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Original Paper

MicroRNA-29a-3p Downregulation Causes Gab1 Upregulation to Promote Glioma Cell Proliferation

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Key Words
Glioma • Gab1 • MicroRNA-29a-3p • Cell proliferation

Abstract
Background/Aims: Glioma causes significant human mortalities annually. Molecularly-targeted therapy is a focus of glioma research. ***Methods:*** Grb2-associated binding 1 (Gab1) expression and microRNA-29a-3p ("miR-29a-3p") expression in human glioma cells and tissues were tested by Western blotting assay and qRT-PCR assay. shRNA/siRNA strategy was applied to silence Gab1 in human glioma cells. miR-29a or anti-sense miR-29a construct was transfected to human glioma cells. Cell proliferation was tested by BrdU ELISA assay and cell counting assay. ***Results:*** We show that expression of Gab1 was significantly elevated in human glioma tissues and cells, which correlated with downregulation of its putative microRNA: miR-29a-3p. In A172 glioma cells and primary human glioma cells, Gab1 shRNA/siRNA inhibited Akt-Erk activation and cell proliferation. Forced-expression of miR-29a-3p downregulated Gab1, inhibiting glioma cell proliferation, whereas miR-29a-3p was in-effective on cell proliferation in Gab1-silenced A172 cells. Furthermore, introduction of a 3'-untranslated region (3'-UTR) mutant Gab1 (UTR-G160A) blocked miR-29a-3p-induced inhibition on Akt signaling and A172 cell proliferation. ***Conclusions:*** miR-29a-3p downregulation leads to Gab1 upregulation to promote glioma cell proliferation.

Introduction

As one common brain tumor, glioma causes significant annual human mortalities [1-3]. Glioblastoma and other high-grade (grade III-IV) gliomas have an extremely poor prognosis [1-3], due to the ineffectiveness of the current treatments, including the surgical

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glioma resection, postoperative radiation and temozolomide chemotherapy [4-6]. Gliomas are characterized by extensive molecular heterogeneity [1], resulting in dysregulated and hyperactivation of multiple signaling pathways, including phosphatidylinositol 3-kinase (PI3K)-Akt and Erk-MAPK cascades, which positively contribute to glioma tumorigenesis and progression [7-9].

Grb2-associated binding (Gab) proteins are scaffolding/adapter family proteins, which include at least three members, Gab1, Gab2, and Gab3 [10-13]. Gab1 is the most predominant Gab family protein [10-13], and can dock with SH2- and SH3-containing signaling proteins [10-13]. Gab1 associates with p85, the PI3K regulatory subunit, to mediate downstream Akt activation [14, 15], whereas Gab1-SHP2 association is necessary for Erk-MAPK activation [15, 16]. It has previously been shown that Gab1 expression was elevated in human glioma cells [17]. However, the underlying mechanisms of Gab1 upregulation, as well as the potential functions of Gab1 in glioma cell progression have not been extensively studied.

microRNAs (miRNA), a class of endogenous small non-coding regulatory RNAs, can alter gene expression at both translational and post-transcriptional levels [18, 19]. The 19-24 nucleotide single-stranded noncoding RNAs bind to the 3' untranslated region (UTR) of their targeted genes, causing mRNA decay and/or translational block [18, 19]. miRNA are often dysregulated in human glioma [20-22]. Through searching multiple miRNA databases we identified a novel putative Gab1-targeting miRNA: microRNA-29a-3p ("miR-29a-3p"). Experimental validation shows that miR-29a-3p downregulation leads to Gab1 upregulation to promote glioma cell proliferation.

Materials and Methods

Ethics approval

The protocols of this study were according to the principles of Declaration of Helsinki, and were approved by the Soochow University's Ethics Review Board (ERB) and Internal Review Board (IRB).

Reagents

All the antibodies utilized in this study were described previously [14, 23-26]. Cell culture reagents were from Gibco (Shanghai, China). Puromycin was purchased from Sigma Chemicals (Shanghai, China).

A172 glioma cell line

The established human glioma A172 cell line was purchased from the Cell Bank of Shanghai Biological Institution (Shanghai, China). Cells was cultivated as described [26, 27]. Every four months, DNA fingerprinting and profiling were performed to confirm the cell line's origin. Cells were subjected to mycoplasma and microbial contamination examination every month. Population doubling time, colony forming efficiency, and morphology were also examined.

Human glioma tissues

As previously described [28], a total of thirty-two (32) different glioma patients (16 low-grade and 16 high-grade) at the Affiliated Hospitals of Soochow University (Suzhou, China) and Shanghai Xinchao (Shanghai, China), were enrolled. The preparations and examination of fresh human tissue specimens were also described early [26, 28]. Written informed-consent was obtained from each participant. The protocols of this study were according to the principles of Declaration of Helsinki, and were approved by the Soochow University's ERB and IRB.

Primary culture of human glioma cells and astrocytes

Establishment and culture of primary human glioma cells were described in detail in our previous studies [26-28]. In short, fresh human glioma tissues were thoroughly washed (in cold PBS), minced and filtered. Single-cell suspensions were obtained through digestion [26], and primary glioma cells were cultured in the described medium [25]. One primary human glioma cell line was established. The origin and culture of primary human astrocytes were also described in our previous study [26].

Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) assay

qRT-PCR was performed using an ABI7600 Prism equipment through the SYBR Green PCR kit. The detailed protocol was described previously [26, 28-32]. Quantification of *Gab1* mRNA was through the $^{\Delta\Delta}$Ct method [33]. *Gab1* mRNA primers were described in previous study [34]. *GAPDH* was tested as the internal reference gene [26]. The detection of mature hsa-miR-29a/b/c-3p and hsa-miR-29a-5p was examined by the TaqMan microRNA assay using the primers described [35-38]. For each preparation, ten (10) ng of RNA was reverse-transcribed using the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, Shanghai, China).

Forced-expression of miR-29a and anti-sense miR-29a ("antagomiR-29a")

The pre-miR-29a, obtained from Applied Biosystems, was sub-cloned into the HpaI and XhoI sites of pSuper-puro-GFP [29, 39] to generate the miR-29a expression vector. The vector was then co-transfected with the packaging plasmids VSVG and Hit-60 [14] via Lipofectamine 2000 to construct the viral particles in HEK293T cells. The viral particles were added to cultured glioma cells. Cells were then subjected to puromycin (1.0 μg/mL) selection for three passages. Control cells were constructed with non-sense scramble microRNA-control ("miR-C") [28]. For permanent inhibition of miR-29a, vectors with an anti-miR-29a sequence [40] was packaged into the virus.

Gab1 mRNA 3'-UTR luciferase assay

The reporter vector with the 3-UTR of *Gab1* carrying a putative miR-29a-3p binding site (Position 159-165) was constructed by Genepharm (Shanghai, China). The complementary oligonucleotides for the selected region were hybridized to form double-stranded DNA and inserted into pmIR-ReporterTM firefly luciferase vector (Genepharm). The construct was further co-transfected with the above miR-29a expression vector to A172 glioma cells. Cells were then lysed and analyzed by the luciferase assay kit (Promega, Shanghai, China). β-galactosidase luciferase was utilized as an internal control.

Western blotting assay

The detailed protocol for Western blotting assay has been extensively described in our previous studies [23, 24, 26]. Same set of lysate samples were run in sister gels to examine different proteins. For each lane, the exact amount of protein lysates were loaded. Image J software (NIH) was always employed to quantify the intensity of each band.

Gab1 stable knockdown

Two non-overlapping GV248 lentiviral shRNAs ("-a/-b", with non-overlapping sequences) against human Gab1 (NM_002039) were described previously [41]. The detailed protocol for shRNA infection and stable cell selection were described in our previous studies [14, 23-26, 31]. The scramble non-sense lentiviral shRNA ("scr-shRNA") [26] was added to the control cells. Gab1 knockdown in the stable cells was verified by Western blotting assay and/or qRT-PCR assay.

Gab1 siRNA

In the primary human glioma cells, siRNA method was applied to knockdown Gab1. Gab1 siRNA (sc-35431) and a negative control scramble siRNA were purchased from Santa Cruz Biotech (Shanghai, China). siRNA (200 nM each, 24 hours) transfection was performed via the described Lipofectamine 2000 (Invitrogen) method [42].

Gab1 mutation

Through *in vitro* site-directed mutagenesis system, a miRNA-29a-binding 3'-UTR-mutant Gab1 (Gab1-UTR-G160A, tagged with Flag) vector was constructed and verified by Genepharm (Shanghai, China). The construct was sub-cloned into the GV248 lentiviral vector, and was added to A172 glioma cells. Stable cells were again selected by puromycin (for 3-4 passages). Expression of the mutant Gab1 (UTR-G160A, tagged with Flag) in stable cells was tested by Western blotting assay.

Cell proliferation assay

Cell counting assay and BrdU incorporation ELISA assay of cell proliferation were described early in our previous studies [14, 23, 24, 26, 31].

Statistical analysis

Statistical analysis was performed as previously described [26, 43].

Results

Gab1 upregulation and microRNA-29a-3p downregulation in human glioma tissues and cells

To examine Gab1 upregulation in human gliomas, qRT-PCR was employed to test *Gab1mRNA* expression. As shown in Fig. 1A, *Gab1 mRNA* levels were significantly elevated in glioma tumor tissues ("T"), as compared to the surrounding normal brain tissues ("N"). Notably, *Gab1 mRNA* upregulation was greater in late-stage gliomas (Grade III-IV, n=16) than that in early-stage gliomas (Grade I-II, n=16) (Fig. 1A). Western blot analysis confirmed Gab1 protein upregulation in glioma tumor tissue (Fig. 1B, see quantification), results further confirmed greater Gab1 protein upregulation in late-stage gliomas than that in early-stage gliomas (Fig. 1B).

Using computational analysis of microRNA databases (TargetScan v7.1), we identified that the 3-UTR of human Gab1 (at position 159-165) (Fig. 1C) is a virtual target of microRNA-29a-3p ("miR-29a-3p"). miR-29a-3p showed highest percentile scores and low Context+ scores against Gab1 (TargetScan v7.1). Testing the expression of miR-29a-3p in human glioma tissues, we found that miR-29a-3p levels were significantly decreased in both early-stage and late-stage (Fig. 1D) gliomas, compared to the relatively high levels in normal brain tissue (Fig. 1D). Notably, miR-29a-3p downregulation was again more significant in late-stage gliomas (Fig. 1D). In contrast, the expression of other miR-29 family members, miR-29b-3p and miR-29c-3p, were unchanged between glioma tissues and normal brain tissues (Fig. 1E). Thus, miR-29a-3p is downregulated in human glioma tissues.

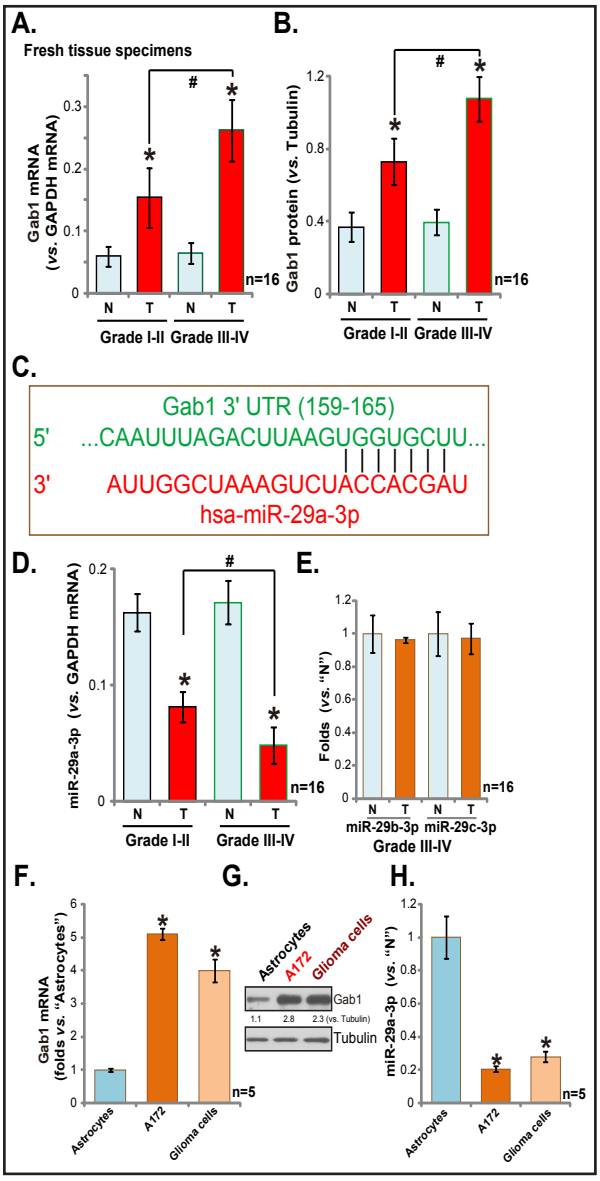

Fig. 1. Gab1 upregulation and microRNA-29a-3p depletion in human glioma tissues and cells. Fresh human glioma tissues ("T") and paired surrounding normal brain tissues ("N") were homogenized and dissolved in the tissue lysis buffer, Gab1 mRNA expression (vs. GAPDH mRNA) (A), microRNA-29a/b/c-3p expression (vs. GAPDH mRNA, D-E) were examined by qRT-PCR assay; Western blotting assay results showed expression of Gab1 in above patients' tissues (results were quantified, B). (C) microRNA-29a-3p ("miR-29a-3p") putatively targets the 3-UTR of human Gab1 (at position 159-165). Expressions of Gab1 mRNA (F), Gab1/Tubulin protein (G) and miR-29a-3p (H) in the listed cells were also shown. "MW" stands for molecular weight (Same for all Fig.). Bars stand for mean ± SD (Same for all Fig.). * $p<0.05$ vs. "N" group (A, B and D) or "Astrocytes" (F and H). # $p<0.05$ (A, B and D).

The upregulation of Gab1 expression in human glioma cells was correlated with down-regulation of miR-29a-3p. As compared to the primary human astrocytes ("Astrocytes") [26, 28], Gab1 (protein and *mRNA*) level was again elevated in established A172 glioma cells [26] and primary human glioma cells ("Glioma cells") (Fig. 1F and G) , whereas miR-29a-3p levels were decreased (Fig. 1H). These results suggest that miR-29a-3p is a putative anti-Gab1 miRNA.

Gab1 knockdown inhibits Akt-Erk activation and glioma cell proliferation

In order to study the function of Gab1 in human glioma cells, two distant lentiviral Gab1 shRNAs with non-overlapping sequences , "shGab1a" and "shGab1b" [41], were applied. Performing qRT-PCR (Fig. 2A) and Western blotting assays (Fig. 2B), we show that both shRNAs potently knocked down Gab1 in A172 glioma cells (Fig. 2A and B). Consequently, activation of Akt (pAkt at Thr-308) and Erk (p-Erk1/2 Thr202/Tyr204), two major Gab1 downstream pro-cancerous cascades [10, 12], were largely inhibited (Fig. 2B). As expected, expression of miR-29a-3p was not changed with Gab1 knockdown (Fig. 2C). Examining cell proliferation, employing viable cell counting assay and BrdU ELISA assay, we show that Gab1 shRNA knockdown significantly suppressed A172 cell proliferation, as the number of cells (at Day-4, Fig. 2D) and BrdU ELISA OD (Fig. 2E) were both decreased in Gab1-silenced cells.

To study the effect of Gab1 on the primary human glioma cells we utilized the siRNA strategy. The applied Gab1 siRNA dramatically decreased *Gab1* mRNA (Fig. 2F) and protein expression (Fig. 2G) in the primary cancer cells. Gab1 silence inhibited Akt and Erk activation (Fig. 2G) and proliferation of the primary cancer cells (Fig. 2H and I). These in vitro studies in glioma cells demonstrate that Gab1 knockdown inhibits Akt-Erk activation and cancer cell proliferation.

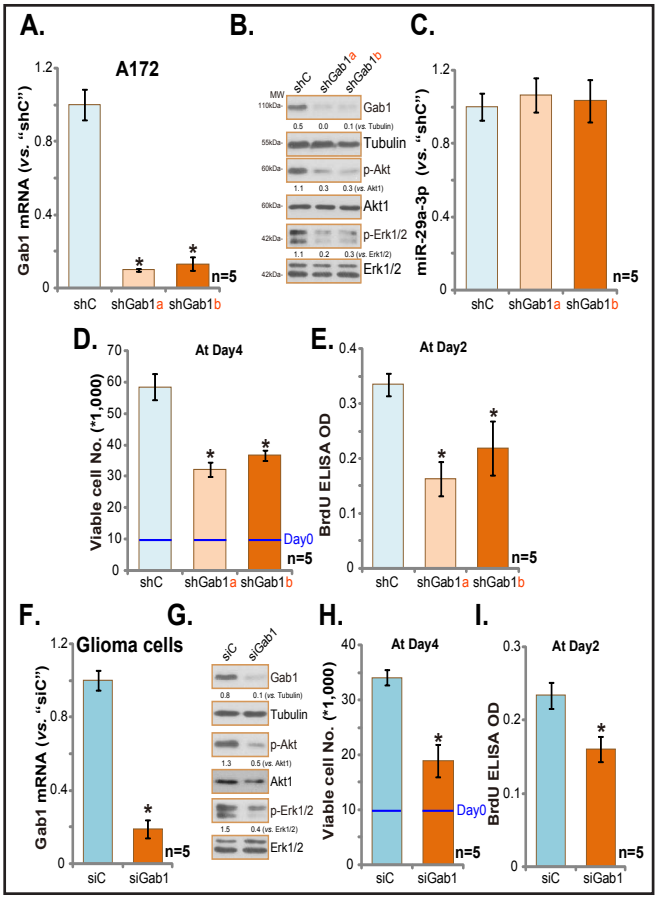

Fig. 2. Gab1 knockdown inhibits Akt-Erk activation and glioma cell proliferation. Stable A172 cells, constitutively expressing Gab1 shRNA ("-a/-b") or scramble control shRNA ("shC"), were subjected to qRT-PCR assay (A, to test Gab1 mRNA) and Western blotting analysis of listed proteins (B); miR-29a-3p expression was also tested (C). Above cells were also subjected to listed proliferation assays (D and E). Expressions of Gab1 mRNA (F) and listed proteins (G) in primary human glioma cells ("Glioma cells"), transfected with 200 nM of Gab1 siRNA ("siGab1") or scramble control siRNA ("siC"), were shown. Proliferation of these cells was also tested (H and I). Gab1 expression and Akt-Erk phosphorylations were quantified (B and G). * $p<0.05$ vs. "shC" or "siC" group. Experiments in this Fig. were repeated three times, with similar results obtained.

Forced-expression of miR-29a-3p silences Gab1, inhibiting glioma cell proliferation

To study the potential effect of miR-29a on Gab1 expression,

we constructed a miR-29a expression vector ("pSuper-puro-miR-29a", named as "Vec-miR-29a"). Using this vector, we established stable A172 cells constitutively-expressing "Vec-miR-29a". A qRT-PCR assay (Fig. *3A*) confirmed that miR-29a-3p levels were significantly elevated in the stable cells. Significantly, forced-expression of miR-29a-3p depleted Gab1 (mRNA and protein) in A172 cells (Fig. 3B and C). Activation of Akt (Fig. 3C) was also inhibited in "Vec-miR-29a"-expressing cells. More importantly, A172 cell proliferation, tested using cell counting (Fig. 3D) and BrdU ELISA (Fig. 3E) assays, was suppressed with forced-expression of miR-29a-3p. Expression of microRNA-control ("miR-C") had no effect on miR-29a-3p or Gab1 expression, as well as cell proliferation (Fig. 3A-E).

Exogenous expression of the anti-sense miR-29a ("antagomiR-29a") caused further miR-29a-3p downregulation (Fig. 3F), resulting in increased Gab1 expression and Akt activation (Fig. 3G), and enhanced cell proliferation (Fig. 3H). In the primary human glioma cells, forced-expression of "Vec-miR-29a" similarly induced miR-29a-3p upregulation (Fig. 3I), Gab1 silence (Fig. 3J), Akt suppression (Fig. 3J) and cell proliferation inhibition (Fig. 3K). These studies demonstrate that forced-expression of miR-29a-3p can silence Gab1 to inhibit glioma cell proliferation.

Gab1 is the primary target of miR-29a-3p in glioma cells

As recent studies have shown that miR-29a-3p could possibly have other targets [44, 45], we next examined if Gab1 is the direct and the primary target of miR-29a-3p in human glioma cells. Luciferase activity assay results demonstrate that *Gab1* mRNA 3-UTR luciferase activity was decreased significantly with forced-expression of miR-29a-3p, but was increased after antagomiR-29a expression (Fig. 4A). These results suggest that Gab1 is likely the direct target of miR-29a-3p.

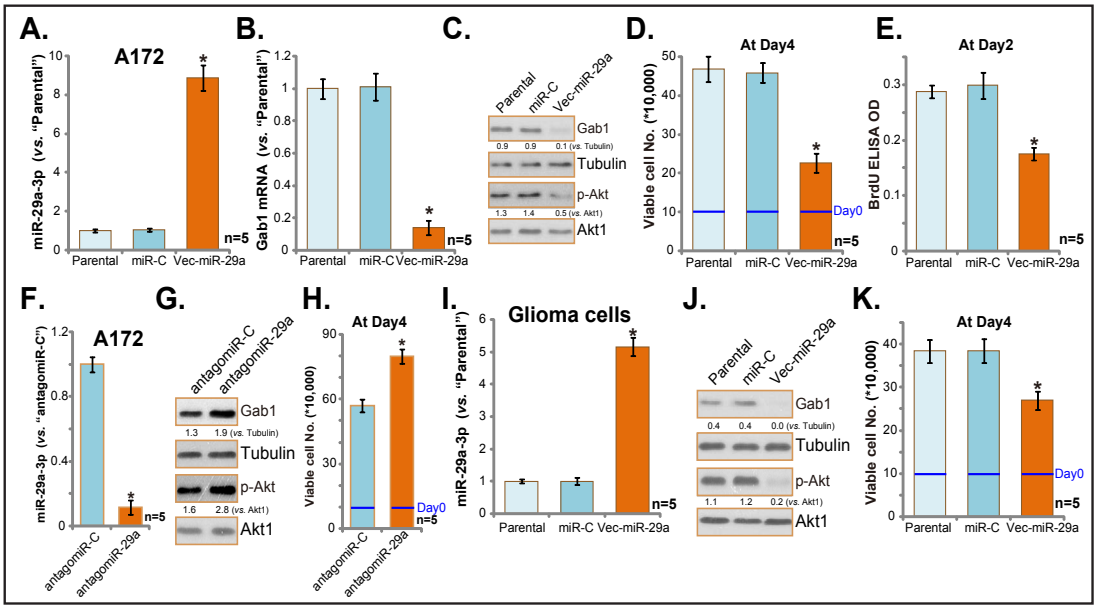

Fig. 3. Forced-expression of miR-29a-3p silences Gab1, inhibiting glioma cell proliferation. A172 glioma cells, expressing non-sense control miRNA ("miR-C"), miR-29a-pSuper-puro ("Vec-miR-29a"), as well as the anti-sense miR-29a ("antagomiR-29a") or antagomiR-control ("antagomiR-C"), were subjected to qRT-PCR assay to test expression of miR-29a-3p (A and F) and Gab1 mRNA (B); Listed proteins were tested by Western blotting assay (C and G); Cell proliferation was also tested (D, E, H). The primary human glioma cells, infected with "miR-C" or "Vec-miR-29a", were also subjected to above assays to test miR-29a-3p expression (I), listed protein expression (J) and cell proliferation (K). "Parental" stands for the parental control cells. Gab1 expression and Akt-Erk phosphorylations were quantified (C, G and J). * $p<0.05$ vs. "miR-C" or "antagomiR-C" group. Experiments in this Fig. were repeated three times, with similar results obtained.

If Gab1 is the primary target of miR-29a-3p, then forced-expression of miR-29a-3p should not affect cells with depleted Gab1. To test this hypothesis, we exogenously expressed "Vec-miR-29a" (see Fig. 3) in Gab1-shRNA-expressing A172 cells. As demonstrated, miR-29a-3p expression (Fig. 4C) failed to further inhibit proliferation of A172 cells with Gab1 shRNAs (-a/-b) (See Fig. 2). Thus, miR-29a-3p was unable to inhibit proliferation of Gab1-silenced cells, suggesting that Gab1 is the primary target of miR-29a-3p in A172 cells.

In further support of this hypothesis, we constructed a 3'-UTR-mutant Gab1 (UTR-G160A), that was not targeted by miR-29a-3p(Fig. 4D). Introduction of the 3'-UTR-mutant Gab1 almost completely blocked miR-29a-3p-induced inhibition of Akt activity (Fig. 4D) and proliferation (Fig. 4F and G). miR-29a-3p levels were equivalent between cells with/out the 3'-UTR-mutant Gab1 (Fig. 4E). These results suggest that Gab1 is the direct and primary target of miR-29a-3p in mediating its anti-glioma cell activity.

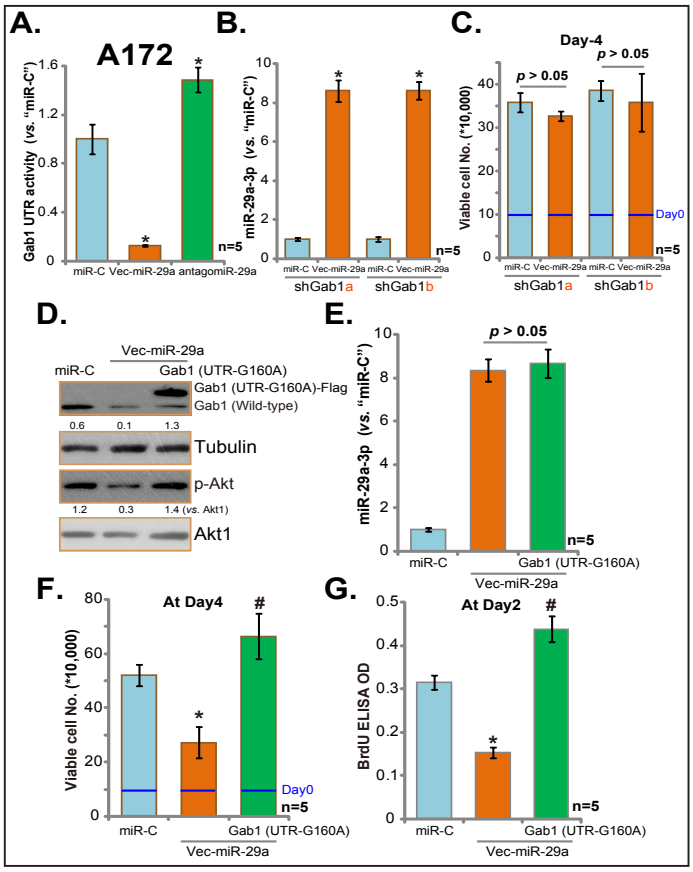

Fig. 4. Gab1 is the primary target of miR-29a-3p in glioma cells. A172 cells, expressing non-sense control miRNA ("miR-C"), miR-29a-pSuper-puro ("Vec-miR-29a") or the anti-sense miR-29a ("antagomiR-29a"), were subjected to luciferase activity to test Gab1 mRNA 3'-UTR activity (Fig. A). Stable A172 cells with indicated Gab1 shRNA ("-a/-b") were further constructed with "Vec-miR-29a" or "miR-C", miR-29a-3p expression (B) and cell proliferation (C) were tested. A172 cells with "miR-C" or "Vec-miR-29a" were further constructed with/out the 3'-UTR-mutant Gab1 (Gab1 UTR-G160A, tagged with Flag), listed protein expression (D), miR-29a-3p expression (E), and cell proliferation (F and G) were tested. Gab1 expression and Akt phosphorylation were quantified (D). * $p<0.05$ vs. "miR-C" group. # $p<0.05$ vs. "Vec-miR-29a" only group. Experiments in this Fig. were repeated three times, with similar results obtained.

Discussion

Simultaneous activation of multiple receptor tyrosine kinases (RTKs) in human glioma is believed to cause constitutive activation of multiple oncogenic downstream signalings, including PI3K-Akt and Erk-MAPK cascades [46, 47]. RTKs, including EGFR, VEGFR, FGFR and PDGFR, are important oncotarget proteins of glioma [47]. Thus, inhibition of one of these RTKs results in partial or no inhibition of downstream signaling and weak anti-glioma activity [46, 47]. Remarkably, the signaling transduction from these surface RTKs to downstream cascades relies heavily on adaptor/scaffold proteins, of which, Gab1 is a key adaptor protein [10, 12, 48]. We show that shRNA/siRNA knockdown of Gab1 largely attenuated Akt and Erk-MAPK activation in human glioma cells. More importantly, glioma cell proliferation was also significantly suppressed with Gab1 knockdown. Therefore, targeting

the adaptor protein Gab1 should be a reasonable strategy again glioma cells, especially as its expression is significantly elevated in human glioma tissues and cells.

miR-29s are well-established anti-tumor miRNAs [44, 45]. There are at least three members of miR-29 family miRNAs, including miR-29a, miR-29b and miR-29c [44, 45]. Aberrant expression (mainly downregulation) of miR-29 family miRNAs in multiple cancers is associated with tumorigenesis and cancer progression [44, 45]. miR-29 family miRNAs are known to target multiple tumor-related signaling proteins, including cell proliferation, cell cycle progression, cell survival, apoptosis-resistance and metastasis as well as epigenetic and immune-response [44, 45]. Therefore, miR-29s are well-established anti-tumor miRNAs [44, 45]. We show that miR-29a is downregulated in human glioma tissues. Meanwhile, forced-expression of miR-29a induced downregulation of Gab1 and glioma cell proliferation inhibition.

In the current study, we propose that *Gab1 mRNA* is the target of miR-29a-3p, acting as a tumor suppressor. In both established (A172 cell line) and primary human glioma cells, forced-expression of miR-29a-3p caused Gab1 repression at both mRNA and protein level, leading to downstream Akt-Erk inhibition. Downregulation of miR-29a-3p via antagomiR-29a caused Gab1 upregulation and enhanced downstream signaling activation. Significantly, the *Gab1* mRNA 3-UTR activity was significantly inhibited by miR-29a-3p expression, but was enhanced with antagomiR-29a expression.

Recent studies have proposed that miR-29 could be a novel anti-cancer miR. For example, Park et al., demonstrated that miR-29 activated p53 by targeting p85alpha and CDC42, and promoted cancer cell apoptosis [49]. Furthermore, miR-29 expression led to direct downregulation of Mcl-1, an anti-apoptotic Bcl-2 family member, to promote cancer cell apoptosis [50]. In the current study, we provide evidence to support that Gab1 is the primary target protein of miR-29a-3p in mediating its anti-glioma cell activity. Forced-expression of miR-29a-3p was ineffectual against proliferation in glioma cells when Gab1 was already silenced. Remarkably, introduction of an UTR mutant Gab1 was able to completely block miR-29a-3p-induced inhibition on human glioma cells. Thus, miR-29a-3p inhibits glioma cell proliferation possibly via directly and primarily silencing its target Gab1.

The significance of miRs in diagnostic and prognostic determination has been demonstrated in glioma [20-22] and a number of other human cancers [51, 52]. In the current study, we provided the quantitative description of a potential relationship between decreased miR-29-3p expression and the grade of malignant glioma patients. According to our results, miR-29-3p is expressed at low levels in glioma, and higher WHO grade gliomas tend to have even lower expression of miR-29-3p. It will certainly be interesting to further test the significance of miR-29-3p downregulation in the diagnosis and prognosis of human glioma.

Conclusion

In summary, miR-29a-3p downregulation leads to Gab1 upregulation to promote glioma cell proliferation. Expression of miR-29a-3p to block Gab1 expression could provide a promising therapeutic strategy to inhibit RTK-mediated glioma progression.

Acknowledgements

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of the project conceived of the study, and participated in its design and coordination and helped to draft the manuscript.

Disclosure Statement

None of the authors have any competing interests in the manuscript.

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