

**MOL #76166**

**MiR-335 is required for differentiation of malignant glioma  
cells induced by activation of cAMP/PKA pathway**

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**Running title:** The crucial role of miR-335 in glioma cell differentiation.

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224 words in Abstract

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**Abbreviations:** GBM, glioblastoma multiforme; APL, acute promyelocytic leukemia; PKA , protein kinase A; GFAP, glial fibrillary acidic protein; miRNA , microRNA; qRT-PCR, quantitative real-time PCR; NC, negative control; MEST , mesoderm-specific transcript.

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### **Abstract**

Glioma is the most common malignant cancer affecting the central nerve system, with dismal prognosis. Differentiation-inducing therapy is a novel strategy that has been preliminarily proved effective against malignant glioma. Previously we reported that activation of cAMP/protein kinase A (PKA) pathway is capable of inducing glioma cell differentiation, characterized by astrocyte-like shape and dramatic astrocyte biomarker-gliial fibrillary acidic protein (GFAP) induction. However, little progress has been made on molecular mechanisms related. Here we demonstrate that miR-335 is responsible for the glioma cell differentiation stimulated by activation of cAMP/PKA pathway. In the cAMP elevator cholera toxin-induced differentiation model of rat C6 glioma cells, miR-335 was significantly up-regulated, which was mimicked by other typical cAMP/PKA pathway activators forskolin, dbcAMP and abolished by PKA specific inhibitor KT5720. In a gain and loss of miR-335 function assay, exogenous miR-335 resulted in induction of GFAP, while miR-335 specific inhibitor antagomir-335 violently blocked the cholera toxin-induced GFAP up-regulation. Importantly, in human U87-MG glioma cells and human primary culture glioma cells, miR-335 also mediated cholera toxin-induced differentiation. Taken together, our findings suggest that miR-335 is potentially required for differentiation of malignant glioma cells induced by cAMP/PKA pathway activation and one single microRNA may act as an important fate determinant to control the differentiation status of malignant gliomas, which has provided a new insight into the differentiation-inducing therapy against malignant gliomas.

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### **Introduction**

Gliomas derived from astrocytes or astroglial precursors are the most common primary tumors affecting the adult central nervous system, accounting for >60% of primary brain tumors (DeAngelis, 2001). The majority of gliomas in adults are highly malignant with a poor prognosis, in particular with high-grade tumors such as glioblastoma multiforme (GBM) (Trog et al., 2006). Current therapy with surgery, radiation, and chemotherapy rarely, if ever, cures the disease and infrequently prolongs life for >1 year (Bao et al., 2006; Curran et al., 1993).

Despite recent advances in malignant glioma treatment, truly innovative approaches are required to move beyond the modest benefits achieved to date. One novel strategy is the differentiation-inducing therapy using agents that modify cancer cell differentiation. Previously, all-trans-retinoic acid has been used as an agent to induce cell differentiation in clinical treatment of acute promyelocytic leukemia (APL) (Huang et al., 1988), demonstrating the remarkable efficacy of differentiation therapy in treatment of cancers. Such excellent effects, however, were not reproduced in other hematological and, particularly, solid tumors. Finding potent differentiation agents for malignant gliomas remains a real challenge. Notably, the classic stimulators of cAMP/ protein kinase A (PKA) pathway such as cholera toxin and forskolin, have been proven to induce astrocytic differentiation of malignant glioma cells characterized by the astrocyte-like shape and the accumulation of the reliable biomarker glial fibrillary acidic protein (GFAP) (He et al., 2011; Li et al., 2007; Lu et al., 2009). Nevertheless, a detailed knowledge of the molecular mechanism related is still largely unknown.

MicroRNAs (miRNAs) are small non-coding RNAs (18 to 25 nucleotides) with potential

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roles in regulation of gene expression at posttranscriptional levels (Esquela-Kerscher and Slack, 2006). Cumulative evidences suggest that miRNAs control a wide array of biological processes including cell differentiation, proliferation, and apoptosis whose dysregulation is a hallmark of cancer. MiR-335, transcribed from the genomic region chromosome 7q32.2, has been reported to act as a suppressor of tumor initiation and metastasis in breast cancer (Png et al., 2011; Tavazoie et al., 2008). Furthermore, it is also demonstrated that miR-335 regulates Rb1 and controls cell proliferation in a p53-dependent manner (Scarola et al., 2010). Of note, a recent study has shown that miR-335 orchestrates cell proliferation, migration and differentiation in human mesenchymal stem cells (Tome et al., 2011). These investigations indicate the important roles of miR-335 in tumor initiation and progression; however, the biological role of miR-335 in malignant glioma cell differentiation is still missing and perplexing.

In the present study, we use a traditional biotoxin, cholera toxin, which catalyzes ADP-ribosylation of Gs protein and results in accumulation of cellular cAMP (Guerrant et al., 1994), as a tool to pharmacologically manipulate the activation of cAMP/PKA pathway and induce the differentiation of malignant glioma cells. The results demonstrate that cholera toxin stimulates miR-335 expression via cAMP/PKA pathway and this stimulation is potentially required for cholera toxin-induced astrocytic differentiation of malignant gliomas. Our findings suggest that one single miRNA appears to control the differentiation status of glioma cells, which also provides a new insight into the differentiation therapy against malignant gliomas.

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### **Materials and Methods**

**Cell cultures and drug treatment.** Rat glioma cell line C6 cells and human glioma cell line U87-MG cells were obtained from the American Type Culture Collection (Manassas, USA). Cells were maintained in DMEM supplemented with 10% FBS (Invitrogen, Grand Island, NY) and a humidified atmosphere of 5% CO<sub>2</sub> at 37°C. Human primary culture glioma cells were isolated from three human glioma tissues which were obtained immediately after surgical removal with informed consent from each patient. Tumors were classified according to the WHO classification system as WHO Grade II astrocytoma (1 tumor) and WHO Grade IV astrocytoma (GBM; 2 tumors). The isolation and culture of primary glioma cells were prepared as previously described (Li et al., 2007). C6 cells were treated with cholera toxin, forskolin, dbcAMP and KT5720 (Sigma-Aldrich, St. Louis, MO) in DMEM containing 1% FBS. For U87-MG cells and primary cells, cholera toxin was added to DMEM containing 5% FBS. Control was treated with an equivalent volume of DMEM containing 1% or 5% FBS.

**Morphological evaluation.** The cell morphologies were studied during the indicated time course using an IX71 inverted microscope and a DP70 CCD camera (Olympus, Melville, NY).

**Western blot analysis.** Western blot was performed as described (Akagi et al., 2002; Shu et al., 2011a). The following antibodies were used: antibodies against GFAP (1:1,000; Cell Signaling Technology, Danvers, MA) and  $\beta$ -actin (1:2,000; Neomarkers, Fremont, CA).

**MiRNA microarray assay.** Paraflo miRNA microarray was performed by a service provider (LC Sciences, USA) and data were analyzed. C6 cells were treated with or without 10 ng/ml cholera toxin for indicated times. Total RNA samples (2 to 5  $\mu$ g) were size fractionated using a YM-100 microcon centrifugal filter (Millipore) and small RNAs (<300 nt) were 3'-extended

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with a poly (A) tail using poly (A) polymerase. An oligonucleotide tag was then ligated to the poly (A) tail for later fluorescent dye staining; two different tags were used for the two RNA samples in dual-sample experiments. Hybridization was executed overnight on a  $\mu$ Paraflo™ microfluidic chip using a micro-circulation pump (Atactic Technologies) (Gao et al., 2004). On the microfluidic chip, each detection probe consisted of a chemically modified nucleotide coding segment complementary to target miRNA and a spacer segment of polyethylene glycol to extend the coding segment away from the substrate. Detection probes were made *in situ* using PGR (photogenerated reagent) chemistry. Hybridization melting temperatures were balanced by chemical modifications of the detection probes. Hybridization was performed using 100  $\mu$ l 6 $\times$ SSPE buffer (0.90 M NaCl, 60 mM Na<sub>2</sub>HPO<sub>4</sub>, 6 mM EDTA, pH 6.8) containing 25% formamide at 34°C. After hybridization detection used fluorescence labeling using tag-specific Cy5 dyes. Hybridization images were collected using a laser scanner (GenePix 4000B, Molecular Device) and digitized using Array-Pro image analysis software (Media Cybernetics).

**Quantitative real-time PCR (qRT-PCR).** Total RNA was prepared using TRIZOL reagent (Invitrogen). Expression of mature miR-335 was determined by stem-loop primer SYBR Green qRT-PCR and normalized to U6. The stem-loop primer sequence for reverse transcription was as follows: 5'- CTC AAC TGG TGT CGT GGA GTC GGC AAT TCA GTT GAG ACA TTT TT -3'. The generated cDNA was amplified with primers for miR-335 (5'-ACA CTC CAG CTG GGT CAA GAG CAA TAA CGA AA-3' and 5'- CTC AAC TGG TGT CGT GGA -3'). All qRT-PCR were performed in triplicates.

**Oligonucleotide transfection.** MiR-335 mimics, negative control (NC), antagomir-335 and

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antagomir-NC (RiboBio, Guangzhou, China) were transfected using Lipofectamine RNAiMAX (Invitrogen) according to manufacturer suggested procedures. Transfection efficiency was evaluated by Cy3-labeled oligonucleotides negative control.

**Statistical analysis.** Data are presented as mean  $\pm$  SD of three separated experiments if not noticed. A difference with a  $p < 0.05$  by the two-sample Student's *t* test or one-factor ANOVA analysis was considered statistical significant.

## **Results**

### **Cholera toxin induces differentiation of C6 glioma cells.**

Microscopic observation of C6 glioma cells treated with 10 ng/ml cholera toxin for the indicated times revealed major alterations in morphology. We first observed that the cell numbers were significantly reduced after cholera toxin treatment compared with control group. Interestingly, unlike the mainly polygonal morphology of the control, the shape of cholera toxin-treated cells was similar to that of mature astrocytes, with smaller round cell bodies and much longer, fine, tapering processes (Fig. 1A). Importantly, GFAP protein, an established biomarker of mature astrocytes was dramatically up-regulated as early as 24 h treatment of cholera toxin and kept rising until 72 h (Fig. 1B). These results are consistent with our previous data (Li et al., 2007) and indicate that cholera toxin can induce glioma cell differentiation, characterized with the astrocyte-like shape and the GFAP stimulation.

### **Cholera toxin induces miR-335 expression.**

MiRNAs have been shown to carry out post-transcriptional control of a multitude of cellular processes. To determine whether miRNAs participate in the cholera toxin-induced glioma cell



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differentiation, we first compared the miRNA expression profiles of C6 glioma cells treated with or without cholera toxin for 6, 12 and 24 h, respectively. As shown in Supplemental Table 1, many of the 875 miRNAs analyzed exhibited significantly differential expression in response to cholera toxin, however, only miR-335 displayed up-regulated uniformly in three time spots. The relative expression of miR-335 presented in a Heat Map further revealed this trend (Fig. 2A). Furthermore, data from qRT-PCR analysis, showing that miR-335 expression rose with 6 h of cholera toxin stimulation and kept increased until 24 h, is in line with data from miRNA profile (Fig. 2B). All together, these results indicate that cholera toxin induces miR-335 expression in C6 glioma cells.

### **MiR-335 elevation induced by cholera toxin is mediated by activation of cAMP/PKA pathway.**

To determine the role of cAMP/PKA signaling in induction of miR-335 expression, we used other two cAMP increasing reagents forskolin which can activate adenylate cyclase (AC) to produce more cAMP and dbcAMP which is analogue with cAMP. As expected, 10  $\mu$ M forskolin and 1 mM dbcAMP both significantly induced miR-335 expression (Fig. 3A). Conversely, 5  $\mu$ M PKA specific inhibitor KT5720 almost completely abrogated the miR-335 induction by cholera toxin (Fig. 3B). All the data indicate that activation of cAMP/PKA pathway mediates miR-335 elevation in C6 glioma cells.

### **MiR-335 is essential for cholera toxin-induced differentiation of C6 glioma cells.**

To investigate biological functions of miR-335 in the cholera toxin-induced differentiation of glioma cells, we designed a gain and loss of miR-335 function assay. Obviously, miR-335 over-expression triggers C6 cells to transform into a more mature astrocytic state (Fig. 4A). In

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the paralleled western blot analysis, miR-335 moderately stimulated GFAP expression and cooperated with cholera toxin to promote GFAP accumulation (Fig. 4B). Conversely, miR-335 specific inhibitor antagomir-335 apparently reduced the GFAP expression and strongly abrogated the cholera toxin-stimulated GFAP induction as well as the morphologic change (Fig. 4C and D). Taken together, we can deduce that miR-335 is potentially required for the cholera toxin-induced differentiation of glioma cells.

### **MiR-335 is also required for cholera toxin-induced differentiation in human glioma cell line U87-MG cells and human primary cultured glioma cells.**

To test whether the effects of miR-335 in cholera toxin-induced differentiation is species-specific or not, human glioma cell line U87-MG cells and human primary cultured glioma cells were utilized. As Figure 5A, B and 6A, B show, cholera toxin initiated both U87-MG cells and primary cultures to transform into more mature astrocyte-like ones with a star-shaped cell body, pronounced elongation of filamentous processes and to produce more GFAP, indicating the pro-differentiation effect of cAMP signalling activation in human glioma cells. Data from qRT-PCR revealed that cholera toxin also gave rise to miR-335 accumulation in U87-MG cells and primary cultures from three clinical glioma tissues (Figure 5C and 6C). In the following gain and loss of miR-335 function assay, the pro-differentiation effect of miR-335 similar to cholera toxin (Figure 5D, E and 6D, E) and the abolishment of pro-differentiation capability by antagomir-335 against cholera toxin (Figure 5F, G and 6F, G), were also observed whatever in human glioma cell line or human primary cultured glioma cells, further suggesting that a pivotal role of miR-335 in cAMP elevator-induced glioma cell differentiation, which is evolutionarily conserved from rat to human.

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### **Discussion**

Activation of cAMP/PKA pathway has been proven to be an effective approach to induce differentiation of malignant gliomas. However, molecular mechanisms related are still poorly understood. Here we show that miR-335 plays an essential role in the cAMP/PKA pathway activator-induced differentiation of rat C6 glioma cells, human U87-MG glioma cells and particularly human primary culture glioma cells. Thus, miR-335 as an evolutionarily conserved miRNA might represent a potential therapeutic target for the differentiation therapy of this highly aggressive and, as yet, therapy- refractory tumor.

cAMP is a well-known classic second messenger (Moss and Vaughan, 1979), and ample evidences indicate that cAMP-elevating stimuli such as N-substituted cAMP analogues and cAMP-increasing reagents can induce cell differentiation in gliomas (Takanaga et al., 2004; Van Kolen and Slegers, 2004). Consistently, we previously reported that cAMP elevators cholera toxin, forskolin and analogues dbcAMP are capable of inducing differentiation of glioma cells via cAMP/PKA/CREB pathway (Li et al., 2007). In the present study, we further found that these cAMP/PKA pathway activators dramatically stimulate miR-335 expression whereas PKA specific inhibitor strongly blocks the miR-335 elevation, indicating that activation of the cAMP/PKA pathway makes a critical contribution to the miR-335 accumulation. Furthermore, the gain and loss of miR-335 function assay proved the conclusive role of miR-335 in glioma cell differentiation. Taken together, these data has provided strong evidences that cAMP/PKA pathway initiators act as triggers of miR-335 accumulation to induce glioma cell differentiation.

It is known that miR-335 is an intronic miRNA resided on the intron region of its cognate

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protein-coding gene *MEST* (Ronchetti et al., 2008). Based on evidences that intronic miRNAs are frequently co-expressed with their host gene, we postulate that miR-335 expression might be under the control of the same regulative motif as the host gene *MEST* and processed from the same primary mRNA transcript regulated by RNA polymerase II in malignant glioma cells. In agreement with this postulation, it is reported that miR-335 is coordinately expressed with *MEST* gene in multiple myeloma (Ronchetti et al., 2008). Intriguingly, we also observed that *MEST* mRNA level was significantly increased in a time-dependent manner after cholera toxin stimulation in C6 cells, detected by mRNA microarray (Supplementary Figure 1). All these data support a hypothesis that downstream effectors of PKA may activate *MEST* transcription concomitant with miR-335 expression, which will be investigated in our subsequent study.

GFAP is a member of the family of intermediate filaments specific for astrocyte and a well established astrocytic differentiation biomarker (McKeown-Longo et al., 1984). We have reported that activation of IL-6/JAK2/STAT3 pathway partially mediates the cholera toxin-activated GFAP expression (Shu et al., 2011b), suggesting that endogenous cytokines might be as potential candidates of differentiation agents against malignant gliomas. In this study, we found that exogenous miR-335 clearly induced GFAP expression, whereas knockdown of endogenous miR-335 markedly abrogated GFAP accumulation by cholera toxin, indicating that miR-335 is potently required for cAMP/PKA activation-induced astrocytic differentiation of malignant gliomas and miRNA analogies might work as differentiation agents or synergistic ones against malignant gliomas. In addition, despite no potential targets of miR-335 which negatively regulate the IL-6/JAK2/STAT3 pathway have

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been discovered, all the data mentioned above support a possibility that miR-335 might have a crosstalk with IL-6/JAK2/STAT3 pathway and suggest the occurrence of epigenetic mechanism on GFAP expression. No matter how, to our knowledge, there are no previous report that assess the role of miR-335 and its effect on malignant gliomas in the field of cellular differentiation.

In this study, we designed to concentrate on those miRNAs whose expression patterns are consistent in all tested time spots and found that only one miRNA, miR-335, accord with the defined-criterion. Although we focus on miR-335 in the present investigation, several other miRNAs displaying differentially expression might be involved in cAMP/PKA pathway activation-induced differentiation (Table 1). For example, two well-known oncogenic miRNAs miR-221 and miR-222, which have been proven to promote cell growth by targeting *P27* and *P21* (Fu et al., 2011; Kim et al., 2009; Park et al., 2009), were both reduced within 12 h and 24 h of cholera toxin stimulation (Supplemental Figure 2). Considering the post-transcriptional regulation of miRNA on target genes, the down-regulation of miR-221 and -222 might theoretically lead to the up-regulation of P27 and P21 proteins, which is verified by our previous data showing that cholera toxin increases P27 and P21 proteins expression in a dose-dependent manner (Li et al., 2007). In addition, another increased miR-204 (Supplemental Figure 3), which acts as a tumor suppressor gene by inhibiting anti-apoptotic proteins Bcl-2 and Mcl-1 (Chen et al., 2009), at least in part, facilitates glioma cell differentiation and subsequent apoptosis. All these findings indicate that activation of cAMP/PKA pathway-induced differentiation of malignant glioma might be directed by multi-miRNAs and each miRNA appears to play its unique role.

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In conclusion, our data demonstrated that miR-335 may be a determinant of cellular differentiation induced by activation of cAMP/PKA cascade in malignant tumors, which opens a new insight for our better understanding the molecular mechanism underlying differentiation therapy of malignant gliomas and encourages miR-335 as a novel therapeutic target.

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**Authorship Contributions:**

*Participated in research design:* Yan, Shu, Zhou and Zhu.

*Conducted experiments:* Shu, Zhou, Zhu, Zhang and Wu.

*Contributed new reagents or analytic tools:* Zhang, Wu and Chen.

*Performed data analysis:* Shu, Zhou, Zhu and Chen.

*Wrote or contributed to the writing of the manuscript:* Shu and Zhu.

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### Legends for figures

**Figure 1. Cholera toxin induces differentiation of C6 malignant glioma cells.** A, morphologic transformation (Original magnification:  $\times 200$ ). C6 cells were incubated with 10 ng/ml cholera toxin for indicated time courses. B, Time-dependent effect of GFAP protein expression.

**Figure 2. MiR-335 is highly up-regulated after cholera toxin incubation in C6 glioma cells.** A, Heat map of specific miRNAs expression (up-panel) and the relative expression level of miR-335 (down-panel). Cells were incubated with 10 ng/ml cholera toxin for indicated time courses, and then detected by miRNA microarray. B, The relative expression level of miR-335. Cells were treated with 10 ng/ml cholera toxin for indicated time courses, and then detected by qRT-PCR. \*\*,  $p < 0.01$ .

**Figure 3. Activation of cAMP/PKA pathway mediates the cholera toxin-induced miR-335 accumulation in C6 glioma cells.** A, The effect of cholera toxin, forskolin and dbcAMP on miR-335 expression. Cells were treated with 10 ng/ml cholera toxin, 10  $\mu$ M forskolin or 1mM dbcAMP for 24 h, respectively. B, The effect of PKA inhibitor KT5720 on miR-335 expression. Cells were treated with 10 ng/ml cholera toxin and /or 5  $\mu$ M KT5720 for 24 h. MiR-335 expression was determined by qRT-PCR. \*\*,  $p < 0.01$ .

**Figure 4. MiR-335 is involved in cholera toxin-induced differentiation of C6 glioma cells.** A and B, The effect of miR-335 on morphology transformation (Original magnification:  $\times 200$ ) and the protein expression of GFAP. C and D, The effect of antagomir-335 on morphology transformation (Original magnification:  $\times 200$ ) and the protein expression of GFAP. C6 cells were transfected with 50 nM miR-335, 50 nM antagomir-335 and/or 10 ng/ml

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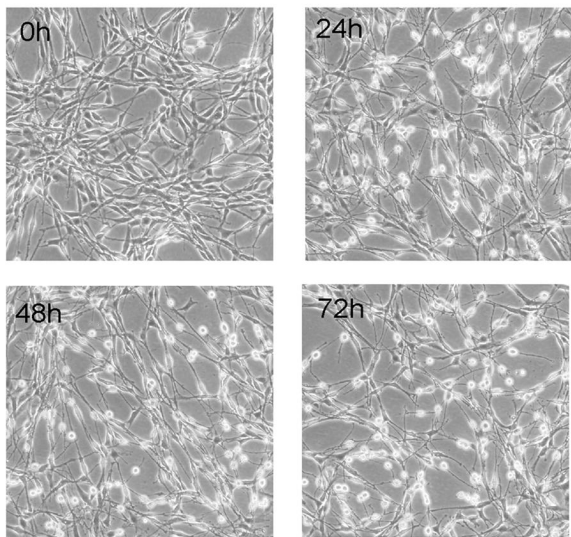
cholera toxin for 48 h.

**Figure 5. MiR-335 is also responsible for cholera toxin-induced differentiation in human glioma cell line U87-MG cells.** A, B and C, The effect of cholera toxin on morphology transformation (Original magnification:  $\times 200$ ), the protein expression of GFAP and the expression of miR-335. U87-MG cells were treated with 100 ng/ml cholera toxin for 24 h. \*\*,  $p < 0.01$ . D and E, The effect of miR-335 on morphology transformation (Original magnification:  $\times 200$ ) and the protein expression of GFAP. F and G, The effect of antagomir-335 on morphology transformation (Original magnification:  $\times 200$ ) and the protein expression of GFAP. Cells were transfected with 50 nM miR-335, 50 nM antagomir-335 and/or 100 ng/ml cholera toxin for 48 h.

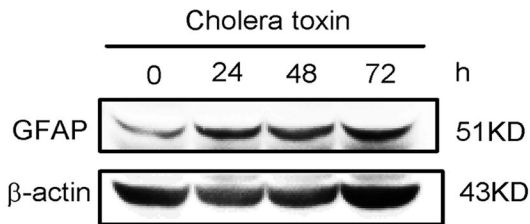
**Figure 6. MiR-335 is also required for cholera toxin-induced differentiation in human primary cultured glioma cells.** A, B and C, The effect of cholera toxin on morphology transformation (Original magnification:  $\times 200$ ), the protein expression of GFAP and the expression of miR-335. Primary culture cells were isolated from three clinical glioma tissues, and were treated with 100 ng/ml cholera toxin for 48 h. D and E, The effect of miR-335 on morphology transformation (Original magnification:  $\times 200$ ) and the protein expression of GFAP. F and G, The effect of antagomir-335 on morphology transformation (Original magnification:  $\times 200$ ) and the protein expression of GFAP. Cells were transfected with 50 nM miR-335, 50 nM antagomir-335 and/or 100 ng/ml cholera toxin for 48 h.

Figure 1

A

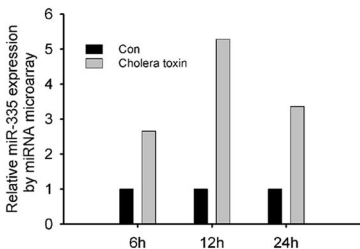
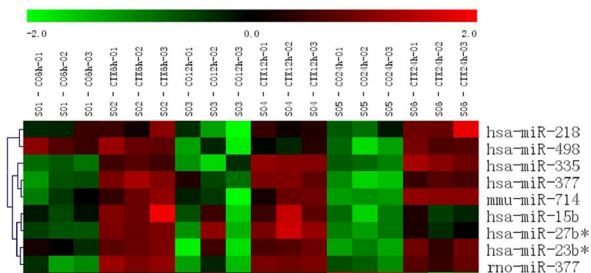


B



# Figure 2

## A



## B

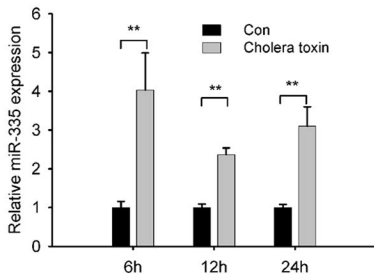


Figure 3

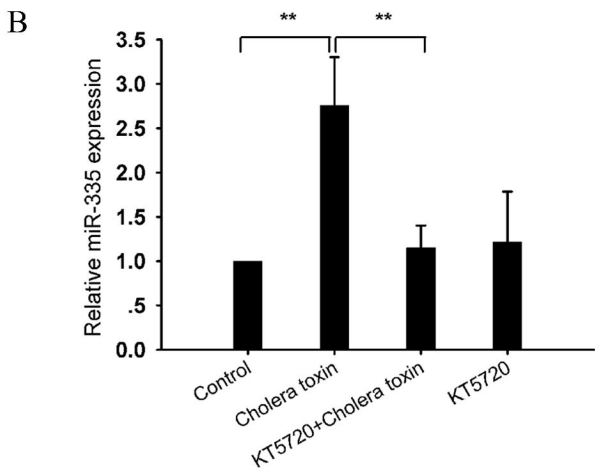
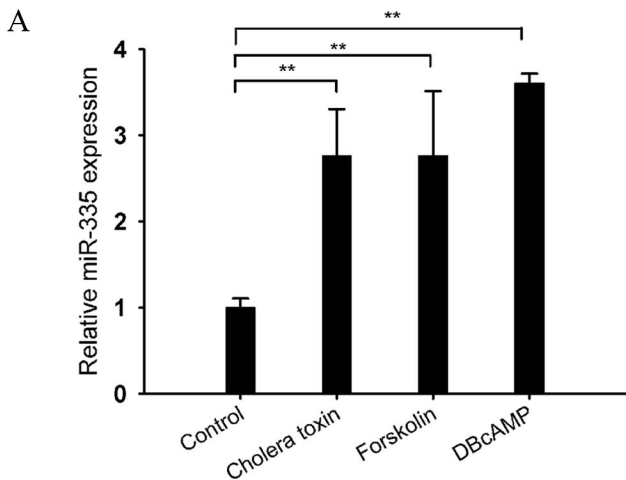


Figure 4

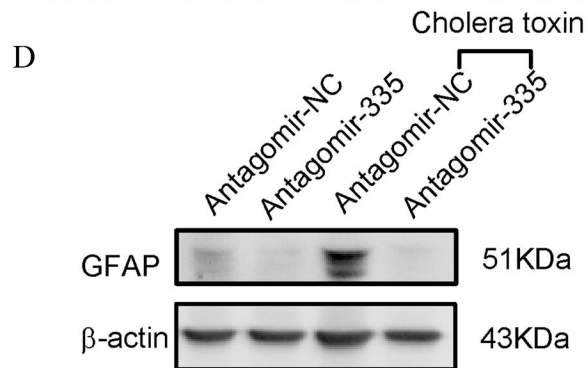
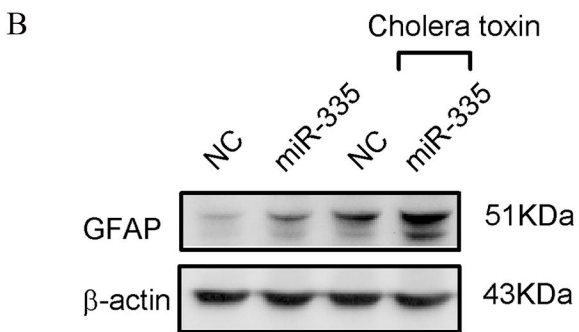
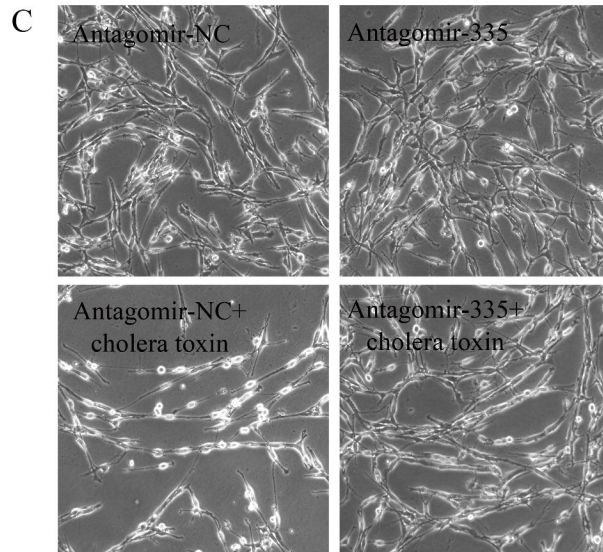
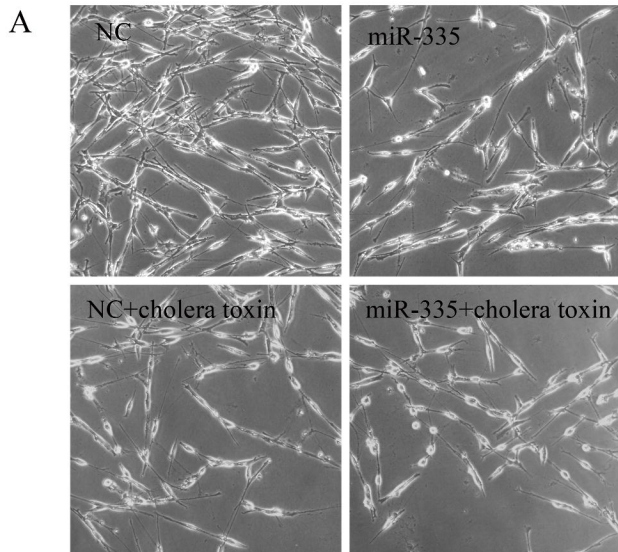




Figure 5

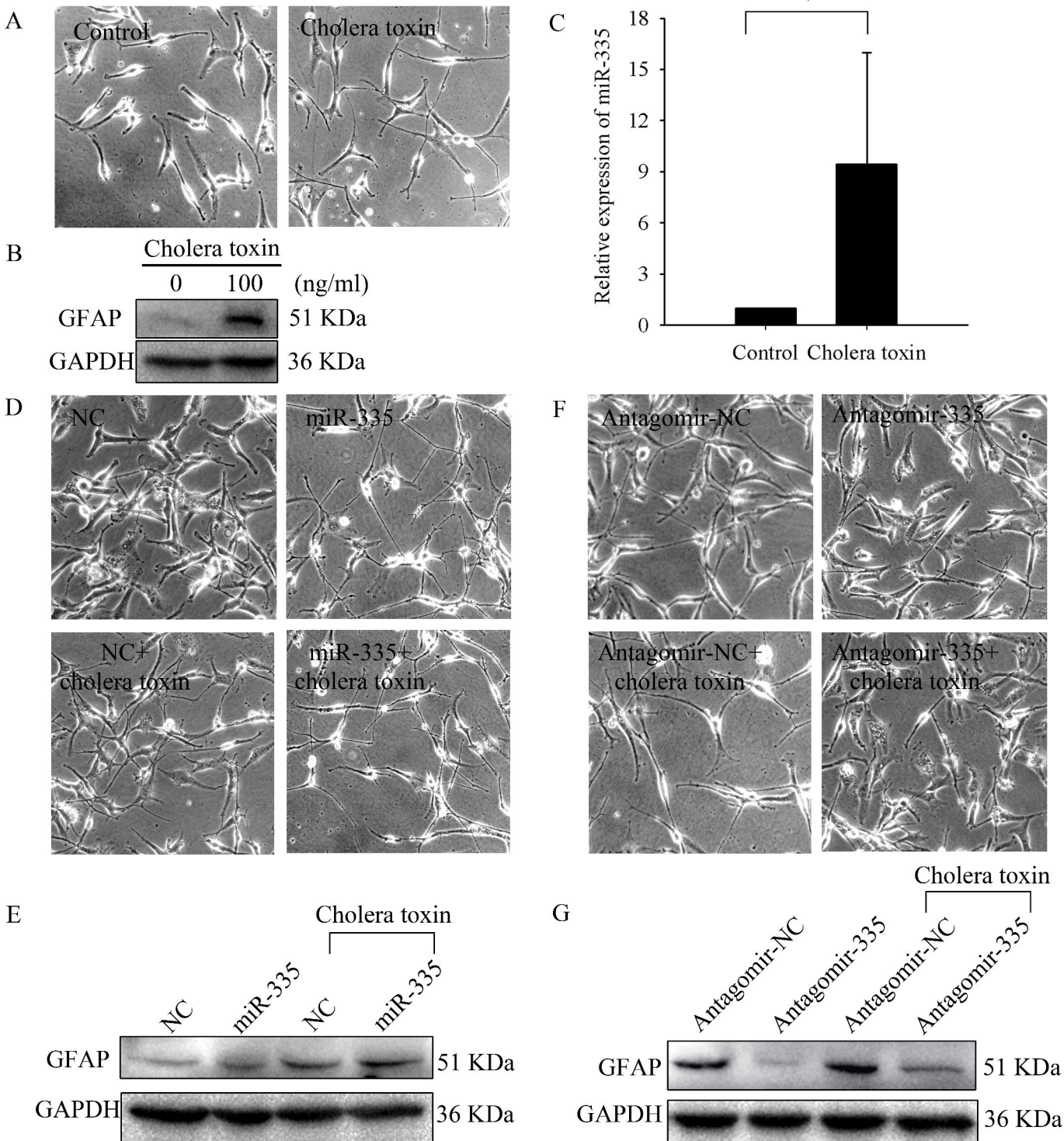


Figure 6

