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MiR-218 sensitizes glioma cells to apoptosis and inhibits tumorigenicity by regulating ECOP-mediated suppression of NF-κB activity

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Introduction. Malignant gliomas are the most common and deadly primary brain tumors in adults. Increasing evidence has indicated that microRNAs (miRNAs) have an influence on the regulation of apoptotic cell signaling. Downregulation of miRNA 218 (miR-218) has been indicated in human glioma specimens. Here, we investigate the function of miR-218 in apoptosis and tumor growth of glioma cells.
Methods. The expression of miR-218 was detected by real-time quantitative reverse transcriptase PCR. The effects of miR-218 on glioma cell proliferation and tumorigenicity were investigated by in vitro clonogenicity and in vivo xenograft assay. Apoptosis was evaluated by flow cytometric analysis and assay by terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling. The downstream targets of miR-218 were identified by bioinformatics analysis and further validated by Western blot and luciferase reporter assay.
Results. Overexpression of miR-218 induces glioma cell apoptosis and inhibits glioma cell viability, proliferation, and tumorigenicity. Epidermal growth factor receptor–coamplified and overexpressed protein (ECOP) was identified as a functional downstream target of miR-218, which can regulate transcriptional activity of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and associated with apoptotic response. Ectopic expression of ECOP rescued the glioma cells from miR-218–induced apoptosis and increased NF-κB activity.
Conclusion. These results suggest that miR-218 sensitizes glioma cells to apoptosis by regulating ECOP-mediated suppression of NF-κB activity, which may provide novel opportunities for glioma therapy.

Keywords: apoptosis, ECOP, glioma, miR-218, NF-κB.

Malignant gliomas, including astrocytomas, ependymomas, oligodendrogliomas, and mixed gliomas, are the most common and deadly primary brain tumors worldwide.[1] Among them, glioblastoma multiforme (GBM; World Health Organization grade IV) is the most malignant and usual type of glioma, which comes from astrocytes with poor differentiation.[2] The current standard of care for newly diagnosed GBM patients includes maximal safe surgical resection, followed by a combination of radiation and chemotherapy with temozolomide.[3,4] However, disease recurrence is still inevitable. The median survival for GBM patients is only around 10–14 months.[1,4] Therefore, new strategies are urgently needed to complement the current standard therapies in order to improve clinical outcomes.

Micro(mi)RNAs are small noncoding single-stranded RNA molecules that have been found in various species and play essential roles in posttranscriptional regulation of gene expression.[5,6] Growing evidence has indicated an important role for miRNAs in the development of different cancers, including gliomas, and miRNAs have

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been associated with tumor suppressor and oncogenic activities.[7,8] Increasing numbers of miRNAs abnormally expressed in gliomas have been identified by miRNA-expression profiling methods.[9] Among these miRNAs, miRNA 218 (miR-218) has been shown to be downregulated in human GBM specimens versus adjacent brain devoid of tumor.[10–13] However, the role of miR-218 in the regulation of glioma cell apoptosis has not been reported.

In this study, we demonstrated that miR-218 sensitizes glioma cells to apoptosis. We further identified a functional target of miR-218: epidermal growth factor receptor–coamplified and overexpressed protein (ECOP), which can regulate transcriptional activity of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and associated apoptotic response. We also found that stable overexpression of miR-218 suppresses tumorigenicity of glioma cells in vivo. Taken together, our results suggest that miR-218 sensitizes glioma cells to apoptosis by regulating ECOP-mediated suppression of NF-κB activity.

Material and Methods

Cell Lines and Cell Culture

Human glioma cell lines (U87, U118, U138, U373, SW1088, SW1783) and an immortalized glial cell line (SVG p12) were purchased from American Type Culture Collection. U87, U373, U138, and SVG p12 cells were cultured in minimal essential medium (Invitrogen), U118 cells in Dulbecco's modified Eagle's medium (Invitrogen), and SW1088 and SW1783 cells in Leibovitz's L-15 medium (Invitrogen). The primary glioma cells were cultured as follows: patient biopsies were immediately dissociated by trypsinization and subsequently cultured in neurobasal medium (Invitrogen), with 1× B-27, 1× GlutaMax I (Invitrogen). All media were supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 100 U/mL penicillin/streptomycin (Sigma).

RNA Extraction and Real-time Quantitative PCR

Total RNA was extracted using TRIzol reagent (Invitrogen). The isolated total RNA was polyadenylated for 2-step real-time quantitative reverse transcriptase (qRT) PCR using the NCode miRNA First-Strand Synthesis and qRT-PCR kits (Invitrogen) according to the manufacturer's instructions. The sequence-specific forward primers for mature miR-218 and U6 internal control were CGTTGTGCTTGATCTAACCATGT (23 bp, guanine–cytosine = 43.49%, melting temperature = 60.4°C) and 5′- CTCGCTTCGGCAGCACA-3′, respectively.

MTS Assays

Cells were transfected with pLL3.7 pre–miR-218 (P-miR-218) or pLL3.7 control vector (P-miR-control). Twenty-four hours after transfection, transfected cells were seeded into 96-well plates at a density of 5×10^3 per well (100 μL). For assay by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), we used the CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega) following the manufacturer's instructions. Briefly, at 2 h before each of the desired time points (24 h, 48 h, 72 h, and 96 h), 20 μL of the MTS reagent was added into each well and cells were incubated at 37°C for 3 h. Absorbance was detected at 490 nm using a Wallac Victor 1420 Multilabel plate reader. All experiments were repeated 3 times.

Establishment of Glioma Stable Cell Lines

One day before transfection, U87 cells were seeded into 6-well plates at about 60% confluence. Cells were transfected with P-miR-218 or P-miR-control vectors using FuGENE HD transfection reagent (Roche Diagnostics) in the absence of antibiotic. After 48 h, cells were subcultured to 10% confluence in medium containing 1 μg/mL of puromycin (Sigma). When all cells in the nontransfected control culture were killed, antibiotic-resistant clones were picked and passaged in medium containing half concentration of puromycin, as in the first round of selection. The expression of miR-218 was confirmed by real-time qRT-PCR.

Clonogenicity Assay

The transfected glioma stable cells were seeded into 6-well plates and cultured in cell culture medium for ~2 weeks to allow colony formation. The culture medium was changed every third day. The colonies were then fixed in 100% methanol and stained with crystal violet solution. The number of macroscopically detectable colonies was registered.

Soft Agar Assay

Twenty-four-well plates were coated with 0.5 mL/well of complete medium containing 0.6% melted bacto-agar. Then an appropriate number of transfected glioma stable cells (10^3 cells/well) were suspended in 0.5 mL of a 0.3% agar-medium solution. The plates were kept in the incubator, and 3–5 weeks later the number of colonies formed was counted under the microscope. Three experiments were performed in triplicate. Mean ± standard deviation (SD) were calculated from 3 independent experiments.

Flow Cytometric Analysis

The transfected stable cells were collected and washed with cold phosphate buffered saline (PBS) twice. Cells were then resuspended in 1× binding buffer (BD Pharmingen) at a concentration of 1×10^6 cells/mL. One hundred microliters of the solution (1×10^5 cells) was transferred to a 5- mL culture tube. Five microliters

of phycoerythrin Annexin V and 5 μL propidium iodide (PI; BD Pharmingen) were added to the samples, which were each incubated for 15 min at room temperature (25°C) in the dark. Then 400 μL of 1 × binding buffer was added to each tube and the sample was analyzed by flow cytometry (FACSCanto II, BD Biosciences) within 1 h. The data were analyzed using FlowJo software. Three experiments were performed in triplicate.

Luciferase Reporter Assay

The 3′ untranslated region (UTR) sequence of ECOP predicted to interact with miR-218 or a mutated sequence with the predicted target sites was synthesized and inserted into the XbaI and FseI sites of pGL3 control vector (Promega). These constructs were named pGL3-ECOP-3′UTR and pGL3-ECOP-3′UTR-mut. The NF-κB luciferase reporter plasmid was purchased from Stratagene. For reporter assay, U87 cells were plated onto 24-well plates and transfected with pGL3-ECOP-3′UTR or pGL3- ECOP-3′UTR-mut and P-miR-218 or P-miR-control vectors using FuGENE HD. In the NF-κB luciferase reporter assay, P-miR-218 or P-miR-control was transfected into U87 cells using FuGENE HD. Then NF-κB luciferase reporter vector was cotransfected into pretreated U87 cells. A Renilla luciferase vector pRL-SV50 (Promega) was cotransfected to normalize the differences in transfection efficiency. After transfection for 48 h, cells were harvested and assayed with the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Transfection was repeated 3 times in triplicate.

Western Blotting

The transfected cells or tissue samples were harvested for immunoblot analysis according to our previously described protocol.[14] Rabbit polyclonal antibodies against human ECOP (E-13, sc-138921; 1:500 dilution) and glyceraldehyde-3-phosphate dehydrogenase (FL-335, sc-25778; 1:2500 dilution) were products from Santa Cruz Biotechnology.

Animal Studies

Stable transfected U87 cells were resuspended in PBS and implanted into the right and left flanks (1.5×10^6 cells per flank) of BALB/c athymic mice by subcutaneous injection. Tumor volumes were determined by measuring the length (a) and the width (b). The tumor volume (V) was calculated according to the formula $V = ab^2/2$. Statistical significance between P-miR-218 and P-miR-control transfected group animals was evaluated using Student's t-test.

TUNEL Assay

Glioma tissues were fixed in 4% paraformaldehyde in PBS embedded in paraffin and cut into ∼5- mm sections through the center of the tissue. The diameter of the tumor slices examined varied according to the tumor volume. Then the sections were stained with hematoxylin and eosin. The presence of apoptotic cells within the tumor sections was evaluated by assay by terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling (TUNEL) using the DeadEnd Fluorometric TUNEL System (GenScript USA) following the manufacturer's protocol. The percentage of apoptosis was determined by counting the number of apoptotic cells divided by the total number of cells in the field.[15]

Statistical Analysis

Experimental data were presented as mean ± SDs. All statistical analyses were performed using a 2-tailed Student's t-test (SPSS v12.0). Differences were considered to be statistically significant at $P < .05$.

Results

MiR-218 Is Downregulated in Glioma Cells and Inhibits Glioma Cell Viability

MiR-218 was downregulated in human GBM specimens versus adjacent brain devoid of tumor.[10–13] To investigate the functional role of miR-218 in glioma carcinogenesis, we first analyzed the expression of miR-218 in various glioma cell lines (U87, U118, U138, U373, SW1088, SW1783) and an immortalized glial cell line (SVG p12) by real-time qRT-PCR. When compared with immortalized glial cells, the expression of miR-218 was significantly lower by at least 2- to 5-fold in all glioma cell lines examined (Fig. 1A). We further analyzed the expression of miR-218 in 20 cases of glioma and nonglioma tissue samples by real-time qRT-PCR. When compared with nonglioma brain tissues, the expression of miR-218 was significantly lower, by ∼3-fold, in all glioma samples examined (Fig. 1B).

Then we investigated whether expression of miR-218 affects glioma cell viability. We transfected U87, U118, and primary cultured glioma cells with P-miR-218 or P-miR-control to overexpress miR-218. At different time points (24 h, 48 h, 72 h, and 96 h) after transfection, we detected by 2-step qRT-PCR that transfection of P-miR-218 increased the expression level of miR-218 in U87, U118, and primary cultured glioma cells at 48 h and 72 h compared with P-miR-control transfection (Fig. 1C–E). Subsequently, MTS assay showed that overexpression of miR-218 significantly inhibited U87 and U118 cell viability at 48 h and 72 h after transfection (Fig. 1F–H). These results suggest that the expression of miR-218 may be involved in glioma carcinogenesis.

MiR-218 Inhibits Glioma Cell Proliferation In Vitro

Clonogenic assay is an effective method to evaluate the proliferative ability and tumorigenicity of a single

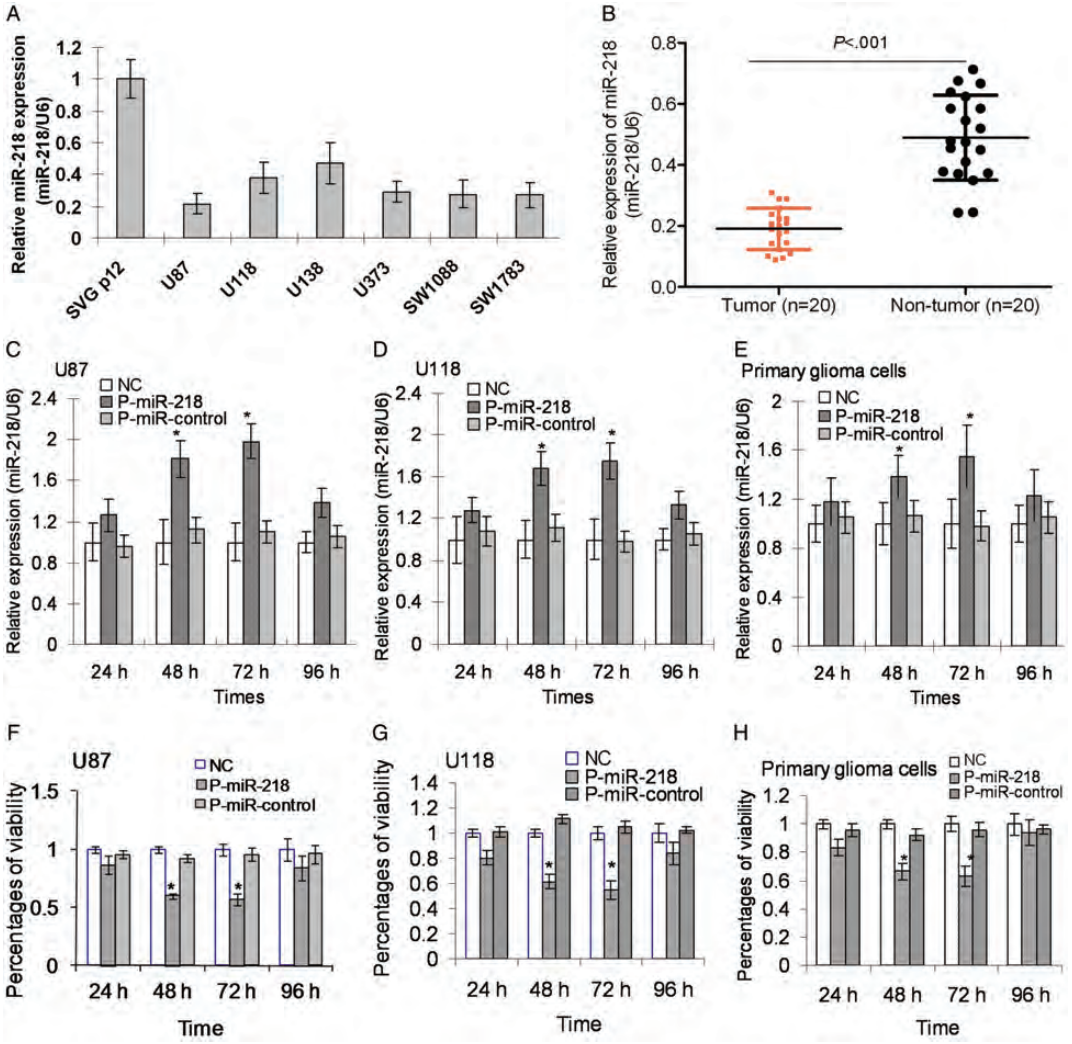

Fig. 1. MiR-218 is downregulated in glioma cells and inhibits glioma cell viability. (A) The expression of miR-218 in various glioma cell lines (U87, U118, U138, U373, SW1088, SW1783) and an immortalized glial cell line (SVG p12) by real-time qRT-PCR. (B) The relative expression level of miR-218 in glioma samples and nontumor brain tissues by real-time qRT-PCR. (C–E) Overexpression of miR-218 in U87, U118, and primary cultured glioma cells by pLL3.7-pre-miR-218 (P-miR-218) or pLL3.7-control vector (P-miR-control) transfection. Total RNA was collected and detected by real-time qRT-PCR at different time points. NC refers to the non-treated cells. (F–H) Ectopic expression of miR-218 significantly inhibited U87, U118, and primary cultured glioma cell viability at different time points. Data are mean ± SDs of 3 independent experiments. $^*P < .05$.

cell in vitro.[16] To determine the effects of miR-218 re-expression on glioma cell proliferation in vitro, we used the plate clonogenic assay. Stable overexpression of miR-218 dramatically reduced the number of surviving colonies from the 2 glioma cell lines compared with the P-miR-control vector transfected cells (Fig. 2A and B).

A hallmark of cellular transformation is the ability of tumor cells to grow in an anchorage-independent way in a semisolid medium.[17] To further investigate the effects of miR-218 expression on anchorage-independent growth of glioma cells, a soft agar assay was performed. The result showed that stable overexpression of miR-218 in both glioma cells was able to strongly reduce the number of colonies growing in soft agar (Fig. 2C and D).

ECOP Is a Functional Downstream Target of MiR-218

To understand the mechanisms by which miR-218 inhibits its glioma cell viability and proliferation, we used several computational methods to identify functional targets of miR-218 in humans. Among hundreds of targets predicted by different miRNA programs, ECOP was of particular interest. According to miRecords online prediction, ECOP can be predicted as a potential target of miR-218 by 6 different miRNA target prediction tools, including miRanda, MirTarget2, PicTar, PITA, RNA hybrid, and TargetScan/TargetScanS.[18]

To validate that ECOP is a direct functional target of miR-218, we investigated whether miR-218 targets the 3′UTR of ECOP mRNA by dual-luciferase reporter assay. According to the predicted target sites from

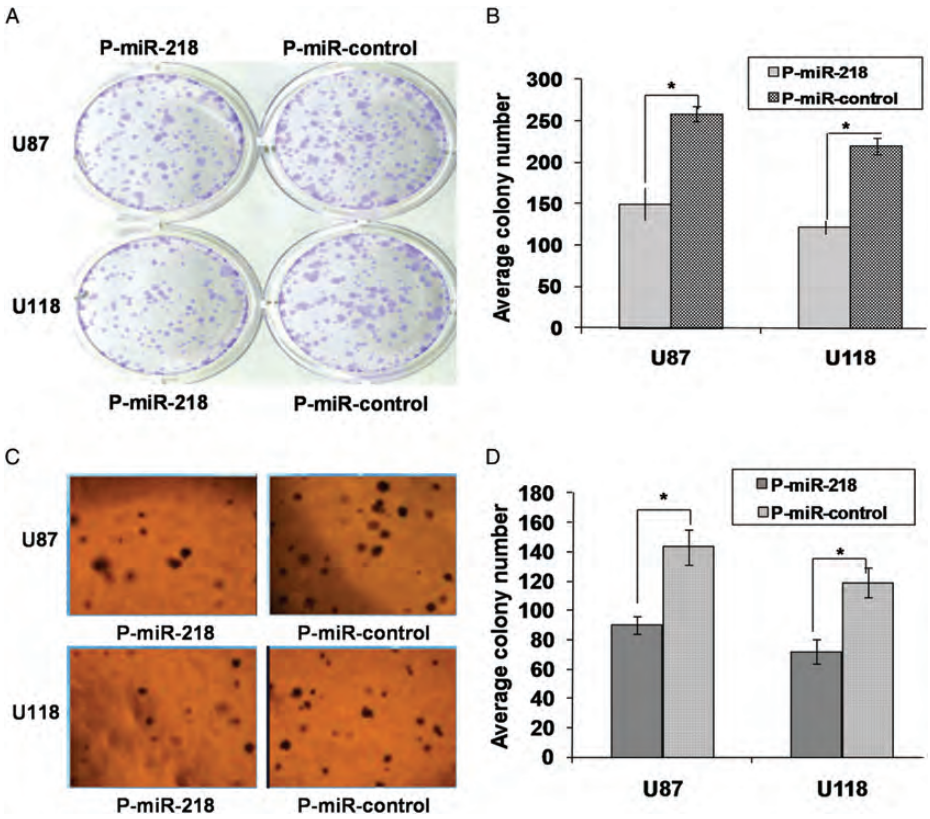

Fig. 2. MiR-218 inhibits glioma cell proliferation in vitro. (A) Representative images of clonogenicity assays. (B) Clonogenicity assays. Transfected cells were seeded into 6-well plates and cultured in cell culture medium for ~2 wk to allow colony formation. The number of macroscopically detectable colonies was registered. (C) Representative images of soft agar assay colony. (D) Soft agar assays. Transfected U87 and U118 cells (1000 cells/well) were suspended in 0.5 mL of a 0.3% agar-medium solution. The number of colonies formed was counted under the microscope. All data are given as mean ± SDs of 3 independent experiments. Significant differences are indicated as follows: 1-sample *t*-test, $*P < .05$.

TargetScan (Fig. 3A), we cloned the wild-type 3′UTR fragment containing these predicted sites into the pGL3 luciferase reporter vector (pGL3-ECOP-3′UTR). Another 3′UTR fragment with a mutation within each seed region was cloned as control (pGL3-ECOP-3′UTR-mut) (Fig. 3A). We observed that only cotransfection of P-miR-218 (not P-miR-control) and pGL3-ECOP-3′UTR (not pGL3-ECOP-3′UTR-mut) suppressed luciferase activity, significantly at ~40% (Fig. 3B). These data confirm that ECOP is a direct downstream target of miR-218. We further measured the protein expression of ECOP in U87 cells transfected with P-miR-218 or P-miR-control. Western blotting showed that overexpression of miR-218 in U87 cells downregulated the endogenous protein expressions of ECOP (Fig. 3C).

MiR-218 Induces Apoptosis in Glioma Cells by Inhibition of NF-κB Activity

Because ECOP has been reported as a key regulator of NF-κB signaling and is associated with tumor cell apoptosis,[7] we further investigated the effect of miR-218 on apoptosis of glioma cells using the Annexin-V/PI assay. Cells that are viable are both Annexin V and PI negative, while cells that are in early apoptosis are Annexin V positive and PI negative, and cells that are in late apoptosis or already dead are both Annexin V and PI positive. We observed in U87 and U118 cells that the proportion of early apoptotic cells induced by transfection of P-miR-218 (U87: 15.5%; U118: 11.7%) was greatly increased compared with that induced by transfection of P-miR-control vector (U87: 4.51%; U118: 4.39%) (Fig. 4A and B). These results suggest that miR-218 can induce apoptosis in glioma cells.

To further investigate whether miR-218 induces apoptosis in glioma cells by inhibition of NF-κB activity, we investigated whether NF-κB activation was affected by overexpression of miR-218. We observed that NF-κB transcriptional activity was decreased in glioma cells that were transfected with P-miR-218 compared with P-miR-control transfection (Fig. 4C). Moreover, the mRNA expression levels of 3 classical NF-κB target genes, BcL-xL, MYC and CCND1, were also found to be decreased in P-miR-218 transfected glioma cells (Fig. 4D). These data suggest that miR-218 induces apoptosis in glioma cells by inhibition of NF-κB activity and downstream target genes.

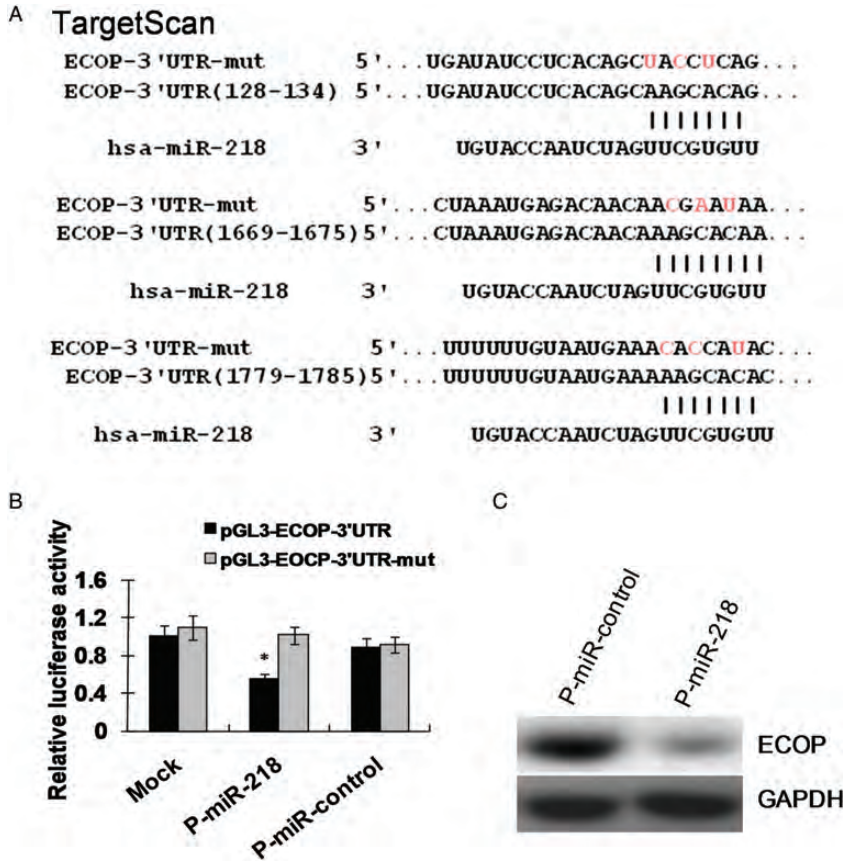

Fig. 3. ECOP is one of the functional downstream targets of miR-218. (A) ECOP mRNA 3′UTR putative sites or mutated sites targeted by miR-218 (TargetScan). (B) U87 cells were transfected with ECOP 3′UTR reporter vector or 3′UTR mutant reporter vector and with P-miR-218 or P-miR-control. (C) Western blot analysis of total cell lysates extracted from P-miR-218 or P-miR-control transfected U87 cells using the indicated antibodies. Data were mean ± SD of 3 independent experiments. *$P < .05$. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Re-expression of ECOP Increases NF-κB Activity and Decreases MiR-218 Induced Apoptosis in Glioma Cells

To further investigate the effects of ECOP in mediation of apoptosis induced by miR-218 in glioma cells, we rescued miR-218–mediated ECOP suppression in U87 cells by cotransfection of a plasmid carrying a wild-type ECOP gene-coding region (pcDNA-ECOP, without 3′UTR). The re-expression of ECOP was validated by western blotting (Fig. 5A). We observed that NF-κB transcriptional activity was increased in stable miR-218 transfected U87 cells that were transfected with the wild-type ECOP gene (Fig. 5B). We also observed that the proportion of early apoptotic cells induced by re-expression of ECOP (27.3%) was greatly decreased compared with pcDNA-control vector transfection (9.61%) in stable transfected U87 cells (Fig. 5C). These observations indicated that miR-218–induced apoptosis and NF-κB activity inhibition in glioma cells was partially, if not totally, recapitulated upon ECOP re-expression (Fig. 5B and C), suggesting that the effects of miR-218 on NF-κB activity and glioma cell apoptosis are mediated mainly by ECOP.

Stable Overexpression of MiR-218 Suppresses Tumorigenicity and Apoptosis of Glioma Cells

To substantiate the role of miR-218 in glioma carcinogenesis, we further assessed the effects of miR-218 overexpression on tumorigenicity of glioma cells in vivo. Stable transfected U87 P-miR-218 or U-87 P-miR-control cells were implanted into the right and left flanks (1.5×10^6 cells per flank) of BALB/c athymic mice by subcutaneous injection. Tumor volumes were determined every week by measuring the length (a) and the width (b). The tumor volume (V) was calculated according to the formula $V = ab^2/2$. At 42 days post-injection, the mean volumes of tumors generated from U87 P-miR-218 cells were significantly smaller than those originating from U87 P-miR-control cells (Fig. 6A and B). Western blotting for the glioma xenograft tissues revealed that expression of ECOP was decreased in tumor tissues derived from U87 P-miR-218 cells compared with those from U87 P-miR-control cells (Fig. 6C). The tissue sections from each group were collected and assayed by TUNEL analysis to detect apoptotic cells. The results showed that the apoptotic percentage was significantly increased in

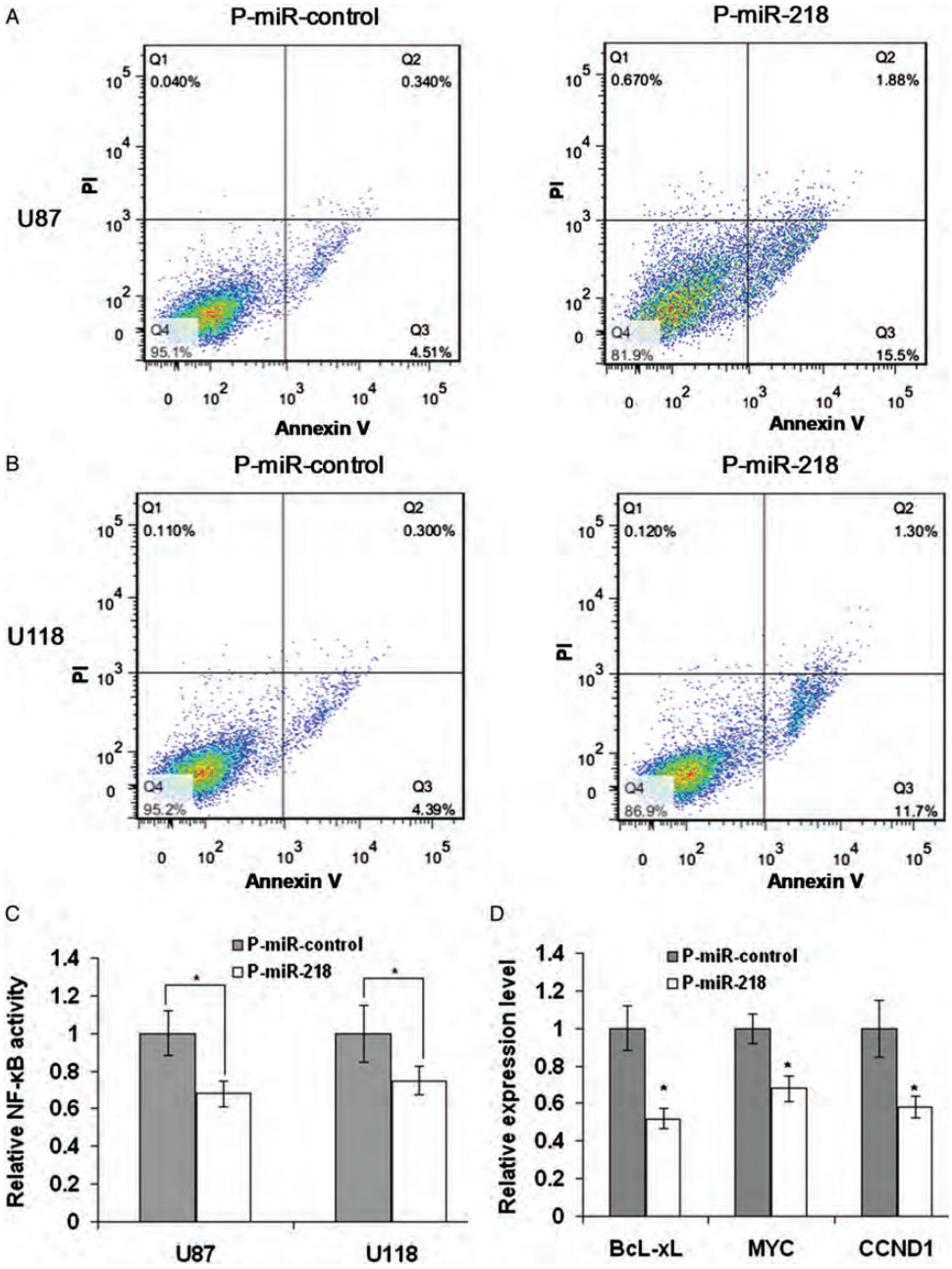

Fig. 4. MiR-218 induces apoptosis in glioma cells by inhibition of NF-κB activity. (A and B) Apoptosis of U87 and U118 cells transfected with P-miR-218 or P-miR-control was assessed using Annexin V/PI staining and flow cytometry analysis. Cells that are viable are both Annexin V and PI negative, while cells that are in early apoptosis are Annexin V positive and PI negative, and cells that are in late apoptosis or already dead are both Annexin V and PI positive. The proportion of early apoptotic cells induced by transfection of P-miR-218 (U87: 15.5%, U118: 11.7%) was greatly increased compared with that induced by transfection of P-miR-control vector (U87: 4.51%, U118: 4.39%) in U87 and U118 cells. (C) U87 cells were transfected with NF-κB luciferase reporter 24 h after transfection of P-miR-218 or P-miR-control vectors. Data were normalized and calculated from 3 independent experiments of 3 replicates each. (D) Real-time qRT-PCR was performed to determine changes in the expression levels of mRNA of classical NF-κB target genes, such as BcL-xL, MYC and CCND1. GAPDH was used as a loading control. Levels of mRNA were normalized to 1. Data are mean ± SDs of 3 independent experiments performed in triplicate. $*P < .05$.

tumor tissues derived from U87 P-miR-218 cells compared with those from U87 P-miR-control cells (42.8% vs 9.8%) (Fig. 6D and E). These results indicate that enforced miR-218 expression restrains glioma tumorigenicity and induces apoptosis in vivo by targeting ECOP.

Discussion

The genetic characteristics and molecular mechanisms of gliomagenesis have become increasingly clear in recent years, but the median survival for patients with glioblastoma is still around 10–14 months.[1] The evasion of

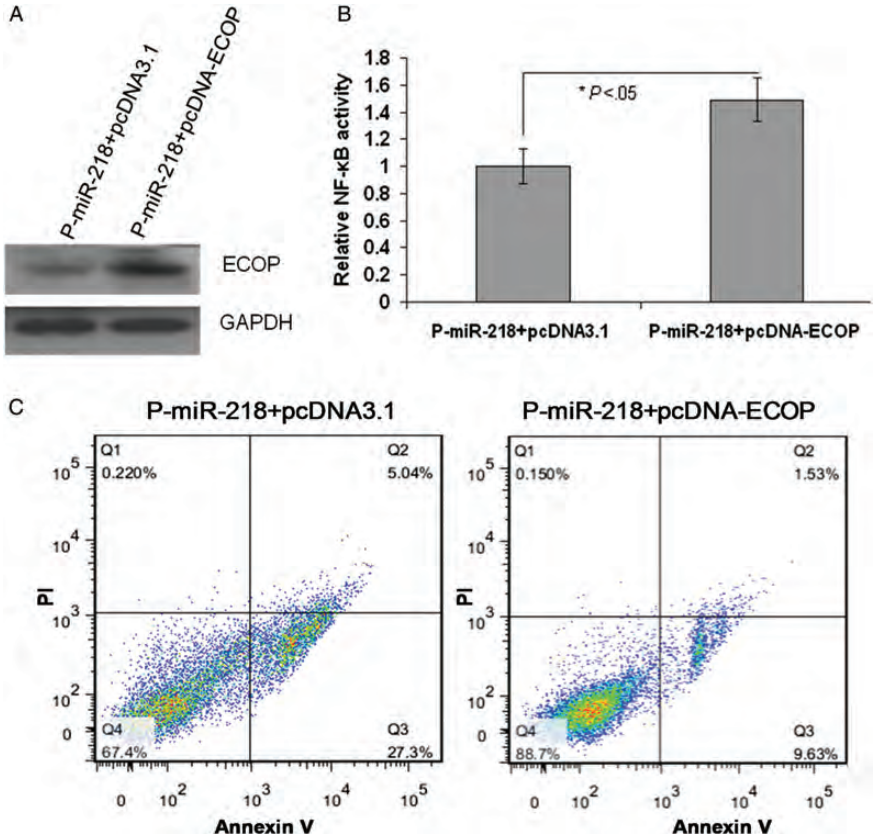

Fig. 5. Re-expression of ECOP increases NF-κB activity and decreases miR-218–induced apoptosis in glioma cells. (A) Western blotting analysis showing re-expression of ECOP by cotransfection of a plasmid carrying a wild-type ECOP gene-coding region (pcDNA-ECOP, without 3′UTR) with P-miR-218. (B) Cells were transfected with NF-κB luciferase reporter for NF-κB activity analysis. (C) Apoptosis of U87 cells transfected with P-miR-218 and pcDNA-ECOP or pcDNA3.1 control vector were assessed using Annexin V/PI staining and flow cytometry. Data are mean ± SDs of 3 independent experiments performed in triplicate. $*P < .05$. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

apoptosis is a hallmark of most cancer development and progression, including glioma. Many studies have shown that the balance between cell proliferation and apoptosis is influenced by protein coding oncogenes or tumor suppressor genes.[19,20] Recently, increasing evidence has also indicated that small noncoding RNAs, such as miRNAs, are associated with apoptosis by negative regulation of the expression of oncogenes or tumor suppressor genes.[21] Many miRNAs have been reported to have an influence on the regulation of apoptotic cell signaling. For example, miR-15 and miR-16 have been shown to induce apoptosis by targeting BCL2 in chronic lymphocytic leukemia.[22] MiR-21 is an anti-apoptotic factor that is strongly overexpressed in human glioblastoma tumor tissues and cell lines compared with nonneoplastic fetal or adult brain tissues and cultured nonneoplastic glial cells.[23]

In this study, we observed the downregulation of miR-218 in human glioma cells compared with immortalized glial cells. This is consistent with previous reports showing that miR-218 is downregulated in human GBM specimens versus adjacent brain devoid of tumor.[10–13] The study by Song et al[13] investigated mainly the role of miR-218 on invasive ability of glioma cells by direct downregulation of IκB kinase–beta. Here, we investigated its role on glioma cell apoptosis and tumorigenicity. By gain of function study, overexpression of miR-218 can inhibit glioma cell viability and proliferation and induce apoptosis in vitro. We further identified that ECOP is a directly functional target of miR-218 by bioinformatics analysis and experimental confirmation using a luciferase reporter assay and Western blotting analysis. It is possible that different targets of each miRNA mediate different biological functions.

ECOP, the epidermal growth factor receptor–coamplified and overexpressed protein, is also called a glioblastoma-amplified secreted protein or vesicular overexpressed-in-cancer prosurvival protein 1.[24] ECOP has been shown to be overexpressed in human GBM and squamous cell carcinoma.[24–26] There is also a study[7] suggesting that ECOP is a key regulator of NF-κB signaling and that the expression of ECOP could contribute to apoptosis resistance. In gliomas, NF-κB is constitutively activated, and the levels of many NF-κB–regulated genes are elevated.[27] NF-κB is thought to upregulate expression of genes that cause suppression of the apoptotic response in cancer cells.[28]

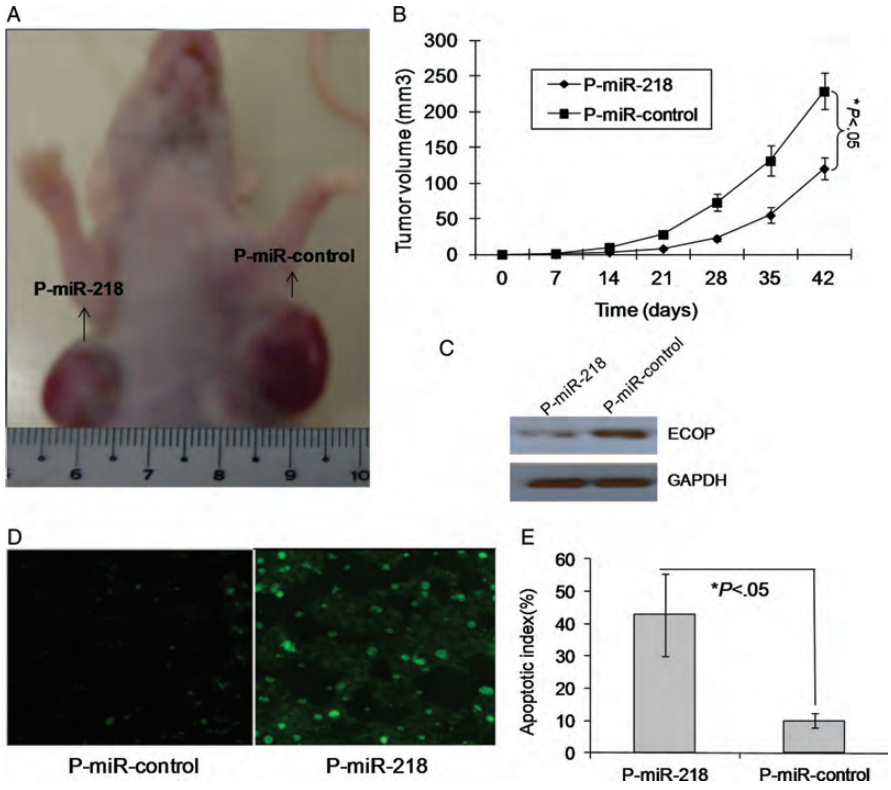

Fig. 6. Stable overexpression of miR-218 suppresses tumorigenicity and induces apoptosis of glioma cells. (A) Representative image for tumor growth is shown. Nude mice were subcutaneously injected with 1.5×10^6 P-miR-218 or P-miR-control stable transfected U87 cells. (B) Determination of the tumor growth. Tumor volume was calculated every week after injection. Data are mean $\pm$ SDs of 3 independent experiments. $^*P < .05$. (C) Western blotting analysis of ECOP protein expressions in xenograft tumor tissues. (D and E) TUNEL staining of tumor tissues. Staining shows the representative fields from tumor tissues in the P-miR-218 or P-miR-control stable transfected U87 cell group. The graph shows the percent apoptosis in each group. Values are expressed as mean $\pm$ SDs ($^*P < .05$). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Our study confirmed that miR-218 can induce apoptosis in glioma cells by targeting ECOP to inhibit NF-κB activity and the expression of downstream target genes such as BcL-xL, MYC, and CCND1.

Furthermore, re-expression of ECOP increases NF-κB activity and decreases miR-218–induced apoptosis in glioma cells. This further indicates that miR-218 sensitizes glioma cells to apoptosis by regulating ECOP-mediated suppression of NF-κB activity. Finally, stable overexpression of miR-218 showed suppression of glioma cell tumorigenicity and apoptosis in vivo and ECOP expression in tumor tissue, suggesting that enforced miR-218 expression restrains glioma tumorigenicity and induces apoptosis by targeting ECOP. These results indicate that miR-218 and ECOP play important roles in glioma carcinogenesis. It is very promising to develop miR-218 as a biomarker for glioma patients or as a potential therapeutic candidate for miRNA replacement therapy.[29,30]

Conflict of interest statement. None declared.

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