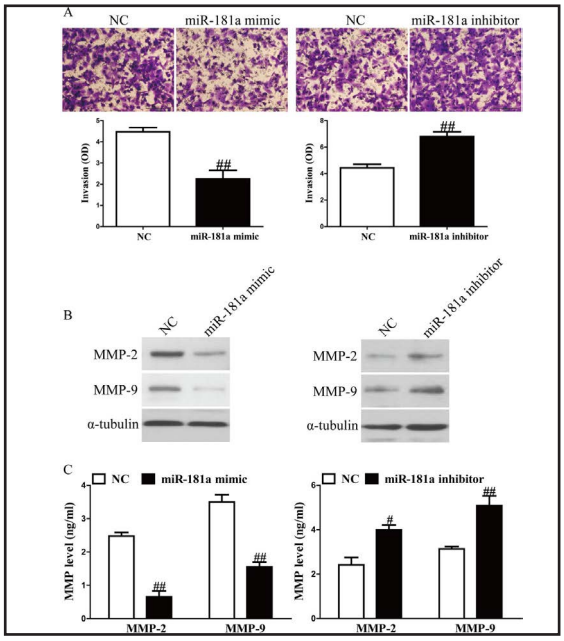



**Fig. 2.** Effects of miR-181a on the proliferation, cell cycle, and expression of related genes in glioma cells. U251 cells were transfected with a miR-181a mimic or inhibitor for 48 h. (A) Levels of miR-181a in glioma cells were determined by qRT-PCR. (B) Cell proliferation was assessed by a BrdU-ELISA assay. (C) Cell cycle was detected by flow cytometry. (D) mRNA expression of PCNA, CDK2, CDK4, cyclin D1, cyclin E1, p21, and p27 was determined by qRT-PCR. All data are presented as the mean ± SEM, n = 6. $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. NC.

**Fig. 3.** Effects of miR-181a on cell invasion and expression of related proteins in glioma cells. U251 cells were transfected with a miR-181a mimic or inhibitor for 48 h. (A) Invasion of glioma cells was assessed by the Transwell assay. (B) Protein expression of MMP-2 and MMP-9 was determined by western blotting. (C) Levels of total MMP-2 and MMP-9 in the culture supernatants were detected with ELISA. All data are presented as the mean ± SEM, n = 6. $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. NC.



(Fig. 4A). Finally, we evaluated the expression of EMT-related transcription factors in U251 cells. Introduction of miR-181a significantly decreased the protein and mRNA expression of Twist, Slug, and ZEB1 in U251 cells (Fig. 4B), whereas transfection of the miR-181a inhibitor had the opposite effects (Fig. 4A, B). Together, our data showed that miR-181a upregulation dramatically inhibited the invasion and EMT of glioma cells.

*miR-181a directly targeted the Kaiso 3′UTR*

According to TargetScan 6.2, we identified a miR-181a-binding site in the 3′UTR of Kaiso (Fig. 5A). To determine whether Kaiso is a direct target of miR-181a, luciferase plasmids containing potential Kaiso miR-181a binding sites (WT) or a mutated Kaiso 3′UTR were constructed (Fig. 5A). Overexpression of miR-181a inhibited WT Kaiso reporter activity but not the activity of the mutated reporter construct in U251 cells, demonstrating that miR-181a could specifically target the Kaiso 3′UTR by binding to the seed sequence (Fig. 5B). Next, we confirmed the results at the mRNA and protein levels. Transfection of miR-181a significantly decreased the expression of Kaiso, whereas knockdown of miR-181a increased Kaiso expression in U251 cells (Fig. 5C). These data indicated that miR-181a directly regulated Kaiso expression in glioma cells through 3′UTR sequence binding.

*Downregulation of Kaiso by siRNA inhibited the proliferation, invasion, and EMT of glioma cells*

To explore the functional roles of Kaiso in glioma cells, U251 cells were transfected with siRNA-NC or siRNA-Kaiso for 48 h, and then the proliferation, invasion, and EMT of glioma cells were evaluated. Western blot analysis showed that Kaiso expression was significantly decreased in U251 cells transfected with si-Kaiso for 48 h compared with that in the si-

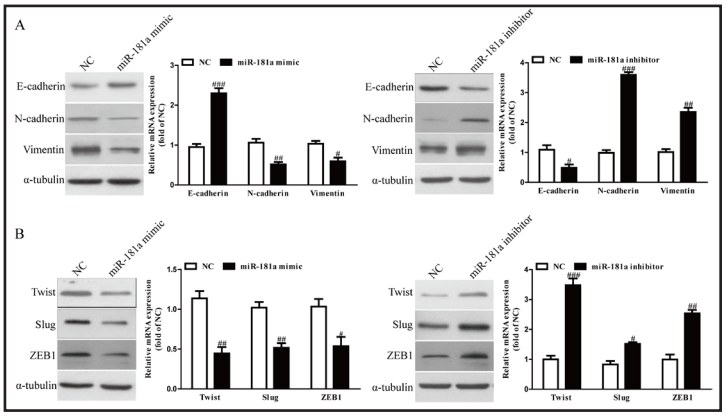



**Fig. 4.** Effects of miR-181a on the expression of EMT-related molecules in glioma cells. U251 cells were transfected with miR-181a mimic or inhibitor for 48 h. (A) Protein and mRNA expression of E-cadherin, N-cadherin, and vimentin was determined by western blotting and qRT-PCR, respectively. (E) Protein and mRNA expression of Twist, Slug, and ZEB1 were determined by western blotting and qRT-PCR, respectively. All data are presented as the mean ± SEM, n = 6. #P<0.05, ##P<0.01, ###P<0.001 vs. NC.

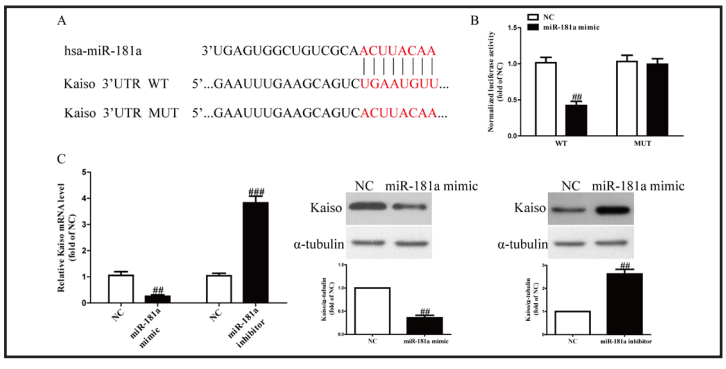



**Fig. 5.** Kaiso is a direct target of miR-181a. U251 cells were transfected with a miR-181a mimic or inhibitor for 48 h. (A) Schematic representation of Kaiso 3′UTRs showing the putative miRNA target site. (B) Analysis of the relative luciferase activities of Kaiso-WT and Kaiso-MUT. (C) mRNA and protein expression of Kaiso were determined by qRT-PCR and western blot analysis, respectively. All data are presented as the mean ± SEM, n = 6. ##P<0.01, ###P<0.001 vs. NC.

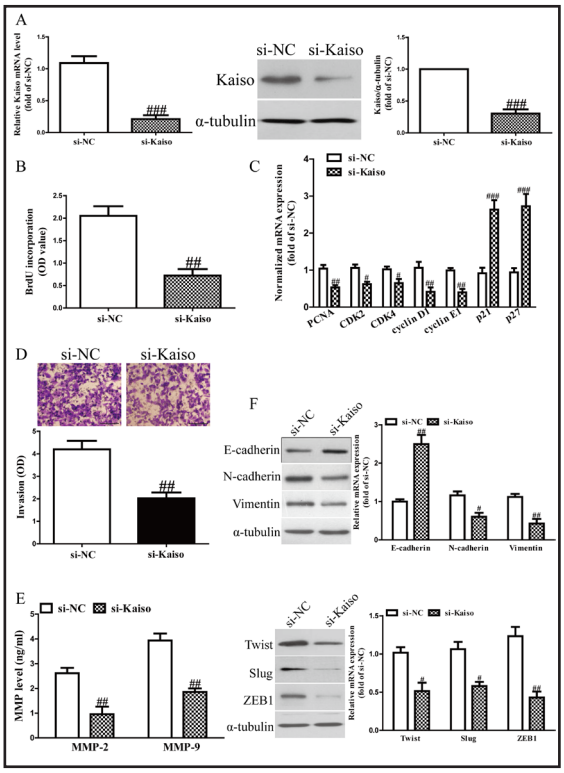



**Fig. 6.** Effects of Kaiso silencing on the proliferation, invasion, and EMT of glioma cells. U251 cells were transfected with si-Kaiso or si-NC. (A) mRNA and protein expression of Kaiso was determined by qRT-PCR and western blot analysis, respectively. (B) Cell proliferation was assessed by the BrdU-ELISA assay. (C) mRNA expression of PCNA, CDK2, CDK4, cyclin D1, cyclin E1, p21, and p27 were determined by qRT-PCR. (D) Invasion of glioma cells was assessed by the Transwell assay. (E) Total secretion of MMP-2 and MMP-9 in the culture supernatants was detected by an ELISA assay. (F) The expression of E-cadherin, N-cadherin, vimentin, Twist, Slug, and ZEB1 was determined by western blotting and qRT-PCR, respectively. All data are presented as the mean ± SEM, n = 6. $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. si-NC.

NC group (Fig. 6A). The Brdu-ELISA assay showed that Kaiso knockdown significantly suppressed the proliferation of glioma cells (Fig. 6B), and the qRT-PCR assay showed that downregulation of Kaiso decreased the mRNA levels of PCNA, CDK2, CDK4, cyclin D1, and cyclin E1 and increased those of p21, p27 (Fig. 6C). Furthermore, both the Transwell and ELISA assays suggested that decreased Kaiso expression inhibited the invasive ability of glioma cells and dramatically downregulated the expression of MMP-2 and MMP-9 (Fig. 6D, E). Finally, knockdown of Kaiso resulted in inhibition of the EMT and downregulation of related transcription factors (Fig. 6F). Consequently, Kaiso silencing had similar effects as miR-181a overexpression in glioma cells.

*Overexpression of Kaiso significantly inhibited the effects of miR-181a mimic on proliferation, invasion, and EMT in glioma cells*

To determine whether miR-181a targeting Kaiso was responsible for inhibition of the proliferation, invasion, and EMT of glioma cells. We constructed an expression vector that encoded the entire Kaiso coding sequence but lacked the 3′UTR. Then, we co-transfected pcDNA-Kaiso or its NC (pcDNA3.1) with the miR-181a mimic or NC into U251 cells (Fig. 7A). The cell proliferation assay showed that concomitant overexpression of miR-181a and Kaiso abrogated the inhibitory effects of the miR-181a mimic (Fig. 7B). Meanwhile, the mRNA levels of PCNA, CDK2, CDK4, cyclin D1, and cyclin E1 were increased and the mRNA levels of p21 and p27 were decreased in miR-181a-overexpressing glioma cells after exogenous introduction of Kaiso (Fig. 7C). Kaiso overexpression also reversed the inhibitory effects of the miR-181a mimic on the invasion of glioma cells (Fig. 7D) and upregulated the expression of MMP-2 and MMP-9, which was inhibited by the miR-181a mimic (Fig. 7E). Moreover, increased Kaiso expression promoted the EMT of glioma cells transfected with miR-181a mimic (Fig. 7F). Therefore, the inhibitory effects of miR-181a were reversed by Kaiso overexpression. Taken together, these results clearly confirmed that miR-181a inhibited the proliferation, invasion, and EMT in glioma cells by targeting and downregulating Kaiso (Fig. 8).

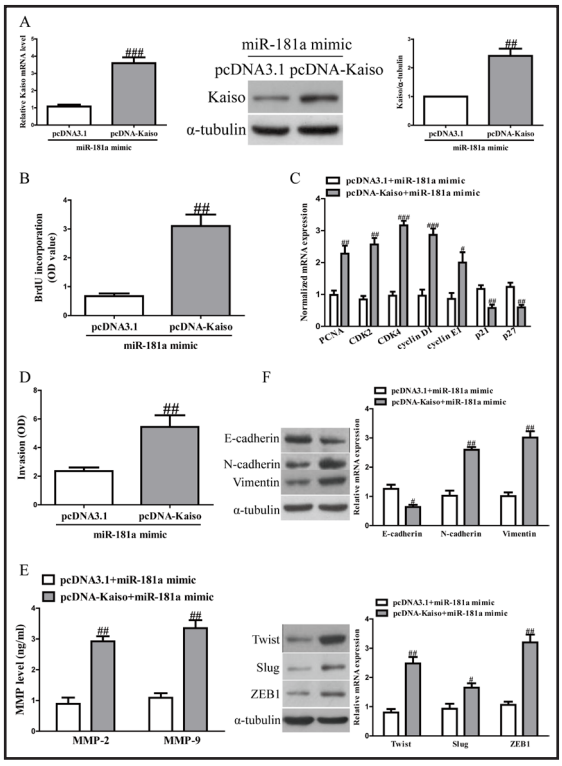



**Fig. 7.** Overexpression of Kaiso partially promoted the cell proliferation, invasion, and EMT in miR-181a-overexpressing glioma cells. U251 cells were transfected with a miR-181a mimic with or without the pcDNA-Kaiso vector. (A) mRNA and protein expression of Kaiso were determined by qRT-PCR and western blotting, respectively. (B) Cell proliferation was assessed by the BrdU-ELISA assay. (C) mRNA expression of PCNA, CDK2, CDK4, cyclin D1, cyclin E1, p21, and p27 was determined by qRT-PCR. (D) Invasion of glioma cells was assessed by the Transwell assay. (E) Total secretion of MMP-2 and MMP-9 in the culture supernatants was detected by the ELISA assay. (F) Expression of E-cadherin, N-cadherin, vimentin, Twist, Slug, and ZEB1 was determined by western blotting and qRT-PCR, respectively. All data are presented as the mean ± SEM, n = 6. $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. miR-181 mimic + pcDNA3.1.

## Discussion

Because the involved molecular mechanisms underlying glioma remain largely unknown, the prognosis of patients with glioma is poor. Therefore, identifying the critical genes that are abnormally expressed in glioma tissues and elucidating the precise mechanisms that lead to dysregulated genes accelerating the progression of glioma are needed to develop effective glioma treatments. Several studies have reported that Kaiso expression is closely associated with many cancer types. However, some studies have considered Kaiso a pro-oncogene [8, 12, 24], and others have suggested that it is a tumor suppressor [25–27]. In this study, both the mRNA and protein levels of Kaiso were significantly higher in glioma tissues and cells compared to those in non-cancerous tissues and normal cells. We also showed that the expression of Kaiso in HGG was significantly higher than that in LGG.

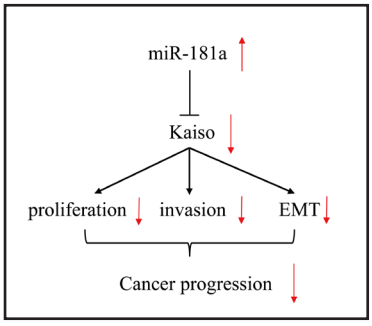



**Fig. 8.** Schematic diagram showing miR-181a regulation of Kaiso and subsequent changes in cell cycle progression and cell invasion.

Accumulating evidence has shown that dysregulated miRNAs are closely associated with malignant biological behaviors, including proliferation, cell cycle progression, migration, invasion, EMT, apoptosis, angiogenesis, and chemoresistance [28]. Previous studies have shown that miR-181a plays both prooncogenic and tumor suppressive roles in several cancers, including cervical [29], ovarian [30], colorectal [31], and hepatocellular cancers [32], acute myeloid leukemia [33], and gastric cancer [34]. Two recent studies demonstrated that miR-181a downregulation in glioma is closely associated with a poor prognosis [16, 20]. Accordingly, we also found that the level of miR-181a was significantly downregulated in glioma and its level was higher in HGGs than in LGGs. To determine the biological function of miR-181a in glioma, we increased or decreased the level of miR-181a in U251 cells by transfecting them with a miR-181a mimic or inhibitor, respectively. We demonstrated that

miR-181a overexpression significantly inhibited the proliferation of glioma cells. To confirm this result, transfection of miR-181a decreased the mRNA levels of PCNA, CDK2, CDK4, cyclin D1, and cyclin E1 and increased the mRNA levels of p21 and p27 in glioma cells.

Invasion is one process of metastasis. In this study, the Transwell assay showed that the overexpression or knockdown of miR-181a significantly suppressed or promoted the invasive ability of U251 cells compared with the NC group. The degradation of extracellular matrix (ECM) components by proteolytic enzymes is very important for the invasion of cancer cells [35], and MMPs that degrade the ECM are closely associated with the invasion, metastasis, and angiogenesis of cancer cells [36–38]. In particular, both MMP-2 and MMP-9 are responsible for the invasion and EMT of malignant tumors by degrading components of the basement membrane [37, 39, 40]. We found that the expression of MMP-2 and MMP-9 was significantly decreased in U251 cells after transfection with a miR-181a mimic, whereas a miR-181a inhibitor markedly enhanced MMP-2 and MMP-9 expression. The EMT process is another key molecular step in the process of distant metastasis, as it allows cancer cells to acquire invasive properties and metastatic growth characteristics, and its activation is found in many types of malignant tumors including glioma [41, 42]. Proteins including E-cadherin, N-cadherin, vimentin, fibronectin, and β-catenin have been confirmed as the markers of EMT [43]. After transfection with a miR-181a mimic or inhibitor, we assessed the changes of the EMT markers in U251 cells. Transfection of miR-181a dramatically upregulated expression of the epithelial marker E-cadherin and downregulated expression of the mesenchymal markers vimentin and N-cadherin. Furthermore, there are many types of transcription factors, including Twist 1, Twist 2, Slug, Snail, ZEB1, and ZEB2, that are key EMT inducers [43–46]. Our data indicated that upregulation of miR-181a resulted in the decreased expression of Twist1, Slug, and ZEB1. In addition, the increased expression of miR-181a inhibited the invasion and EMT of glioma cells, potentially resulting in the suppression of glioma metastasis.

Two miRNAs, namely, miR-31 and miR-181a, have been reported to inhibit cancer cells by targeting Kaiso [47, 48]. Although miR-31 was shown to inhibit Kaiso expression in prostate cancer cells [48], no studies have demonstrated the relationship between miR-181a and Kaiso in glioma. Based on previous studies and our findings, we hypothesized that the overexpression of miR-181a would decrease the expression of Kaiso and inhibit cancerous signals such as proliferation, invasion, and EMT. Accordingly, our study showed that the overexpression of miR-181a in U251 cells enhanced the inhibition of Kaiso at both the mRNA and protein levels, and these effects were also observed with Kaiso knockdown. Restoration of Kaiso reversed the inhibitory effects of miR-181a, indicating that Kaiso may play critical roles in the progression and metastasis of glioma.

In conclusion, our results showed that miR-181a expression was significantly downregulated in glioma cells, and its level was the lowest among miR-181 family members. Overexpression of miR-181a inhibited the proliferation, invasion, and EMT of glioma cells by directly downregulating Kaiso expression. Therefore, our study provides compelling evidence supporting the roles of miR-181a and Kaiso as prognostic factors for glioma.

**Acknowledgements**

This work was supported by the National Nature Science Foundation of China (Grant No. 81401009) and Nature Science Foundation of Heilongjiang Province, China (Grant No. QC2013C088).

**Disclosure Statement**

The authors have no conflict of interests to declare.

## References

1 Taylor LP: Diagnosis, treatment, and prognosis of glioma: five new things. Neurology 2010;75:S28-32.
2 Dunbar E, Yachnis AT: Glioma diagnosis: immunohistochemistry and beyond. Adv Anat Pathol 2010;17:187-201.
3 Huang W, Wang J, Zhang D, Chen W, Hou L, Wu X, Lu Y: Inhibition of KIF14 Suppresses Tumor Cell Growth and Promotes Apoptosis in Human Glioblastoma. Cell Physiol Biochem 2015;37:1659-1670.
4 Ostrom QT, Gittleman H, Stetson L, Virk SM, BarnholtzSloan JS: Epidemiology of gliomas. Cancer Treat Res 2015;163:1-14.
5 Musgrove EA, Caldon CE, Barraclough J, Stone A, Sutherland RL: Cyclin D as a therapeutic target in cancer. Nat Rev Cancer 2011;11:558-572
6 Adachi Y, Yamamoto H, Itoh F, Hinoda Y, Okada Y, Imai K: Contribution of matrilysin (MMP-7) to the metastatic pathway of human colorectal cancers. Gut 1999;45:252-258
7 Onder TT, Gupta PB, Mani SA, Yang J, Lander ES, Weinberg RA: Loss of E-cadherin promotes metastasis via multiple downstream transcriptional pathways. Can Res 2008;68:3645-3654
8 Jones J, Wang H, Zhou J, Hardy S, Turner T, Austin D, He Q, Wells A, Grizzle WE, Yates C: Nuclear kaiso indicates aggressive prostate cancers and promotes migration and invasiveness of prostate cancer cells. Am J Pathol 2012;181:1836-1846
9 Pierre CC, Longo J, Mavor M, Milosavljevic SB, Chaudhary R, Gilbreath E, Yates C, Daniel JM: Kaiso overexpression promotes intestinal inflammation and potentiates intestinal tumorigenesis in Apc(Min/+) mice. Biochim Biophys Acta 2015;1852:1846-1855
10 Dai SD, Wang Y, Miao Y, Zhao Y, Zhang Y, Jiang GY, Zhang PX, Yang ZQ, Wang EH: Cytoplasmic Kaiso is associated with poor prognosis in non-small cell lung cancer. BMC Cancer 2009;9:178
11 Jones J, Mukherjee A, Karanam B, Davis M, Jaynes J, Reams RR, Dean-Colomb W, Yates C: African Americans with pancreatic ductal adenocarcinoma exhibit gender differences in Kaiso expression. Cancer Lett 2016;380:513-522
12 Bassey-Archibong BI, Kwiecien JM, Milosavljevic SB, Hallett RM, Rayner LG, Erb MJ, Crawford-Brown CJ, Stephenson KB, Bédard PA, Hassell JA, Daniel JM: Kaiso depletion attenuates transforming growth factor-β signaling and metastatic activity of triple-negative breast cancer cells. Oncogenesis 2016;5:e208
13 Lewis BP, Burge CB, Bartel DP: Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 2005;120:15-20.
14 Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, Sweet-Cordero A, Ebert BL, Mak RH, Ferrando AA, Downing JR, Jacks T, Horvitz HR, Golub TR: MicroRNA expression profiles classify human cancers. Nature 2005;435:834-838.
15 Zhang BL, Dong FL, Guo TW, Gu XH, Huang LY, Gao DS: MiRNAs Mediate GDNF-Induced Proliferation and Migration of Glioma Cells. Cell Physiol Biochem 2017;44:1923-1938.
16 Huang SX, Zhao ZY, Weng GH, He XY, Wu CJ, Fu CY, Sui ZY, Zhong XM, Liu T: The correlation of microRNA-181a and target genes with poor prognosis of glioblastoma patients. Int J Oncol. 2016;49:217-224.
17 Zhu-mei Shi, Xie-feng Wang, Xu Qian, Tao Tao, Lin Wang, Qiu-dan Chen, Xi-rui Wang, Lei Cao, Ying-yi Wang, Jun-xia Zhang, Tao Jiang, Chun-sheng Kang, Bing-hua Jiang, Ning Liu, Yong-ping You: MiRNA-181b suppresses IGF-1R and functions as a tumor suppressor gene in gliomas. RNA 2013;19:552-560.
18 Erandi Ayala-Ortega, Rodrigo Arzate-Mejía, Rosario Pérez-Molina, Edgar González-Buendía, Karin Meier, Georgina Guerrero, Félix Recillas-Targa: Epigenetic silencing of miR-181c by DNA methylation in glioblastoma cell lines. BMC Cancer 2016;16:226.
19 Wang XF, Shi ZM, Wang XR, Cao L, Wang YY, Zhang JX, Yin Y, Luo H, Kang CS, Liu N, Jiang T, You YP: MiR-181d acts as a tumor suppressor in glioma by targeting K-ras and Bcl-2. J Cancer Res Clin Oncol 2012;138:573-584.
20 Shi L, Cheng Z, Zhang J, Li R, Zhao P, Fu Z, You Y: hsa-mir-181a and hsa-mir-181b function as tumor suppressors in human glioma cells. Brain Res. 2008;1236:185-193.
21 Liu Y, Li Y, Liu J, Wu Y, Zhu Q: MicroRNA-132 inhibits cell growth and metastasis in osteosarcoma cell lines possibly by targeting Sox4. Int J Oncol. 2015;47:1672-1684.
22 Zeng Z, Zhu BH: Arnebin-1 promotes the angiogenesis of human umbilical vein endothelial cells and accelerates the wound healing process in diabetic rats. J Ethnopharmacol. 2014;154:653-662.
23 Zhong F, Chen H, Han L, Jin Y, Wang W: Curcumin attenuates lipopolysaccharide-induced renal inflammation. Biol Pharm Bull. 2011;34:226-232.
24 Vermeulen JF, van de Ven RA, Ercan C, van der Groep P, van der Wall E, Bult P, Christgen M, Lehmann U, Daniel J, van Diest PJ, Derksen PW: Nuclear Kaiso expression is associated with high grade and triple-

negative invasive breast cancer. PLoS ONE 2012;7:e37864.
25 van de Ven RA, Tenhagen M, Meuleman W, van Riel JJ, Schackmann RC, Derksen PW: Nuclear p120-catenin regulates the anoikis resistance of mouse lobular breast cancer cells through Kaiso-dependent Wnt11 expression. Dis Model Mech 2015;8:373-384.
26 Spring CM, Kelly KF, O'Kelly I, Graham M, Crawford HC, Daniel JM: The catenin p120ctn inhibits Kaiso-mediated transcriptional repression of the beta-catenin/TCF target gene matrilysin. Exp Cell Res 2005;305:253-265.
27 Koh DI, Han D, Ryu H, Choi WI, Jeon BN, Kim MK, Kim Y, Kim JY, Parry L, Clarke AR, Reynolds AB, Hur MW: KAISO, a critical regulator of p53-mediated transcription of CDKN1A and apoptotic genes. Proc Natl Acad Sci USA 2014;111:15078-15083.
28 Areeb Z, Stylli SS, Koldej R, Ritchie DS, Siegal T, Morokoff AP, Kaye AH, Luwor RB: MicroRNA as potential biomarkers in Glioblastoma. J Neurooncol 2015;125:237-248.
29 Luo C, Qiu J: miR-181a Inhibits Cervical Cancer Development via Downregulating GRP78 Oncol Res 2017;25:1341-1348.
30 Parikh A, Lee C, Joseph P, Marchini S, Baccarini A, Kolev V, Romualdi C, Fruscio R, Shah H, Wang F, Mullokandov G, Fishman D, D'Incalci M, Rahaman J, Kalir T, Redline RW, Brown BD, Narla G, DiFeo A: microRNA-181a has a critical role in ovarian cancer progression through the regulation of the epithelial-mesenchymal transition. Nat Commun 2014;5:2977.
31 Ji D, Chen Z, Li M, Zhan T, Yao Y, Zhang Z, Xi J, Yan L, Gu J: MicroRNA-181a promotes tumor growth and liver metastasis in colorectal cancer by targeting the tumor suppressor WIF-1 Mol Cancer 2014;13:86.
32 Korhan P, Erdal E, Atabey N: MiR-181a-5p is downregulated in hepatocellular carcinoma and suppresses motility, invasion and branching-morphogenesis by directly targeting c-Met. Biochem Biophys Res Commun 2014;450:1304-1312.
33 Liu X, Liao W, Peng H, Luo X, Luo Z, Jiang H, Xu L: miR-181a promotes G1/S transition and cell proliferation in pediatric acute myeloid leukemia by targeting ATM. J Cancer Res Clin Oncol 2016;142:77-87.
34 Lin F1, Li Y, Yan S, Liu S, Qian W, Shen D, Lin Q, Mao W: MicroRNA-181a inhibits tumor proliferation, invasiveness, and metastasis and is downregulated in gastric cancer. Oncol Res 2015;22:75-84.
35 Simpson-Haidaris PJ, Rybarczyk B: The role of fibrinogen as an extracellular matrix protein. Ann N Y Acad Sci 2001;936:406-425.
36 Bogenrieder T, Herlyn M: Axis of evil: molecular mechanisms of cancer metastasis. Oncogene 2003;22:6524-6536.
37 Vihinen P1, Kähäri VM: Matrix metalloproteinases in cancer: prognostic markers and therapeutic targets. Int J Cancer 2002;99:157-166.
38 Sounni NE, Janssen M, Foidart JM, Noel A: Membrane type-1 matrix metalloproteinase and TIMP-2 in tumor angiogenesis. Matrix Biol 2003;22:55-61.
39 Hornebeck W, Emonard H, Monboisse JC, Bellon G: Matrix-directed regulation of pericellular proteolysis and tumor progression. Semin Cancer Biol 2002;12:231-241.
40 Klein G1, Vellenga E, Fraaije MW, Kamps WA, de Bont ES: The possible role of matrix metalloproteinase (MMP)-2 and MMP-9 in cancer, e.g. acute leukemia. Crit Rev Oncol Hematol 2004;50:87-100.
41 Guarino M: Epithelial-mesenchymal transition and tumour invasion. Int J Biochem Cell Biol 2007;39:2153-2160.
42 Thiery JP, Acloque H, Huang RY, Nieto MA: Epithelial-mesenchymal transition in development and disease. Cell 2009;139:871-890.
43 Kalluri R, Weinberg RA: The basics of epithelial-mesenchymal transition. J Clin Invest 2009;119:1420-1428.
44 Khan MA, Chen HC, Zhang D, Fu J: Twist: a molecular target in cancer therapeutics. Tumor Biol 2013;34:2497-2506.
45 Peinado H, Olmeda D, Cano A: Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? Nat Rev Cancer 2007;7:415-428.
46 De Craene B, Berx G: Regulatory networks defining EMT during cancer initiation and progression. Nat Rev Cancer 2013;13:97-110.
47 Feng J: Upregulation of MicroRNA-4262 Targets Kaiso (ZBTB33) to Inhibit the Proliferation and EMT of Cervical Cancer Cells. Oncol Res DOI: 10.3727/096504017X15021536183526.
48 Wang H, Liu W, Black S, Turner O, Daniel JM, Dean-Colomb W, He QP, Davis M, Yates C: Kaiso, a transcriptional repressor, promotes cell migration and invasion of prostate cancer cells through regulation of miR-31 expression. Oncotarget 2016;7:5677-5689.