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MicroRNA 335 Is Required for Differentiation of Malignant Glioma Cells Induced by Activation of cAMP/Protein Kinase A Pathway[S]

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Received September 30, 2011; accepted December 15, 2011

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. We have reported previously that activation of cAMP/protein kinase A (PKA) pathway is capable of inducing glioma cell differentiation, characterized by astrocyte-like shape and dramatic induction of astrocyte biomarker glial fibrillary acidic protein (GFAP). However, little progress has been made on molecular mechanisms related. Here we demonstrate that microRNA 335 (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 (e.g., forskolin, dibutyryl-cAMP) and abolished by PKA-specific inhibitor (9R,10S,12S)-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-fg:3′,2′,1′-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid, hexyl ester (KT5720). In an assay measuring gain and loss of miR-335 function, exogenetic miR-335 resulted in induction of GFAP, whereas miR-335 specific inhibitor antagomir-335 violently blocked cholera toxin-induced GFAP up-regulation. It is noteworthy that 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 potently required for differentiation of malignant glioma cells induced by cAMP/PKA pathway activation, and a single microRNA may act as an important fate determinant to control the differentiation status of malignant gliomas, which has provided a new insight into differentiation-inducing therapy against malignant gliomas.

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, particularly with high-grade tumors such as glioblastoma multiforme (Trog et al., 2006). Current therapy with surgery, radiation, and chemotherapy rarely, if ever, cures the disease and infrequently prolongs life for >1 year (Curran et al., 1993; Bao et al., 2006).

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 differentiation-inducing therapy using agents that modify cancer cell differentiation. All-*trans*-retinoic acid has been used

ABBREVIATIONS: PKA, protein kinase A; GFAP, glial fibrillary acidic protein; miRNA, microRNA; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; WHO, World Health Organization; dbcAMP, dibutyryl cAMP; KT5720, (9R,10S,12S)-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-fg:3′,2′,1′-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid, hexyl ester; qRT-PCR, quantitative real-time polymerase chain reaction; IL, interleukin; JAK, Janus tyrosine kinase; STAT, signal transducer and activator of transcription; MEST, mesoderm-specific transcript.

as an agent to induce cell differentiation in clinical treatment of acute promyelocytic leukemia (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, in particular, solid tumors. Finding potent differentiation agents for malignant gliomas remains a real challenge. It is noteworthy that classic stimulators of the 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 astrocyte-like shape and the accumulation of the reliable biomarker glial fibrillary acidic protein (GFAP) (Li et al., 2007; Lu et al., 2009; He et al., 2011). Nevertheless, details of the related molecular mechanism remain largely unknown.

MicroRNAs (miRNAs) are small noncoding RNAs (18–25 nucleotides) with potential roles in regulation of gene expression at post-transcriptional levels (Esquela-Kerscher and Slack, 2006). Cumulative evidence suggests that miRNAs control a wide array of biological processes, including cell differentiation, proliferation, and apoptosis, the dysregulation of which 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 (Tavazoie et al., 2008; Png et al., 2011). Furthermore, miR-335 has also been demonstrated to regulate Rb1 and to control cell proliferation in a p53-dependent manner (Scarola et al., 2010). It is noteworthy that miR-335 has been shown to orchestrates cell proliferation, migration, and differentiation in human mesenchymal stem cells (Tomé 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, that catalyzes ADP-ribosylation of G_s protein and results in accumulation of cellular cAMP (Guerrant et al., 1994) as a tool to pharmacologically manipulate the activation of the cAMP/PKA pathway and induce the differentiation of malignant glioma cells. The results demonstrate that cholera toxin stimulates miR-335 expression via the cAMP/PKA pathway and that this stimulation is potentially required for cholera toxin-induced astrocytic differentiation of malignant gliomas. Our findings suggest that a single miRNA seems to control the differentiation status of glioma cells, which also provides a new insight into the differentiation therapy against malignant gliomas.

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, VA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA) and a humidified atmosphere of 5% CO_2 at 37°C. Human primary culture glioma cells were isolated from three human glioma tissues that were obtained immediately after surgical removal; each patient provided informed consent. Tumors were classified according to the World Health Organization (WHO) classification system as WHO grade II astrocytoma (one tumor) and WHO grade IV astrocytoma (glioblastoma multiforme; two tumors). The isolation and culture of primary glioma cells were prepared as described previously (Li et al.,

2007). C6 cells were treated with cholera toxin, forskolin, dibutyryl cAMP (dbcAMP), and (9*R*,10*S*,12*S*)-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-*fg*:3′,2′,1′-*kl*]pyrrolo[3,4-*i*][1,6]benzodiazocine-10-carboxylic acid, hexyl ester (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 different time courses using an IX71 inverted microscope and a DP70 CCD camera (both from Olympus, Melville, NY).

Western Blot Analysis. Western blotting was performed as described previously (Akagi et al., 2002; Shu et al., 2011a). The following antibodies were used: antibodies against GFAP (1:1000; Cell Signaling Technology, Danvers, MA) and β-actin (1:2000; NeoMarkers, Fremont, CA).

miRNA Microarray Assay. Paraflo miRNA microarray was performed by a service provider (LC Sciences, Houston, TX), and data were analyzed. C6 cells were treated with or without 10 ng/ml cholera toxin for 6, 12, and 24 h. Total RNA samples (2–5 μg) were size-fractionated using a Microcon centrifugal filter unit with Ultracel YM-100 membrane (Millipore, Billerica, MA), and small RNAs (<300 nucleotides) were 3′-extended 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 μParaflo microfluidic chip using a microcirculation pump (Atactic Technologies, Houston, TX) (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 photogenerated reagent chemistry. Hybridization melting temperatures were balanced by chemical modifications of the detection probes. Hybridization was performed using 100 μl of 6× sodium chloride/sodium phosphate/EDTA buffer (0.90 M NaCl, 60 mM Na_2HPO_4, and 6 mM EDTA, pH 6.8) containing 25% formamide at 34°C. Fluorescence labeling with tag specific Cy5 dyes was used for after-hybridization detection. Hybridization images were collected using a laser scanner (GenePix 4000B; Molecular Devices, Sunnyvale, CA) and digitized using Array-Pro image analysis software (Media Cybernetics, Inc., Bethesda, MD).

Quantitative Real-Time PCR. Total RNA was prepared using TRIzol reagent (Invitrogen). Expression of mature miR-335 was determined by stem-loop primer SYBR Green quantitative real-time PCR (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 assays were performed in triplicate.

Oligonucleotide Transfection. miR-335 mimics, miR-negative control, antagomir-335, and antagomir-negative control (RiboBio, Guangzhou, China) were transfected using Lipofectamine RNAiMAX (Invitrogen) according to manufacturer-suggested procedures. Transfection efficiency was evaluated by Cy3-labeled oligonucleotide negative control.

Statistical Analysis. Data are presented as mean ± S.D. of three separate experiments if not otherwise noted. A difference of $p < 0.05$ (by two-sample Student's *t* test or one-factor analysis of variance) was considered statistically significant.

Results

Cholera Toxin Induces Differentiation of C6 Glioma Cells. Microscopic observation of C6 glioma cells treated with 10 ng/ml cholera toxin for 24, 48, and 72 h revealed major alterations in morphology. We first observed that the cell numbers were significantly reduced after cholera toxin

treatment compared with control group. It is noteworthy that, 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). It is noteworthy that GFAP, an established biomarker of mature astrocytes, was dramatically up-regulated as early as after 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 by 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 differentiation, we first compared the miRNA expression profiles of C6 glioma cells treated with or without cholera toxin for 6, 12, and 24 h. 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 uniform up-regulation at three time points. The relative expression of miR-335, presented in a heat map, further reveals this trend (Fig. 2A). Furthermore, data from qRT-PCR analysis showing that miR-335 expression rose with 6 h of cholera toxin stimulation and remained 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 to produce more cAMP, and dbcAMP, which is analogous with cAMP. As expected, 10 μM forskolin and 1 mM dbcAMP both signifi-
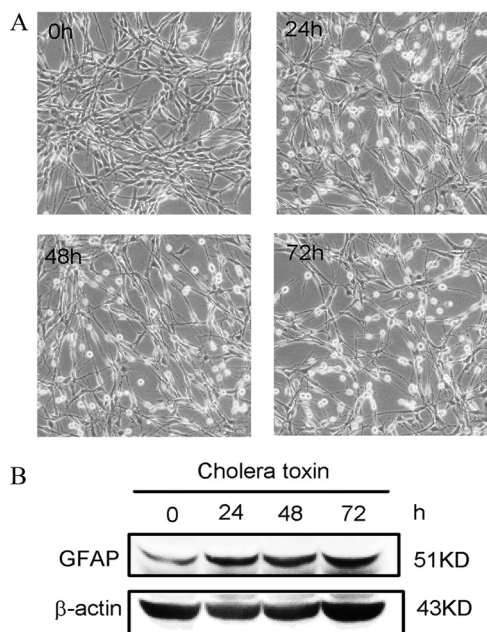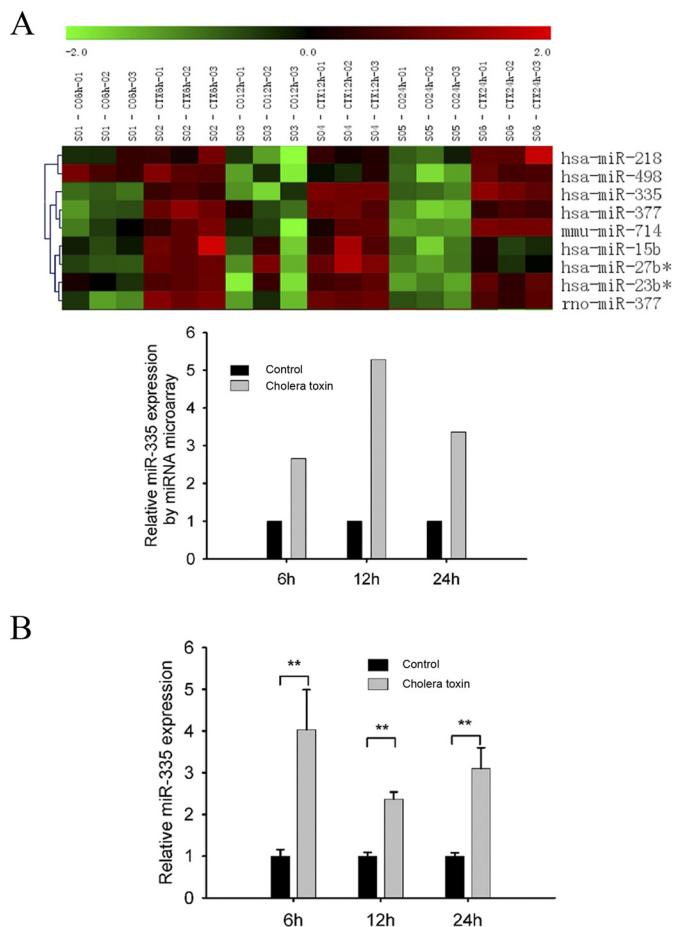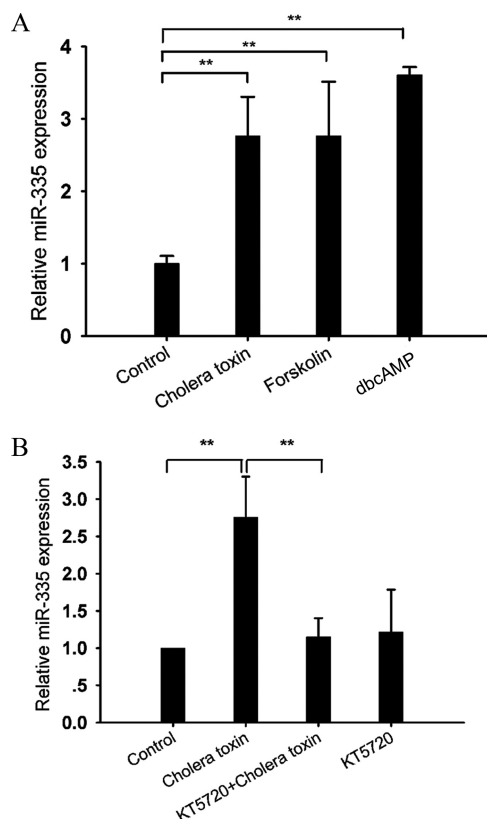
Fig. 2. MiR-335 is highly up-regulated after cholera toxin incubation in C6 glioma cells. A, heat map of specific miRNAs expression (top) and the relative expression level of miR-335 (bottom). Cells were incubated with 10 ng/ml cholera toxin for the indicated time courses and then detected by miRNA microarray. B, the relative expression level of miR-335. Cells were treated with 10 ng/ml cholera toxin for the indicated time courses, and then detected by qRT-PCR. $**$, $p < 0.01$.

cantly induced miR-335 expression (Fig. 3A). Conversely, 5 μM KT5720, a PKA-specific inhibitor, almost completely abrogated the miR-335 induction by cholera toxin (Fig. 3B). All 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 an assay intended to measure gain and loss of miR-335 function. Obviously, miR-335 overexpression triggers C6 cells to transform into a more mature astrocytic state (Fig. 4A). In the parallel Western blot analysis, miR-335 moderately stimulated GFAP expression and cooperated with cholera toxin to promote GFAP accumulation (Fig. 4B). Conversely, the miR-335–specific inhibitor antagomir-335 apparently reduced GFAP expression and strongly abrogated cholera toxin-stimulated GFAP induction as well as morphologic change (Fig. 4, C and D). Taken together, these data allow us to 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

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

Fig. 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 μM forskolin, or 1 mM 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 μM KT5720 for 24 h. MiR-335 expression was determined by qRT-PCR. ∗∗, $p < 0.01$.

differentiation is species-specific, human glioma cell line U87-MG cells and human primary cultured glioma cells were used. As shown in Figs. 5, A and B, and 6, A and B, cholera toxin prompted both U87-MG cells and primary cultures to transform into more mature astrocyte-like cultures, with star-shaped cell bodies and pronounced elongation of filamentous processes, and to produce more GFAP, indicating the prodifferentiation effect of cAMP signaling 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 (Figs. 5C and 6C). In the following assay to measure gain and loss of miR-335 function, exogenic miR-335 induced glioma cell differentiation (Figs. 5, D and E, and 6, D and E) and antagomir-335 abolished the differentiation-inducing activity of cholera toxin (Figs. 5, F and G, and 6, F and G), further suggesting a pivotal role for miR-335 in cAMP elevator-induced glioma cell differentiation, which is evolutionarily conserved from rat to human.

Discussion

Activation of the cAMP/PKA pathway has been proven to be an effective approach to induce differentiation of malignant gliomas. However, related molecular mechanisms remain 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 therapy-refractory tumor.

cAMP is a well known classic second messenger (Moss and Vaughan, 1979), and ample evidence indicates that cAMP-elevating stimuli such as N-substituted cAMP analogs and cAMP-increasing reagents can induce cell differentiation in
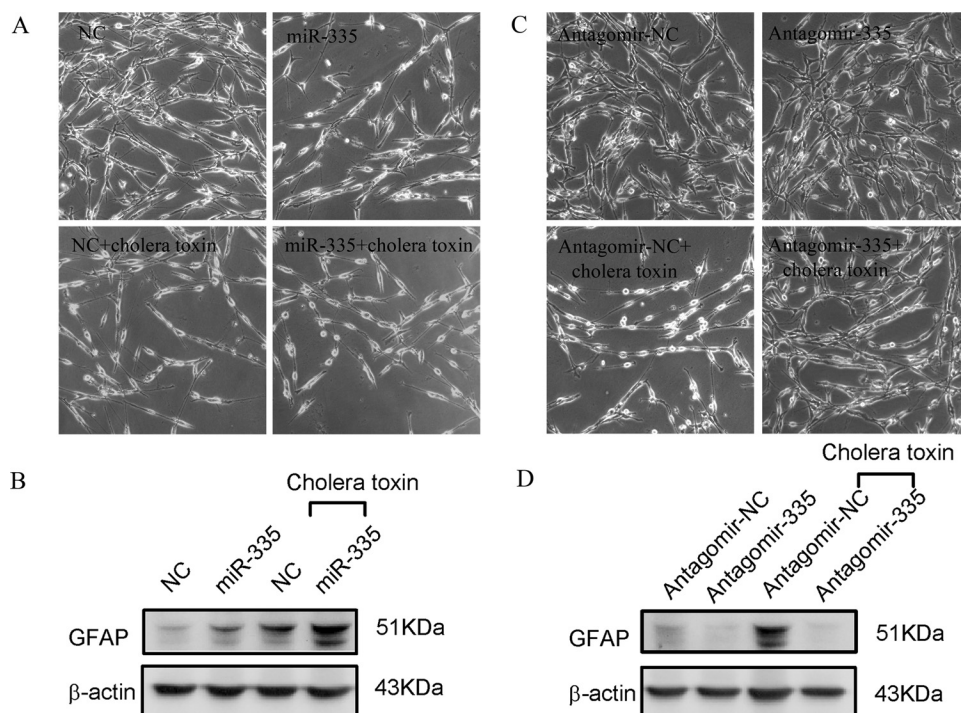
Fig. 4. MiR-335 is involved in cholera toxin-induced differentiation of C6 glioma cells. A and B, the effect of miR-335 on morphology transformation (original magnification, 200×) and the protein expression of GFAP. C and D, the effect of antagomir-335 on morphology transformation (original magnification, 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 cholera toxin for 48 h.
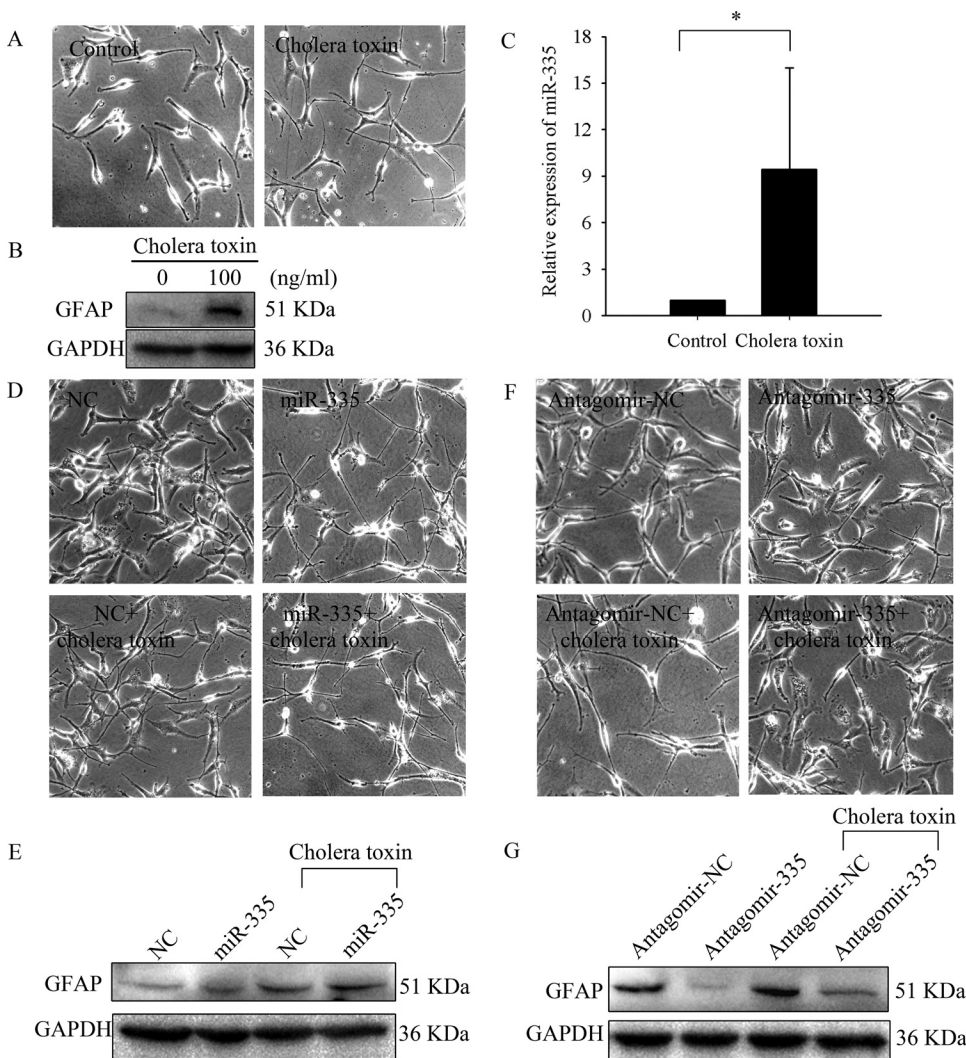
Fig. 5. miR-335 is also responsible for cholera toxin-induced differentiation in human glioma cell line U87-MG cells. A to C, the effect of cholera toxin on morphology transformation (original magnification, 200×), the protein expression of GFAP, and the expression of miR-335. U87-MG cells were treated with 100 ng/ml cholera toxin for 24 h. ∗, $p < 0.05$. D and E, the effect of miR-335 on morphology transformation (original magnification, 200×) and the protein expression of GFAP. F and G, the effect of antagomir-335 on morphology transformation (original magnification, 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. NC, negative control; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

gliomas (Takanaga et al., 2004; Van Kolen and Slegers, 2004). We previously reported that cAMP elevators cholera toxin, forskolin, and the analog dbcAMP are consistently capable of inducing differentiation of glioma cells via cAMP/PKA/cAMP response element-binding protein pathway (Li et al., 2007). In the present study, we further found that these cAMP/PKA pathway activators dramatically stimulate miR-335 expression, whereas the PKA-specific inhibitor KT5720 strongly blocks the miR-335 elevation, indicating that activation of the cAMP/PKA pathway makes a critical contribution to miR-335 accumulation. Furthermore, the assay to measure gain and loss of miR-335 function proved the conclusive role of miR-335 in glioma cell differentiation. Taken together, these data have provided strong evidence that cAMP/PKA pathway initiators act as triggers of miR-335 accumulation to induce glioma cell differentiation.

miR-335 is known to be an miRNA residing on the intron region of its cognate protein-coding gene mesoderm-specific transcript (MEST) (Ronchetti et al., 2008). Based on evidence that intronic miRNAs are frequently coexpressed with their host genes, we postulate that miR-335 expression might be under the control of the same regulative motif as the host gene MEST and might be 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 (Supplemental Fig. 1). All these data support the hypothesis that downstream effectors of PKA may activate MEST transcription concomitant with miR-335 expression, which will be investigated in our subsequent studies.

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 interleukin-6 (IL-6)/Janus tyrosine kinase2 (JAK2)/ signal transducer and activator of transcription3 (STAT3) pathway partially mediates cholera toxin-activated GFAP expression (Shu et al., 2011b), suggesting that endogenous cytokines might be potential candidates for 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 analogs might work as differentiation agents or synergistic ones against malignant gliomas.
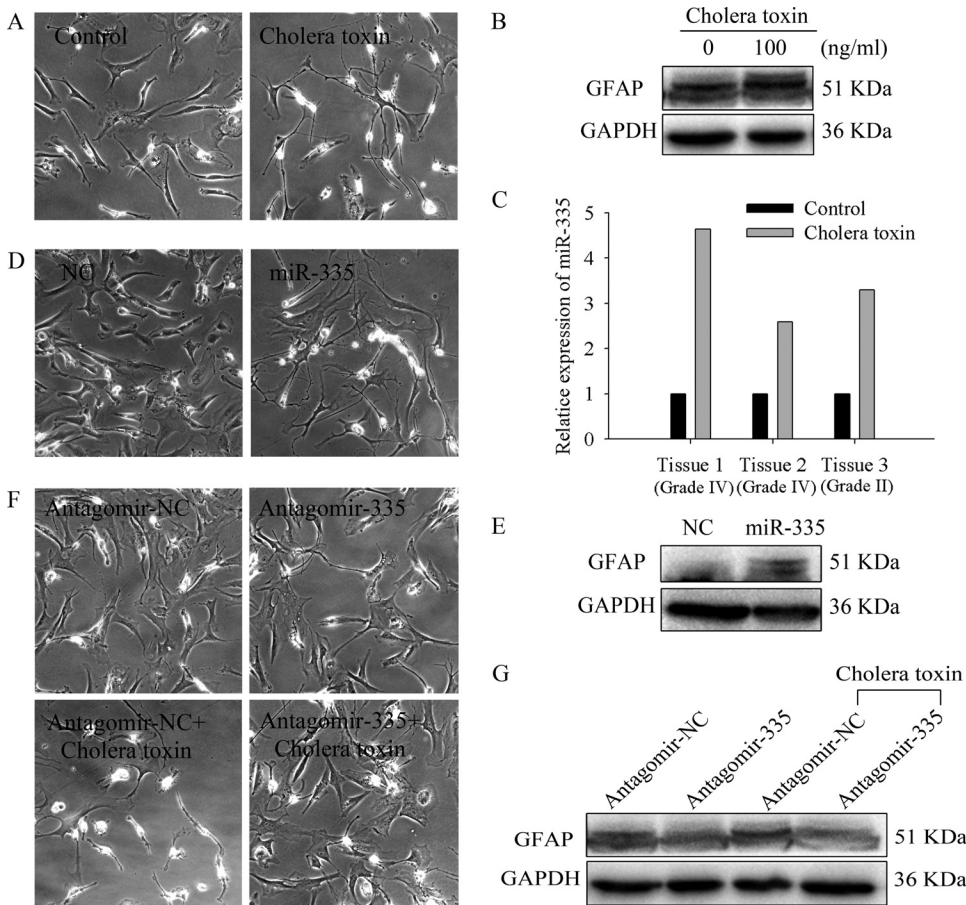
Fig. 6. MiR-335 is also required for cholera toxin-induced differentiation in human primary cultured glioma cells. A to C, the effect of cholera toxin on morphology transformation (original magnification, 200×), the protein expression of GFAP and the expression of miR-335. Primary culture cells were 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, 200×) and the protein expression of GFAP. F and G, the effect of antagomir-335 on morphology transformation (original magnification, 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. NC, negative control; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

In addition, despite the fact that no potential targets of miR-335 that negatively regulate the IL-6/JAK2/STAT3 pathway have been discovered, all the data mentioned above support the possibility that miR-335 might have cross-talk with the IL-6/JAK2/STAT3 pathway and suggest the occurrence of an epigenetic mechanism on GFAP expression. In any case, to our knowledge, no previous reports assess the role of miR-335 and its effect on malignant gliomas in the field of cellular differentiation.

In this study, we concentrated on those miRNAs in which expression patterns are consistent in all tested time points and found that only one miRNA, miR-335, agreed 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 (Supplemental 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 (Kim et al., 2009; Park et al., 2009; Fu et al., 2011), were both reduced within 12 and 24 h of cholera toxin stimulation (Supplemental Fig. 2). Considering the post-transcriptional regulation of miRNA on target genes, the down-regulation of miR-221 and miR-222 might theoretically lead to the up-regulation of the P27 and P21 proteins, which is verified by our previous data showing that cholera toxin increases P27 and P21 protein expression in a dose-dependent manner (Li et al., 2007). In addition, another increased miR-204 (Supplemental Fig. 3), which acts as a tumor suppressor gene by inhibiting antiapoptotic proteins BCL-2 and MCL-1 (Chen et al., 2009), facilitates glioma cell

differentiation and subsequent apoptosis, at least in part. All these findings indicate that activation of cAMP/PKA pathway-induced differentiation of malignant glioma might be directed by multimiRNAs and that each miRNA seems to play its unique role.

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 new insights for better understanding of the molecular mechanism underlying differentiation therapy of malignant gliomas and encourages miR-335 as a novel therapeutic target.

Authorship Contributions

Participated in research design: Shu, Zhou, Zhu, and Yan.
Conducted experiments: Shu, Zhou, Zhu, Zhang, and Wu.
Performed data analysis: Shu, Zhou, Zhu, and Chen.
Wrote or contributed to the writing of the manuscript: Shu and Zhu.

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