miR-340 suppresses the stem-like cell function of glioma-initiating cells by targeting tissue plasminogen activator

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Abstract

Glioma-initiating cells (GICs) have stem-like cell properties thought to be sufficient for recurrence, progression and drug resistance in glioblastomas. In the present study, we defined miR-340 as a differentially expressed microRNA (miRNA) in human GICs that inhibit GIC-mediated tumorigenesis. Further, we defined tissue plasminogen activator (PLAT) as a critical direct target of miR-340 for inhibition. Among miRNAs screened we found that miR-340 expression was decreased in all human GICs and in human glioblastoma tissues, compared to human neural stem cells and normal brain tissues. miR-340 overexpression in GICs suppressed their proliferative, invasive and migratory properties \textit{in vitro}, triggering cell senescence \textit{in vitro} and inhibiting GIC-induced tumorigenesis in mouse brains. shRNA-mediated silencing of PLAT in GICs phenocopied the effects of miR-340 overexpression \textit{in vitro} and \textit{in vivo}, suggesting a potential role for tissue factor in stem-like cell function. Taken together, our results identified miR-340 as a tumor suppressor that functions in GIC to enforce PLAT blockade and ablate their stem-like functions.
Introduction

Glioblastoma multiforme (GBM) is the most common and aggressive malignant brain tumor. Despite the most intensive current therapeutic efforts, the median survival time (~14 months) has not changed significantly in decades (1). The extremely poor prognosis of GBM patients is likely because of the presence of glioma-initiating cells (GICs; also known as glioma stem-like cells), which are the equivalent of the cancer stem cells (CSCs) observed in other cancer types (2-4). CSCs are not only highly resistant to chemo- and radiotherapy (5-7) but are also highly tumorigenic; thus, these cells are thought to be the primary cause of tumor recurrence and progression. To improve the poor prognosis of GBM patients, it is important to understand the mechanism that GICs are activated to have high tumorigenesis and invasion.

MicroRNAs (miRNAs/miRs) are small, non-coding RNAs of approximately 22 nucleotides in length that mediate the posttranscriptional silencing of specific target mRNAs and that are currently recognized as important regulators of tumorigenesis and development (8). Many miRNAs have been reported to be aberrantly expressed in malignant gliomas and play a role in determining the degree of malignancy. These include miR-21, miR-221/222, miR-124 and miR-128 (9-12). These miRNAs can act as either oncogenes or tumor suppressors in gliomas, depending on their effects on cell proliferation and apoptosis. However, there are few studies on integrated functions of miRNAs that significantly regulate tumorigenesis and tumor development initiated by GICs.

In the present study, we comprehensively analyzed the miRNA expression profiles of human GICs (hGICs) and identified miR-340 as a novel miRNA that is significantly down-regulated in hGICs compared with human neural stem cells.
(NSCs). Functional analyses revealed that miR-340 suppressed hGIC proliferation, invasion, and migration in vitro, as well as hGIC tumorigenesis in vivo, in the mouse brain. Furthermore, we defined tissue plasminogen activator (PLAT) gene as a direct target of miR-340. These results indicate that GICs with the decreased level of miR-340 promote glioblastoma formation in the mouse brain and that miR-340 down-regulation can induce a variety of malignant processes, such as cell proliferation and diffuse invasion. We also demonstrated that these effects are primarily mediated by increased levels of the target molecule PLAT. These findings reveal miR-340 and its target gene PLAT as potentially useful therapeutic candidates for the treatment of glioblastoma.
Materials and Methods

Animals and chemical reagents

The mice were obtained from CLEA Japan, Inc. The mouse experiments were performed according to protocols approved by the Animal Care and Use Committees of Ehime University and of Hokkaido University. Chemicals and growth factors were purchased from Invitrogen and PeproTech, respectively, except where otherwise indicated.

Glioma-initiating cell culture and cell lines

Seven primary human glioma samples [five GBMs (E1-4, 6), one anaplastic oligodendroglioma (AO), and one diffuse astrocytoma (DA)] were used to prepare the hGICs. These seven samples were obtained from Ehime University Hospital with the patients’ consent according to the Research Ethics Committee guidelines and were used in compliance with the research guidelines of the Ehime University Graduate School of Medicine and of the Institute for Genetic Medicine of Hokkaido University. Tumor samples were dissociated using a papain dissociation system (Worthington) according to the manufacturer’s instructions. The dissociated cells were cultured to form tumor spheres in serum-free Dulbecco’s modified Eagle’s medium (DMEM)/Ham’s F-12 (Wako) supplemented with human basic fibroblast growth factor (bFGF; 10 nM), human epidermal growth factor (EGF; 10 nM), heparin (5 μM), N2 supplement (Wako), 10 μg/ml insulin (Wako), GlutaMAX supplement, 100 units/ml penicillin G, and 100 μg/ml streptomycin. For immunohistochemistry, GICs were cultured on poly-D-lysine (15 μg/ml, Sigma)-coated and fibronectin (1 μg/ml)-coated 8-well chamber slides (Nunc). The human glioma cell lines U251 and U87 (obtained from ATCC) were
cultured in DMEM with 10% fetal bovine serum (FBS, Thermo Scientific). The mouse GIC lines NSCL61 and OPCL61, that have been established by overexpression of oncogenic HRas\textsuperscript{L61} in p53-deficient mouse NSC and oligodendrocyte precursor cell (OPC), were cultured as described previously (13, 14). Human NSCs (H9 human embryonic stem cell-derived) (Invitrogen) were cultured according to the supplier’s instructions. The cells were maintained at 37°C under a humidified 5% CO\textsubscript{2}/95% air atmosphere for all of the experiments. The characterization of human GICs is presented in the Supplementary Figure S1.

**Microarray hybridization and data processing**

Total RNA was extracted from GICs, glioma cells and glioma tissues using the TRIzol Plus RNA Purification System (Invitrogen). The miRNA microarrays were manufactured by Agilent Technologies, and 100 ng total RNA was hybridized using the miRNA Microarray Kit protocol for use with Human miRNA Microarray Release 16.0 or Mouse miRNA Microarray Release 16.0. Hybridization signals were detected using a DNA microarray scanner (Agilent Technologies), and the scanned images were analyzed using Agilent Feature Extraction software.

For the gene expression analyses, total RNA was amplified and labeled with cy3 using a one-color Agilent Low Input Quick Amp Labeling Kit (Agilent Technologies) according to the manufacturer’s instructions. Labeled cRNAs were fragmented and hybridized to the Agilent Human GE 8×60K Microarray. After washing, the microarrays were scanned using a DNA microarray scanner. Intensity values for each scanned feature were quantified using Agilent Feature Extraction software, which also performs background subtraction corrections.
Data normalization was conducted using Agilent GeneSpring GX version 11.0.2 software. After normalization, hierarchical sample clustering of the expressed genes was performed using the Euclidean distance and average linkage methods (Agilent GeneSpring GX). We used the freely available GenMAPP 2.1 database (http://www.genmapp.org/) and ConPath Navigator software, in combination with custom software programs, for the pathway analysis.

The microarray data have been submitted to NCBI GEO and available under GSE61078, GSE61079, and GSE61080.

**Real-time (RT) PCR**

Total RNA was extracted from GICs, glioma cells, and glioblastoma and normal brain tissues using Isogen reagent (Nippon Gene). Then, total RNA was reverse-transcribed using MultiScribe Reverse Transcriptase (Applied Biosystems) and MMLV RT (Invitrogen) according to the suppliers’ instructions. miR-340 expression was analyzed using TaqMan small RNA assays (Applied Biosystems) and a MiniOpticon RT-PCR System (Bio-Rad) according to the suppliers’ instructions. PLAT expression was analyzed using FastStart Universal SYBR Green (Roche) with a MiniOpticon RT-PCR System. The PCR conditions were as follows: 10 min at 95°C; followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. All of the expression values were normalized against β-actin mRNA expression levels. The RT-PCR primers for plat and β-actin are listed in Supplementary Table 1.

**Transfections**

Either the precursor form of miR-340 or a control miRNA was
overexpressed in GICs and glioma cells using miRNA lentiviral particles (pEZX-MR03; GeneCopoeia) according to the supplier’s instructions. In certain experiments, cells were transfected with a miR-340 expression vector (pBApocMV-Neo-miR-340, Takara) using a Nucleofector device (Lonza) and cultured in the presence of neomycin (300 μg/ml; Sigma) for 10 days.

**Cell proliferation and cell cycle analyses**

GICs and glioma cells (1×10⁴ cells/well) were transfected with either a control vector or a miR-340 expression vector and cultured for 72 h. Cells were collected on days 1, 2, and 3 and counted using a hemocytometer. BrdU staining was performed as previously described (15). Cell cycle analysis was performed using propidium iodide staining and fluorescence-activated cell sorting analysis (BD FACSCalibur, Cycletest Plus DNA Reagent Kit, BD Biosciences) according to the supplier’s instructions.

**Cell invasion and migration assays**

Cell invasion and migration were assessed using a BioCoat Matrigel Invasion Chamber (Becton-Dickinson) and the BioCoat Cell Culture Inserts (Becton-Dickinson), respectively. Briefly, transfected GICs and glioma cells (5-10×10⁴) were resuspended in DMEM/Ham’s F-12 medium, which was supplemented with 1 mg/ml bovine serum albumin (Sigma), and transferred to the upper chamber of each well. Then, medium containing 10% FBS was added to the lower chamber. After incubation for 12 h, the cells on the upper membrane surface were mechanically removed. Cells that had invaded or migrated to the lower side of the membrane were fixed, stained with 0.1% crystal violet, and
counted the amount of cells in five random fields under a microscope (400x).

**Intracranial cell transplantation into the brains of NOD/SCID mice**

Control and miR-340-overexpressing cells (1×10^5) were suspended in 5 μl of culture medium and injected into the brains of 6-8-week-old female NOD/SCID mice that had been anesthetized with 10% pentobarbital. The stereotactic coordinates of the injection site were 2 mm forward from lambda, 2 mm lateral to the sagittal suture, and 5 mm deep. Magnetic resonance imaging (MRI) was taken to confirm the tumor formation before decapitation of the mice.

**Brain fixation and histopathology**

Dissected mouse brains were fixed in 4% paraformaldehyde at 4°C overnight. After fixation, the brains were embedded in paraffin, cut into 6-μm thick coronal sections, and stained with hematoxylin-eosin.

**Immunohistochemistry**

Immunohistochemistry was performed as previously described (15). The following antibodies were used to detect cellular antigens: mouse monoclonal anti-Nestin (1:200; Pharmingen), mouse monoclonal anti-GFAP (1:200; Sigma), mouse monoclonal anti-Sox2 (1:200; Stem Cell Technology), rabbit polyclonal anti-PLAT (1:50; Sigma) and rabbit monoclonal anti-caspase-3 (cleaved) (1:100; Cell Signaling). The antibodies were detected using either Alexa 488-coupled goat anti-rabbit IgG (1:500; Life Technologies) or sheep anti-rabbit IgG (1:1; Nichirei). The nuclei were counterstained with DAPI (1 μg/ml) or hematoxylin.
Senescence-associated-β-galactosidase (SA-β-gal) staining

Cells transfected with either a control or a miR-340 overexpression vector were cultured in the presence of 400 μg/ml G418 for 10 days. Then, selected cells were fixed and stained using a SA-β-gal staining kit (Calbiochem) according to the supplier’s instructions.

Western blotting analysis

Cell or tissue lysates were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and the proteins were transferred to a polyvinylidene fluoride membrane (Amersham). The membrane was blocked in 5% nonfat milk, and then incubated in an anti-Nestin (1:1000), anti-Sox2 (1:500), anti-GFAP (1:500), anti-PLAT (1:250), or anti-GAPDH antibody (1:2000; Chemicon). After washing, the membrane was incubated in IgG-HRP (1:2000; Santa Cruz). An ECL system (Amersham) was used to detect the protein bands.

Vector construction

Full-length human plat was amplified from human glioma cDNA using RT-PCR and KOD-Plus-Ver.2 polymerase (Toyobo, Japan) according to the manufacturer’s instructions and cloned into the pcDNA3-2×FLAG-c vector, resulting in pcDNA3-hPLAT-2×FLAG-c. The primers used for amplification of the full-length human plat gene are listed in Supplementary Table 2.

To knockdown human plat expression, hairpin sequences were inserted into the psiRNA-h7SKhygro G1 expression vector (InvivoGen) to produce psiRNA-h7SKhygro-platsh. The shRNA target sequence was 5’-GAATTTCGATGATGACACTT-3’.
To construct a firefly luciferase-human plat 3’UTR expression vector, human plat 3’UTR genomic DNA was amplified using the KOD plus DNA polymerase and cloned into the pT7Blue-2 vector (Novagen), according to the manufacturer’s instructions. We also amplified a mutant form of the plat 3’UTR genomic DNA, with a deleted miR-340 binding site, and cloned it into the pT7Blue-2 vector. The wild and mutant types of plat 3’UTR DNA were inserted with the firefly luciferase cDNA into the pcDNA3.1-hyg vector, resulting in pcDNA3.1-hyg-Luc-PLAT 3’UTR (Luc-PLAT) and pcDNA3.1-hyg-Luc-ΔPLAT 3’UTR (Luc-ΔPLAT), respectively. The primers used to generate plat 3’UTR reporter construct are listed in Supplementary Table 3.

**Luciferase assay**

hGICs (E3) were transfected with pEF-Rluc (a kind gift from Koji Shimozaki and Shigekazu Nagata), a luciferase-PLAT vector (Luc-PLAT or Luc-ΔPLAT) and a miR-expression vector (control vector or pBApoCMV-Neo-miR340), and their luciferase activities were measured as previously described (16).

**Statistical analyses**

The Mann-Whitney test was used to compare two-paired groups. Kaplan-Meier curves were used to estimate the unadjusted time-to-event variables. Log-rank tests were applied to compare each time-to-event variable between groups. P values less than 0.05 (two-sided) were considered significant. All statistical analyses were performed using the StatMate software program.
Results

miR-340 is a novel miRNA that is down-regulated in GICs.

To identify novel miRNAs that are aberrantly expressed in GICs, we analyzed differences in miRNA expression between mouse and human GICs and control NSCs using miRNA microarrays. We identified seven miRNAs that were aberrantly expressed in both human and mouse GICs, three of which were up-regulated and four of which were down-regulated, compared with NSCs. Among these miRNAs, miR-340 was identified as a novel miRNA that was significantly down-regulated in all human and mouse GICs and in the human glioma cell lines (Figure 1A). Using quantitative PCR analysis, we confirmed that miR-340 expression was significantly decreased in all examined human and mouse GICs and human glioma cell lines compared with NSCs (Figure 1B). Furthermore, we determined that miR-340 expression was significantly decreased in human GBM tissues compared with normal human brain tissues (Figure 1C).

miR-340 inhibits cell proliferation, invasion, and migration and promotes cellular senescence in GICs.

To examine the function of miR-340 in hGICs, we infected hGICs and glioma cell lines with a recombinant lentivirus encoding either GFP alone or GFP and miR-340 together. Then, we purified the GFP-positive cells using flow cytometry and evaluated miR-340 expression in the infected cells (Supplementary Figure S2). During the cell culture, we observed that miR-340-overexpressing cells became flatter and larger than control cells (Figure 2A). Immunohistochemical analysis and Western blot assays revealed that miR-340-overexpressing hGICs partially decreased Nestin expression and lost the...
expression of Sox2, but remained positive for GFAP, an astrocyte marker (Figure 2B-D). miR-340-overexpressing hGICs ceased proliferating during the first 3 days of culture (Figure 3A). The BrdU-incorporation and cell cycle analyses revealed that miR-340 overexpression significantly arrested the cell cycle at the G1/S transition, as indicated by a marked accumulation of cells in the G1 peak and by a reduction of cells in the S phase (Figure 3B and C). In addition, we examined invasiveness and motility in the miR-340-overexpressing hGICs and confirmed that miR-340 significantly inhibited both invasion and migration in these cells (Figure 3D and E). We further demonstrated that miR-340 overexpression activated the expression of SA-β-gal, a marker of cellular senescence, in hGICs (Figure 3F). With the exception of cellular senescence, similar results were observed in the miR-340-overexpressing human glioma cell lines. Taken together, these data indicate that miR-340 negatively regulates the proliferation and invasiveness of both hGICs and glioma cell lines but induces cellular senescence only in hGICs.

**miR-340 inhibits GIC-mediated tumorigenesis in vivo.**

Next, we investigated whether miR-340 overexpression inhibits GIC-mediated tumorigenesis in vivo. We transplanted either miR-340-overexpressing hGICs or their control hGICs into the brains of immunodeficient mice. The control hGICs without miR-340 overexpression continuously formed malignant gliomas with high invasiveness and many mitotic cells with a histopathology closely resembling that of the original tumor, causing death in mice within 48 days (Figure 4A, upper panels, B-D). In contrast, none of the mice injected with miR-340-overexpressing hGICs formed apparent tumors.
(Figure 4A, lower panels), and these mice survived for more than 80 days (Figure 4D). Histological examination three days after transplantation of miR-340-overexpressing hGICs demonstrated that the injected tumor cells were positive for active form of caspase-3 immunostaining, indicating that miR-340 induced GIC apoptosis (Figure 4E). In contrast, tumor formation of miR-340-overexpressing human glioma cell lines in mouse brains was not completely suppressed, resulting in the death of the mice although the mice survived two or four weeks longer than the control (Supplementary Figure S3A and B). These findings indicate that miR-340 is a strong suppressor of tumorigenesis in GICs, although this anti-tumorigenic effect is less obvious in glioma cell lines.

**PLAT is a direct target of miR-340.**

To identify functional targets of miR-340, we compared the gene expression profiles of miR-340-overexpressing hGICs with the profiles of their control GICs (Figure 5A). We selected candidate target genes that were significantly up-regulated or down-regulated in miR-340-overexpressing hGICs and analyzed these candidates using ConPath Navigator software, which is based on the GenMAPP software program (www.genmapp.org). These data revealed that miR-340 regulates the genes related to two signaling pathways. One is the cell cycle pathway, which involves p21/Cip1 and cyclin A (17, 18). The other is the cell adhesion/ECM remodeling pathway, which includes PLAT and MMPs (matrix metalloproteases) (19, 20) (Supplementary Figure S4A and B). Of the molecules that were strongly regulated by miR-340, we focused on PLAT because its expression was significantly decreased in miR-340-expressing hGICs.
(Supplementary Figure S5A). In addition, the 3’ untranslated regions (UTRs) of both the mouse and human plat mRNAs contained potential miR-340 target sequences (Figure 5B). Immunohistochemical analysis and Western blot assays confirmed that miR-340 overexpression decreased PLAT expression in hGICs (Figure 5C and D). Using a reporter vector encoding the firefly luciferase gene with the wild-type plat 3’UTR, we demonstrated that miR-340 overexpression inhibited luciferase activity in hGICs, whereas a deletion in the predicted binding site of miR-340 in the 3’UTR of the plat gene abrogated the aforementioned inhibitory effect of miR-340 (Figure 5E). Taken together, these data strongly indicate that PLAT is a novel direct target of miR-340 in hGICs. We confirmed that PLAT was more highly expressed in human glioblastomas than in normal brain tissues at the level of both mRNA (Figure 5F) and protein (Figure 5G) and that the tumor cells positive for PLAT immunostaining coexisted with those cells expressing CD15 (SSEA-1, hGIC marker) (21) (Supplementary Figure S5B).

PLAT knockdown inhibits GIC proliferation, invasion, and migration in vitro and GIC tumor growth in vivo.

We subsequently examined the functions of PLAT in hGICs. PLAT knockdown using the plat-sh expression vector (Supplementary Figure S6A and B) inhibited hGIC proliferation and invasion in a manner similar to that induced by miR-340 overexpression (Figure 6A-D). The inhibitory effects were fully recovered by introducing mutant PLAT in which the shRNA target sequences were deleted. (Supplementary Figures S7). Furthermore, miR-340 transfection in hGICs overexpressing the PLAT gene that lacked the 3’UTR recovered the inhibitory effects of miR-340 on cell functions (Supplementary Figures S8).
These results indicate that PLAT is a key molecule that promotes malignancy of GIC by directly mediating the functions of miR-340 down-regulation. In addition, transplanting PLAT-knockdown hGICs into mouse brains resulted in the formation of tiny tumors localized at the injection site, but the growth rate was extremely slow, indicating that PLAT promotes GIC tumor growth in vivo (Figure 6E).
Discussion

In the current study, we identified miR-340 as a novel miRNA whose expression was significantly lower in both hGICs and glioma cell lines than in NSCs. We also observed that miR-340 suppressed not only GIC proliferation and invasion \textit{in vitro} but also GIC-initiated tumor formation in mouse brains. These findings indicate that miR-340 can act as a suppressor of malignant functions in GICs, particularly gliomagenesis and extensive tumor invasion. Furthermore, we determined that PLAT is the most significant target of miR-340 in hGICs from the results of luciferase assays and PLAT-knockdown experiments with \textit{plat-sh}, which phenocopied the suppressive effects of miR-340 overexpression \textit{in vitro} and \textit{in vivo}. In addition to PLAT, miR-340 overexpression in hGICs decreased the expression of Sox2, c-Met, CD44, and DNMT1, which are miR-340 target genes (Supplementary Figure S9). c-Met and CD44 regulate cell invasion and migration (22, 23), and Sox2 and CD44 play crucial roles in the maintenance of CSC stemness (24). Although miR-340 overexpression in hGICs did not markedly decrease Nestin expression, Sox2 and CD44 expression significantly decreased, indicating that miR-340 might reduce GIC stemness.

Transplanting miR-340-overexpressing hGICs into mouse brains did not result in tumor formation. However, the miR-340-overexpressing glioma cell lines generated tumors, although the growth rate was considerably suppressed, finally resulting in tumor-caused death of the mice. The mechanisms underlying the differences in miR-340-induced gliomagenesis inhibition in GICs and in glioma cell lines remain unclear. Histopathologically, the transplanted hGICs with miR-340-overexpression showed apoptosis in the early stage of transplantation, suggesting that anti-tumorigenic effects of miR-340 in GIC might be partly due to
tumor cell apoptosis. Recently, the inhibition of Sox2, which is a stem cell marker, was demonstrated to reduce the tumorigenic potential of human gastric cancer cells (25). In the glioma cell lines, the expression levels of Sox2 were initially quite low; therefore, Sox2 function was likely not highly affected by miR-340 in these cell lines. These findings suggest that the tumorigenic mechanisms differ between GICs and more differentiated glioma cell lines.

Interestingly, the present study demonstrated that miR-340 overexpression in hGICs promoted senescence in these cells, whereas miR-340 introduction into glioma cell lines did not induce senescence (data not shown). Cellular senescence can inhibit tumor formation by modulating the redox state in CSCs and by regulating p27 in glioma cells (26, 27). In addition, GICs derived from wild-type mice expressing the senescence-promoting factor esophageal cancer-related gene 4 (Ecrg4) caused significantly reduced tumor formation in mouse brains compared with cells from Ecrg4-knockdown mice (28). We observed that miR-340 overexpression in hGICs decreased DNA methyltransferase 1 (DNMT1) expression (Supplementary Figure 9D); decreased DNMT1 expression can promote cellular senescence (29). These findings suggest that miR-340-induced cellular senescence might explain the reduced tumorigenic activity of miR-340-overexpressing GICs.

Currently, many miRNAs that are down-regulated in GSCs have been reported, including miR-128, miR-124, miR-137, miR-34a, and miR-451 (11, 30-32). The overexpression of these miRNAs inhibits the proliferation and self-renewal, reduces the viability, or induces the differentiation of GSCs but does not cause the irreversible death of GSCs (31-33). Chan et al. demonstrated that miR-138 acts as a prosurvival oncomiR in GSCs and that functional inhibition of
miR-138 prevents not only tumorsphere formation in vitro but also tumorigenesis in vivo by inducing apoptosis and suppressing the proliferation of GSCs (34). It has been recently reported that miR-218 inhibits the self-renewal of GSCs by targeting Bmi1 and regulates glioma cell development by Wnt pathways (35). Additionally, Wang et al. reported that miR-33a is highly expressed in GICs and that antagonizing miR-33a function in GICs reduces self-renewal and tumor progression in mice by inhibiting the PKA and NOTCH pathways, which promote GIC maintenance (36). Many tumor cell functions, including a high proliferation rate, increased cell motility, and suppressed apoptosis and senescence, may be tightly linked during tumor development. Our findings indicate that miR-340 regulates the expression of several key target genes that participate in cell proliferation, invasion, migration, and senescence, including PLAT, C-Met, CD44, Sox2, and DNMT1. Furthermore, p21 and ANXA2 genes, which have important roles in cell proliferation and in cell invasion, respectively, are downstream of PLAT. Both of these genes were also significantly up-regulated or down-regulated by miR-340 overexpression in hGICs (Supplementary Figure S9), indicating that PLAT plays central roles in cell proliferation, invasion, and migration. The loss of miR-340 as a regulator controlling the expression of these genes in GICs promotes the development of these cells into malignant tumors and advances tumor progression (Figure 7).

The National Cancer Institute’s Repository for Molecular Brain Neoplasia Data (REMBRANDT) database (https://caintegrator.nci.nih.gov/rembrandt/) (37) indicates that the prognosis of glioma patients with increased plat levels is poorer than that of patients with decreased plat levels (Supplementary Figure S10). These findings suggest that the introduction of miR-340, which is capable of directly
suppressing PLAT expression, may be a useful therapeutic tool for improving the
prognosis of GBM patients. Furthermore, future studies of the functional
significance of miR-340 target molecules, including PLAT, might provide a
greater understanding of the complex mechanisms of GBM development and
progression.

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References


Figure Legends

Figure 1. miR-340 expression is decreased in mouse and human GICs. (A) Heatmaps showing the hierarchical clustering of miRNAs expressed in mouse (left panel) and human (right panel) GICs and in human glioma cell lines (U87 and U251). miRNAs showing important functions in gliomas are indicated. (B) Fold changes in miR-340 expression in human GICs (E1-4, E6, AO and DA), human glioma cell lines, and NSCL61 compared with normal NSCs. (C) The relative ratios of miR-340 expression in 20 GBM tissues compared with five normal brain tissues. Values are expressed as mean ± SD. ***P<0.001.

Figure 2. miR-340 overexpression in hGICs changes cell morphology and inhibits expression of stem cell markers, Sox2 and Nestin, but remains positive for GFAP. (A) Fluorescence images of control (upper panels) and miR-340-overexpressing hGICs (lower panels). (B) Immunostaining of Nestin (green) in miR-340-overexpressing hGICs (E3, E6) and in the control. (C) Immunostaining of GFAP (green) and Sox2 (red) in miR-340-overexpressing hGICs (E3, E6) and in the control. The nuclei are counterstained with DAPI (blue). (D) Western blotting analysis of Nestin, Sox2, and GFAP expression in miR-340-overexpressing hGICs (E3) and in the control. Scale bar, 200 μm.

Figure 3. miR-340 overexpression in hGICs inhibits cell proliferation, cell invasion, and migration and induces cell cycle arrest and senescence. (A) The cell growth curves of control and miR-340-overexpressing hGICs. (B) The proportions of BrdU-positive cells in control and miR-340-overexpressing hGICs. (C) DNA histogram plots showing the cell cycle analysis of hGICs transfected
with miR-340 or with the control. Cell cycle distributions were detected 72 h after transfection, and the ratios of each phase are represented as numerical values (shown in the inset of the figures). (D, E) Quantification of cell invasion and migration. The amount was expressed as invasion ratios (D) and migration ratios (E) of miR-340-overexpressing hGICs compared with control cells. (F) SA-β-gal staining (green) in control and miR-340-overexpressing hGICs. The nuclei were counterstained with DAPI (blue). Values are expressed as mean ± SD. Scale bar, 50 μm. **P<0.01, ***P<0.001.

**Figure 4.** miR-340 overexpression completely inhibits hGIC tumorigenesis in vivo. (A) H & E staining of mouse brains with hGIC-xenograft (left panels) and the magnified images of tumor injection sites (center panels) five weeks after transplanting hGICs (E3 control) or miR-340-overexpressing hGICs (E3 miR-340). The corresponding MR images taken immediately before the dissection of the mouse brains (right panels). Dotted circles show the delineation of the extent of the tumor. The tumor volume of E3 control was 75.4±17.8 mm³ and that of E3 miR-340 could not be determined because the tumor was undetectable on MRI. (B) Histopathology showing tumor invasion in the tumor border in control hGICs. (C) Histopathology showing mitotic cells (arrows) in the tumor of control hGICs. (D) Survival curves of the mice injected with control hGICs (E3, E6) (n=5) (black dotted line) or with miR-340-overexpressing hGICs (n=3) (red solid line). (E) Immunostaining for caspase-3 of the mouse brains dissected 3 days after injection of miR-340-overexpressing hGICs (E3) (right panel) or of the control hGICs (left panel). Scale bars: 1 mm (left panels) and 0.1 mm (right panels) in A; 50 μm in B; 20 μm in C, and 25 μm in E.
**Figure 5.** PLAT is a putative target of miR-340. (A) Heatmaps of genes with significantly altered expression between control hGICs and miR-340-overexpressing hGICs and between glioma cell lines (none) and glioma cell lines (miR-340). (B) Sequences of the miR-340 binding sites in the mouse and human *plat* 3’UTRs predicted by TargetScan. Mutant form has a deletion of 20 nucleotides from 588 to 607. (C) Immunofluorescence showing PLAT expression (green) in the control and miR-340-overexpressing hGICs. (D) Western blot analysis of PLAT expression levels in control and miR-340-overexpressing hGICs (E3). (E) Relative luciferase activity in miR340-overexpressing hGICs (E3) transfected with either Luc-PLAT or Luc-ΔPLAT (mutant form with deletion in miR-340 binding sites). (F) PLAT expression levels in 20 GBM tissues and in five normal brain tissues. (G) Western blot analysis of PLAT expression levels in human GBM and normal brain tissues. Values are expressed as mean ± SD. **P<0.01, ***P<0.001.

**Figure 6.** PLAT is a functional target of miR-340. (A) Cell growth curves of control and PLAT-shRNA-expressing hGICs (E3 and E6). (B) The proportions of BrdU-positive cells in control and in PLAT-shRNA-expressing hGICs. (C and D) Quantification of cell invasion and migration. The amount was expressed as relative invasion (C) and migration (D) ratios of PLAT-shRNA-expressing hGICs compared with control cells. (E) H&E staining of whole brain slices 5 weeks after transplantation of PLAT-shRNA-expressing hGICs in the mouse brain. Scale bars: 1mm (upper panels), 25μm (lower panels). Values are expressed as mean ± SD. **P<0.01.
Figure 7. A schematic summary of the function of miR-340 and its target genes during the tumorigenesis process in hGICs, including cell cycle, invasiveness, stemness, and senescence. PLAT is a novel direct target gene suppressed by miR-340. Other target genes, including c-Met, CD44, Sox2, and DNMT1, are also down-regulated by miR-340 in hGICs. Of the downstream genes of PLAT, p21 is up-regulated, and ANXA2 is down-regulated by miR-340 overexpression in hGICs.
Yamashita et al, Figure 1
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
A. 

![Graph A](image1)

B. 

![Graph B](image2)

C. 

![Graph C](image3)

D. 

![Graph D](image4)

E. 

![Images](image5)

Yamashita et al, Figure 6
miR-340 regulates various target genes, including PLAT, c-Met, CD44, Sox2, and DNMT1. These genes in turn control cell cycle progression, proliferation, invasion, migration, stemness, and senescence, ultimately affecting tumorigenesis. Yamashita et al, Figure 7

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