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MiR-122 inhibits cell proliferation and induces apoptosis by targeting runt-related transcription factors 2 in human glioma

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Abstract. – OBJECTIVE: Accumulating evidence has suggested that microRNAs play critical roles in the development and progression of human glioma. The role of miR-122 in glioma tumorigenesis has been poorly defined. The current study is designed to investigate whether and how miR-122 affects proliferation and apoptosis of human glioma cells.

PATIENTS AND METHODS: 8 normal brain tissues and 19 glioma tissues (7 for low grade and 12 for high grade) were collected. The expressions of miR-122 and runt-related transcription factors (RUNX2) in normal brain/glioma tissues and normal astrocytes (NHA)/multiple glioma cell lines (U87, U251, and U118) were analyzed by Real-time polymerase chain reaction (PCR). Western blot and luciferase activity assays were performed to validate the predicted relationship between miR-122 and RUNX2. The effects of miR-122 on cell proliferation and apoptosis were assessed by cell counting kit-8 (CCK-8), colony forming, and Annexin V-FITC/PI apoptosis assays using both gain- and loss-of-function approaches.

RESULTS: MiR-122 expression is downregulated in glioma tissues compared with normal brain tissues, and is negatively correlated with the WHO grade. In contrast, the RUNX2 expression is upregulated in glioma tissues, and is positively correlated with the WHO grade. In glioma cell lines, the miR-122 expression is also constantly downregulated. MiR-122 functions as a tumor suppressor by inhibiting proliferation and inducing apoptosis, which is achieved by directly targeting RUNX2. Overexpression of RUNX2 can partially abrogate the effect of miR-122 on glioma cells.

CONCLUSIONS: These results demonstrate a crucial role of miR-122 in regulating cell proliferation and apoptosis. Identifying the miR-122/RUNX2 signaling provides novel insights into the development of therapeutic targets for glioma.

Key Words:
Glioma, WHO grade, Proliferation, Apoptosis, RUNX2.

Introduction

Glioma accounts for most of the malignant tumors in the central nervous system[1]. The most recent well-established treatment pipeline for newly diagnosed malignant gliomas consists of surgical resection, radiotherapy and adjuvant temozolomide treatment. However, malignant is rarely curable with less than 14-month average survival for the high-grade malignant glioma, the so-called glioblastoma multiforme[2]. Identifying the molecular mechanism may hopefully provide new insights into the novel strategies for glioma treatment. Emerging evidence has shown that microRNAs, a class of non-coding small RNA, about 22 nt in length, are critical factors in determining the tumorigenicity by distinct mechanisms[3]. In general, when binds to the 3' untranslated region (UTR) of the target mRNAs, microRNAs are able to degrade them or block their translation, leading to the functional silence of multiple genes[3,4]. Many microRNAs have been identified to exert critical functions in glioma development and progression[5]. For example, the well-characterized oncomiR, miR-17-92 cluster, inhibits the expression of multiple tumor suppressive genes including TGFβRII, Smad4 and CTGF, thereby promoting cell proliferation in Grade III-IV gliomas[6,7]; other microRNAs such as miR-125b, miR-181a/b/c and miR-7 are downregulated in glioma and suppress cell proliferation, cell migration, cell invasion and angiogenesis[8-11]. Tang et al[12] has shown that in the blood sample of glioma patients, the level of miR-122 is significantly lower, suggesting that miR-122 could be a potential biomarker for its diagnosis or prognosis. The distinct functions of miR-122 depend on a variety of conditions. For example, miR-122 is a well-established tumor suppressor in liver cancer but drives tumor pro-

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gression in breast cancer[13-15]. Wang et al[16] has also shown that miR-122 affected WNT signaling in glioma. However, a microRNA may have many targets in the same scenario, many of the functions and mechanisms of miR-122 are yet to be unveiled.

In exploring the expression and function of miR-122 in glioma, we found that miR-122 is downregulated, and that runt-related transcription factors 2 (RUNX2) is upregulated in glioma. Importantly, their differential expression correlates with the WHO grade. Experimentally, miR-122 directly targets RUNX2 to inhibit cell proliferation and promote apoptosis. Overexpression of RUNX2 can cancel the effect of miR-122 on these cellular processes. Therefore, we identify a novel miR-122/RUNX2 signaling in glioma critical for cell proliferation and apoptosis in glioma. These data suggest that miR-122 may have therapeutic value in the treatment of glioma.

Patients and Methods

Patients

The glioma tissues and normal tissues were obtained from July 2014 to June 2016 by the Affiliated Hospital of Qingdao University. The tumor tissues were collected from 19 patients, 7 of them were classified as WHO grade I-II (low grade) glioma, and 12 of them were WHO grade III-IV (high grade) glioma. 8 normal adult brain tissues were collected from surgery for severe traumatic brain injury. The fresh tissues were frozen in liquid nitrogen and subjected to RNA extraction afterwards. Informed consents were obtained from all patients, and the study protocol was approved by Institutional Ethics Review Board of Affiliated Hospital of Qingdao University.

Cell Lines, Transfection, And Colony Forming Assay

Normal human astrocytes (NHA) and three human glioma cell lines (U87, U251 and U118) were all purchased from Cell Bank of Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM with 10% FBS, and the complete medium contains 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were cultured in a humidified atmosphere with 5% CO2 at 37°C. The mimics for miR-122 and its negative control (miR-NC) and antisense of miR-122 (as-miR-122) and its corresponding negative control (as-miR-NC) were purchased from RiboBio (Guangzhou, China), and transfected into cells at the concentration of 200 nmol/L. The cDNA of RUNX2 was purchased from Origene (Rockville, MD, USA) and subcloned into pcDNA3.1 construct (Invitrogen, Carlsbad, CA, USA). The concentration for the transfection of the plasmids is 1 μg/ml. All the transfection procedures were done by the manufacturer provided protocol using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) reagent. Cells were harvested at the time as described for cell viability assay. For western blot and apoptosis assay, cells were collected 72 h after transfection. For colony forming assay, 500 transfected cells were seeded into each 3.5-cm dish and cultured for 2 weeks with complete medium. Colonies were visualized with 0.1% crystal violet solution and counted manually.

Real-Time PCR

Tissue specimens and cells were harvested using TRIzol reagents (Invitrogen, Carlsbad, CA, USA), followed by a standard protocol to isolate RNA. The RNA samples were then quantified and subsequently reverse-transcribed to generate the first strand of the cDNA using the PrimeScriptTM RT reagent Kit (Takara, Dalian, China). The primer set for miR-122 detection was purchased from RiboBio. The primer sequence for RUNX2 was Forward-TGGTTACTGTCATGGCGGGTA Reverse-TCTCAGATCGTTGAACCTTGCTA. GAPDH was used as internal control for *RUNX2* and the primer sequence was Forward-CTGGGCTACACTGAGCACC Reverse-AAGTGGTCGTTGAGGGCAATG. U6 is used as internal control for miR-122, and its primer set was also purchased from RiboBio (Guangzhou, China).

Western Blot

Cells were harvested using RIPA buffer (Promega, Madison, WI, USA) after treatment, and were sonicated. The lysis was then centrifuged at 15000 g for 15 min at 4°C. Approximately 40 μg protein was loaded to each lane in the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel for electrophoresis. Proteins were electro-transferred onto polyvinylidene difluoride (PVDF) membranes, blocked with 5% milk in phosphate-buffered saline (PBS) for 1 h at room temperature and then incubated with primary antibody for RUNX2 and GAPDH (both from Santa Cruz Biotechnology, Santa Cruz, CA, USA). Horseradish peroxidase (HRP) conjugated goat anti-mouse secondary antibody was from ZSGB (Beijing, China),

the incubation condition is 2 h at room temperature. Finally, membranes were developed using enhanced chemiluminescence Kit (PerkinElmer, Waltham, MA, USA) on GE ImageQuant LAS-4000mini (Pittsburgh, PA, USA).

Luciferase Assay

A 560 bp 3'UTR sequence containing the putative binding site of miR-122 was amplified from the cDNA templates using PCR. The PCR product was then inserted into the 3'UTR region of luciferase reporter vector pGL3 (Promega, Madison, WI, USA). For the construction of the mutant reporter, the sequence was changed as shown in Figure 2A using a site-direct mutagenesis kit (Tiangen, Beijing, China) and inserted into the same site of the pGL3 construct. U87 cells were transfected with luciferase reporter (wild-type or mutant, 1 μg/ml), internal control reporter (pRL-TK, 0.2 μg/ml) and miR-122 (or miR-NC, 200 nmol/L) for 48 h. Luciferase activity was detected using the Dual-Luciferase Reporter Assay system (Promega, Madison, WI, USA).

Cell Viability Assay

Cell viability was measured using CCK-8 kit (Beyotime, Shanghai, China). After transfection, cells were seeded into 96-well plates for 5000 cell per well. 2 h before detection, the CCK-8 solution was added to each well at the volume of 20 μl. Cells were kept in 37°C incubator, and Optical density (OD) value for each well was detected at the wavelength of 450 nm on a microplate reader. The results were from 5 independent repeats.

Apoptosis Assay

Apoptosis was determined using the Annexin V-FITC/PI Apoptosis Detection Kit (Yeasen, Shanghai, China). Cells were treated with trypsin and suspended, and stained with 5 μL Annexin

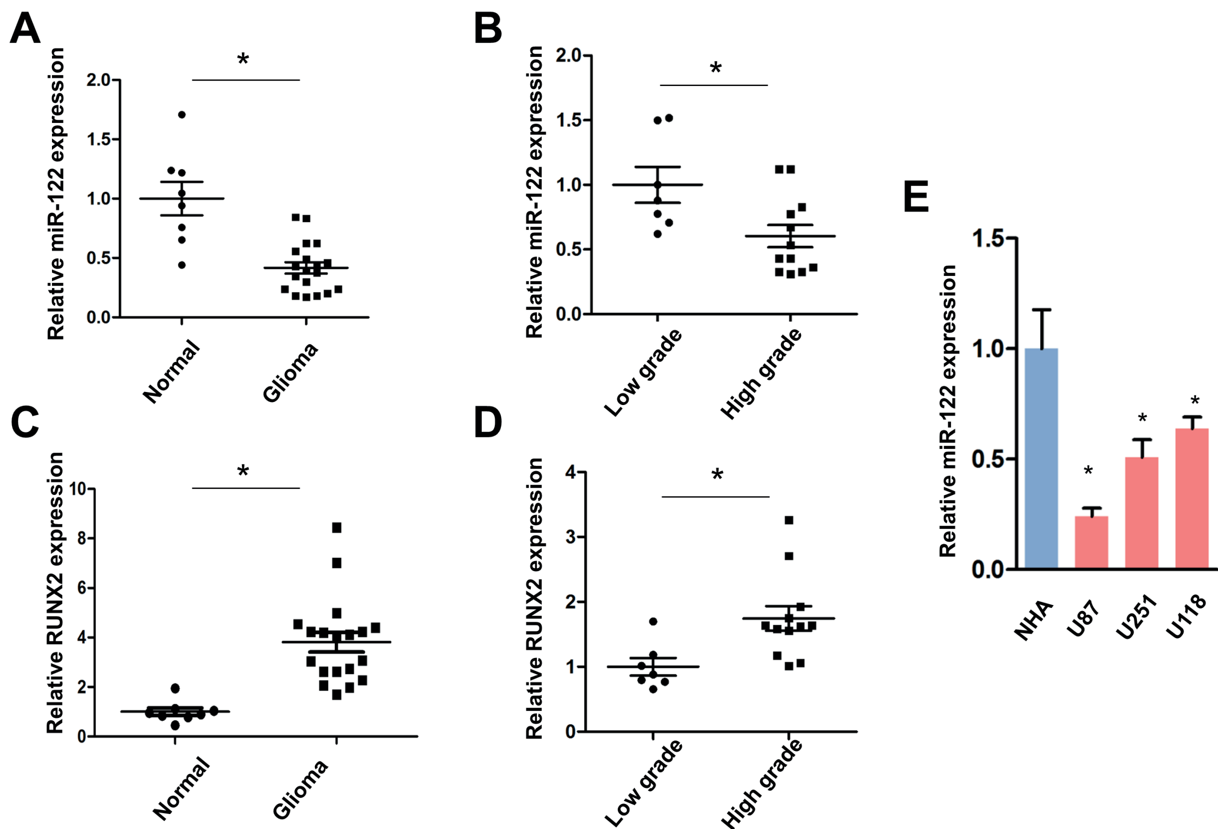

Figure 1. MiR-122 and RUNX2 expression in glioma tissues. ***(A)*** The expression of miR-122 is lower in glioma tissues compared with that in normal brain tissues. ***(B)*** The expression of miR-122 is lower in high-grade glioma tissues compared with that in low-grade glioma tissue. ***(C)*** The expression of RUNX2 is higher in glioma tissues compared with that in normal brain tissues. ***(D)*** The expression of RUNX2 is higher in high-grade glioma tissues compared with that in low-grade glioma tissues. ***(E)*** The expression of miR-122 is significantly downregulated in human glioma cells (U87, U251 and U118) compared with that in normal human astrocytes (NHA). *$p < 0.05$ *vs.* Normal brain tissue or low grade glioma tissues or NHA, $n \geq 3$.

V-FITC and 10 µl PI staining solution provided in the kit for 10 min. Cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Statistical Analysis

Data were presented as means ± standard error of the mean from at least three independent experiments. Independent *t*-test or one-way Analysis of Variance followed by Tukey test was used for determining statistical significance. Correlation between RUNX2 and miR-122 expression was tested by Spearman's correlation. $p < 0.05$ was the criterion for statistical significance.

Results

Analysis of miR-122 and RUNX2 Expression in Glioma Tissues

To understand the role of miR-122 in glioma, we first employed Real-time PCR analysis to compare its expression in normal brain tissues and glioma tissues. We found that miR-122 expression is significantly downregulated in glioma cells (Figure 1A). Importantly, the expression of miR-122 in high-grade glioma tissues is considerably lower than that in low-grade glioma tissues (Figure 1B). The RUNX2 gene is a critical regulator of cell proliferation and apoptosis. Our findings also indicated that the expression of RUNX2 is significantly higher in glioma tissues (Figure 1C), and its expression is higher in high-grade tissues compared with that in low-grade tissues (Figure 1D). Consistent with the expression data obtained in human tissue samples, miR-122 expression was found to be downregulated in multiple human glioma cell lines including U87, U251 and U118 (Figure 1E). Since U87 cells exhibit the lowest level of miR-122, we had chosen this cell line for further experimental analysis.

RUNX2 is a Direct Target of miR-122

The reciprocal expression pattern between *RUNX2* and miR-122 suggested that they might have some functional significance in glioma molecular pathogenesis. Indeed, the online database for microRNA-mRNA binding prediction (www.microrna.org) has revealed that miR-122 potentially targets RUNX2 in its 3'UTR region (Figure 2A). Overexpression of miR-122 in U87 cells significantly suppressed the expression of RUNX2 protein; on the contrary, transfection of the antisense oligonucleotide of miR-122 (as-miR-122) into U87 cells significantly increased the expression of RUNX2 (Figure 2B and C). To confirm whether this change in protein level is a direct effect of miR-122/RUNX2 interaction, we constructed a pGL3 reporter containing the 3'UTR sequence as indicated in Figure 2A and then performed luciferase reporter assay. We found that miR-122 showed a negative effect on reporter activity in RUNX2-wild-type reporter; however, no effect was observed for the RUNX2-mutant reporter (Figure 2D). Furthermore, in glioma tissues, a strong negative correlation between RUNX2 and miR-122 expression was observed (Figure 2E). Collectively, these data suggest that miR-122 negatively regulates RUNX2 expression by directly targeting its 3'UTR.

MiR-122 Inhibits Proliferation and Promotes Apoptosis in Glioma Cells

To functionally test the role of miR-122 in glioma cell malignancy, we first assessed whether miR-122 has an effect on cell proliferative activity using cell viability assay and colony forming assay. Forced expression of miR-122 greatly inhibited cell growth at the indicated time points (48 h, 72 h and 96 h), while inhibition of miR-122 exerts a promoting effect on cell growth (Figure 3A, B and C). The apoptosis is also regulated by miR-122, flow cytometry experiments revealed that apoptosis was significantly enhanced in U87 cells when transfected with miR-122, whereas it was inhibited by as-miR-122 (Figure 3D and E). These data suggest that miR-122 functions as a tumor suppressive gene in human glioma cells by regulating proliferation and apoptosis.

Overexpression of RUNX2 Partially Restores the Effect of miR-122

To confirm whether miR-122 achieved its function through the direct target gene, RUNX2, we cotransfected miR-122 and construct overexpressing RUNX2 into U87 cells. Cell viability and colony forming assay both showed that overexpression of RUNX2 partially reversed the inhibitory effect of miR-122 on cell proliferation (Figure 4A and B). Analysis of the apoptosis also showed that RUNX2 overexpression also partially reduced the pro-apoptotic effect induced by miR-122 (Figure 4C and D). These data indicate that miR-122 inhibits proliferation and induces apoptosis partially through inhibition of RUNX2.

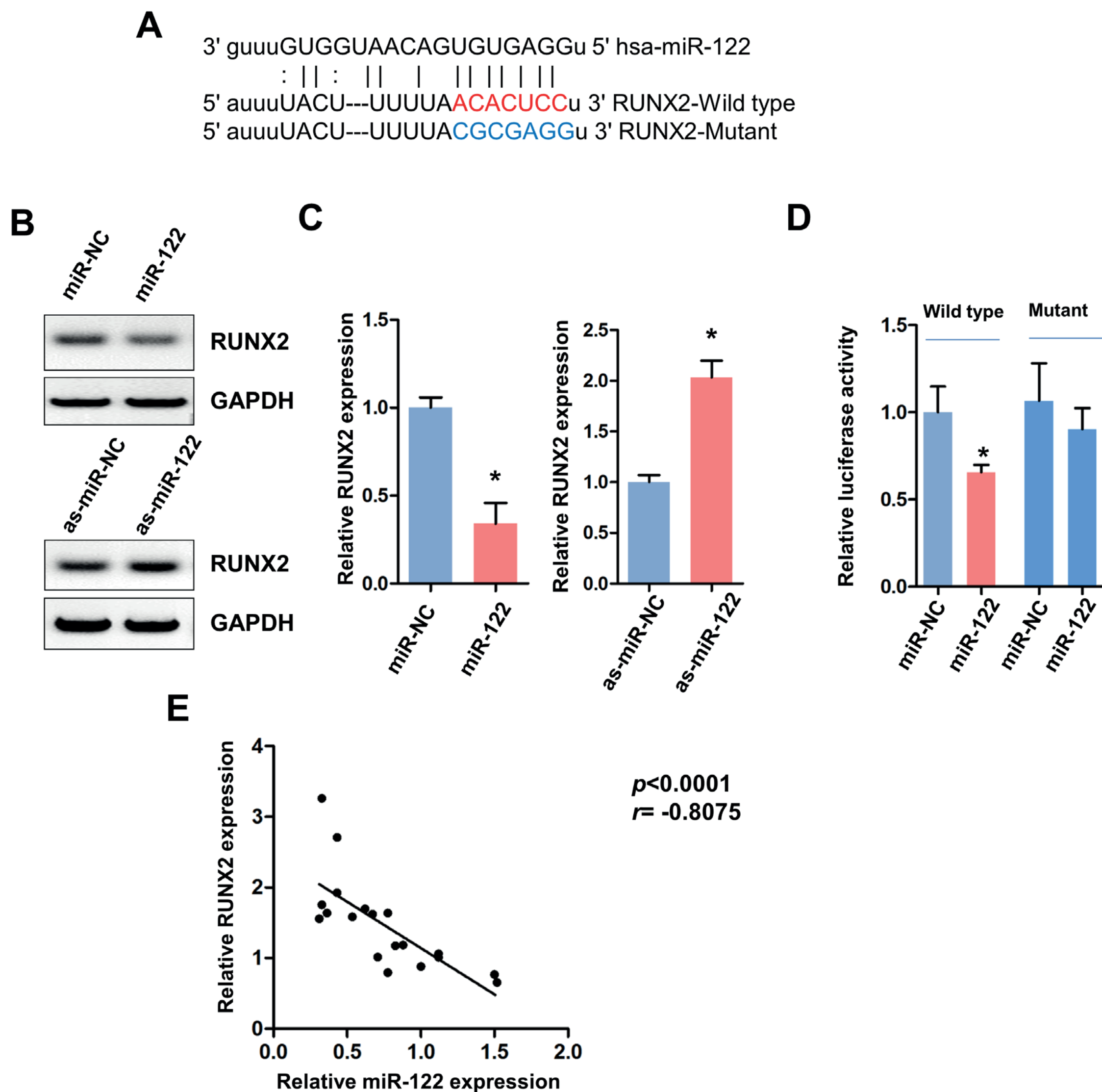

Figure 2. RUNX2 is a direct target of miR-122. ***(A)*** The predicted binding site of miR-122 in RUNX2 3'UTR, mutation of the sequence was marked in blue. ***(B)*** MiR-122 reduced the protein expression of RUNX2, whereas as-miR-122 increased its expression. ***(C)*** The quantification results of Western blot data shown in ***(B)***. ***(D)*** Transfection of miR-122 inhibited luciferase activity of wild type reporter but showed no effect on the activity of mutant reporter in glioma cells. ***(E)*** MiR-122 expression and RUNX2 expression were negatively correlated in glioma tissues. $*p < 0.05$ *vs.* miR-NC or as-miR-NC, $n \geq 3$.

Discussion

The mechanism of the pathogenesis of glioma is still elusive. Thus, identifying its molecular basis greatly benefits the drug development. Herein, we provide novel evidence showing that miR-122 is a tumor suppressor in glioma. MiR-122 is significantly downregulated in tumor specimens. Intriguingly, its expression is negatively associated with the tumor WHO grade, a significantly lower expression of miR-122 is observed in high-grade glioma tissues. On the contrary, RUNX2 is upregulated in glioma and exhibits the highest expression in high-grade glioma. Importantly, we for the first time establish the miR-122/RUNX2 signaling in glioma. We found that RUNX2 serves as a direct target of miR-122. RUNX2 overexpression counteracts the tumor-suppressive action of miR-122 by influencing at least two critical aspects of tumor cell behavior, proliferation, and apoptosis. Our study aids a better understanding towards the molecular pathogenesis of glioma.

The functions of microRNAs in the development and progression of glioma have been exten-

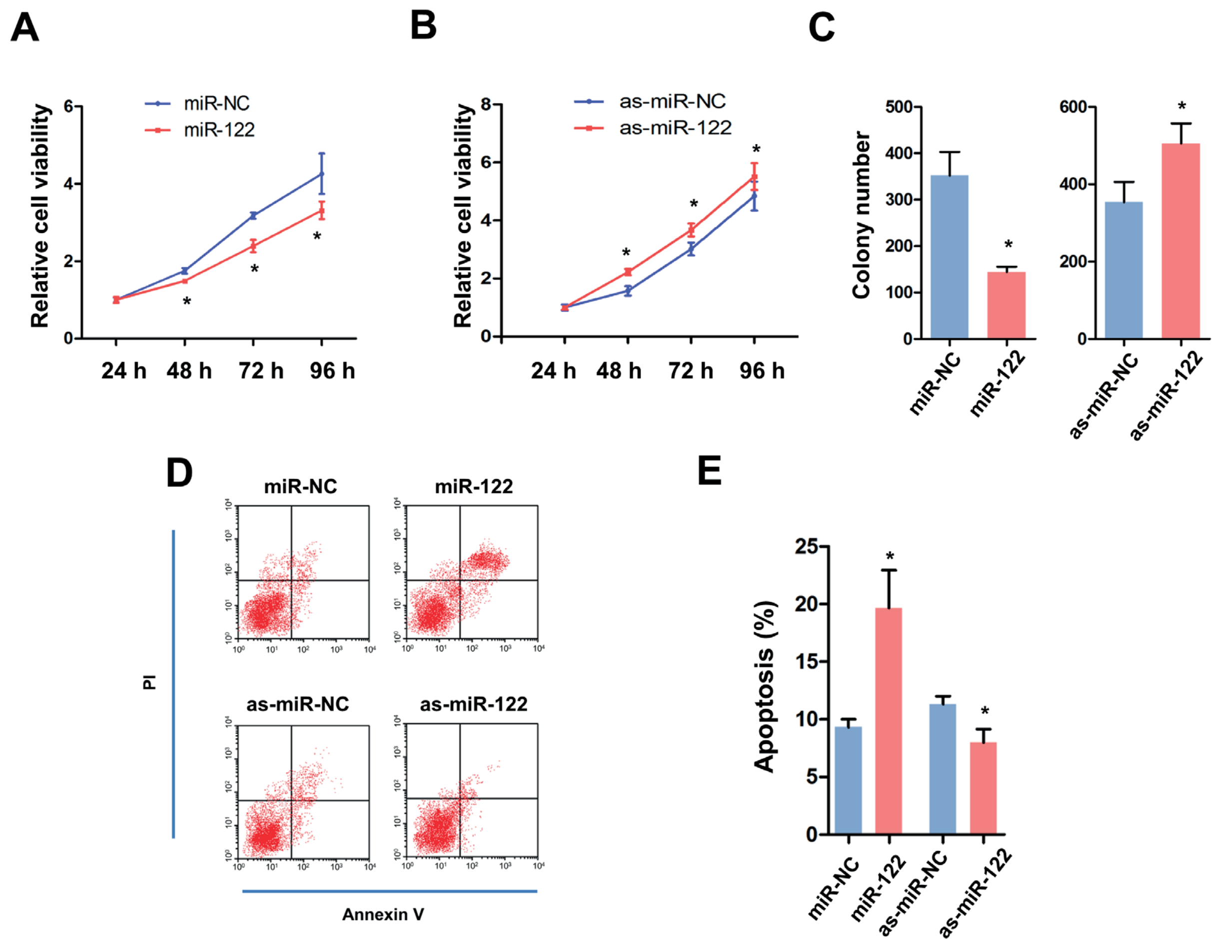

Figure 3. MiR-122 inhibits proliferation and promotes apoptosis in glioma cells. ***(A)*** Overexpression of miR-122 reduced cell viability at 48 h, 72 h and 96 h. ***(B)*** Transfection of as-miR-122 increased cell viability at 48 h, 72 h and 96 h. ***(C)*** MiR-122 inhibited, whereas as-miR-122 promoted colony formation of U87 cells. ***(D)*** MiR-122 promoted, whereas as-miR-122 inhibited apoptosis of U87 cells. (***E)*** The quantification for the results of apoptosis assay shown in ***(D)***. $*p < 0.05$ *vs.* miR-NC or as-miR-NC, $n \geq 3$.

sively studied[5]. A large number of studies have analyzed the differentially expressed microRNAs, and uncovered numerous oncomiRs and tumor suppressive miRs. By targeting distinct targets, these microRNAs are able to function to affect the cell behavior or cell fate via different target genes. For instance, both PDCD4 and TAp63 are reported to be targets of miR-21; and inhibition of miR-21 triggers cell apoptosis and inhibits cell growth activity[17-20]. MiR-17-92 cluster affects the cell proliferation, cell invasion and apoptosis by various targets including TGFβ-RII, Smad4 and CTGF[6,7]. MiR-130b upregulates MAPK/ERK signaling to promote proliferation and to inhibit apoptosis of human glioma cells[21]. On the contrary, miR-181a/b/c and miR-125b are downregulated and have well-defined roles in cellular processes including cell cycle regulation, cell migration and cancer cell stemness[5,8,9,11]. In the present work, we have focused our interest in studying the functional role of miR-122. A recent work[12] has shown that blood miR-122 level was significantly lower in glioma patients. Additional finding demonstrates that miR-122 level is further decreased in high-grade glioma and positively correlated with the prognosis, which suggests that blood miR-122 may serve as a potential diagnostic and prognostic marker[12]. Nevertheless, the function and mechanism of miR-122 in glioma has not been investigated in detail. Wang et al[16] have previously shown that overexpression of miR-122 suppressed proliferation of glioma by suppressing WNT signaling. Our report is in consistent with this work, which

corroborates the tumor suppressive role of miR-122 in glioma.

It is worthy to note that our research provided a novel mechanism by which miR-122 exerts its tumor suppressive action. Spearman correlation suggests that miR-122 and RUNX2 are strongly and negatively correlated. Combined with the bioinformatics analysis, we presumed that RUNX2 could be one of the targets for miR-122. This presumption was then experimentally validated by luciferase assay, which clearly showed that miR-122 binds to the 600 bp site of the RUNX2 3'UTR. RUNX2 is also known as core-binding factor subunit alpha-1 (CBF-alpha-1), and its function is initially identified in the bone. Loss of RUNX2 function leads to bone differentiation defects[22]. RUNX2 transcriptional factor physically interacts with several other factors such as SMADs, c-Myb, HDAC3 and C/EBP, enabling itself to be a critical modulator of cell proliferation[22-25]. The biological functions of RUNX family in glioma are still not well defined. Vladimirova et al[26] found that RUNX2 is expressed in glioma cells, and may contribute to glioma malignancy. It has been recently shown that miR-217 and miR-152 bind to the 3'UTR of RUNX2 directly to alleviate the malignancy of human glioma cells[27,28]. In our study, we demonstrate that RUNX2 is also a target of miR-122, adding complexity to the posttranscriptional regulation of RUNX2 in glioma cells. Of note, the identification of miR-122/RUNX2 signaling does not eliminate other possible mechanisms by which miR-122 regulates cell survival. In other cancer types, several oncogenes

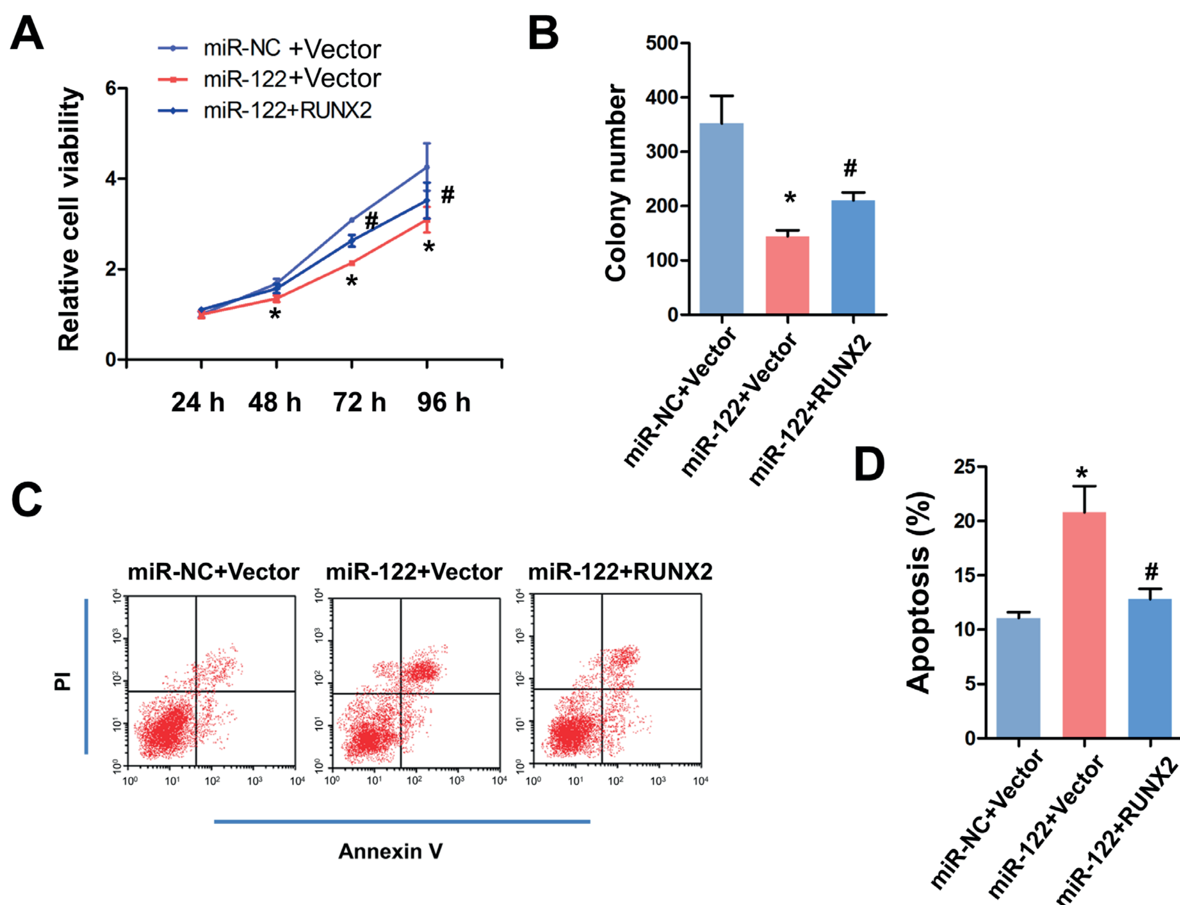

Figure 4. Overexpression of RUNX2 partially restores the effect of miR-122. ***(A)*** Overexpression of RUNX2 partially reversed the inhibitory effect of miR-122 on cell viability. (B) Overexpression of RUNX2 partially reversed the inhibitory effect of miR-122 on colony formation. ***(C)*** Overexpression of RUNX2 partially reversed the promoting effect of miR-122 on apoptosis. ***(D)*** The quantification for the results of apoptosis assay shown in ***(C)***. *$p < 0.05$ *vs.* miR-NC + Vector, # $p < 0.05$ *vs.* miR-122 + Vector n ≥ 3. Vector, the empty pcDNA3.1 construct.

such as SOX3, MMP9 and CREB1 have been established as targets of miR-122[29-31], these factors may work in concert with RUNX2 to mediate the function of miR-122.

Conclusions

We identified that miR-122 is a tumor suppressor in glioma, and its expression and WHO grades are significantly correlated. Downregulation of miR-122 may lead to a higher proliferative potential, which is partially mediated by the direct suppression of the RUNX2 transcriptional factor. Our study may provide novel insights into developing novel strategies for the treatment of malignant gliomas.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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