Supplementary information

Material and methods

Chemicals and Reagents

The glioma U-87 MG, U-118 MG, and patient-derived PDM-123 cells were purchased from American Type Culture Collection (ATCC; Rockville, MD). Dulbecco’s modified Eagle medium (DMEM), GlutaMAX, and fetal bovine serum (FBS) were purchased from GIBCO-BRL (Grand Island, NY). Anti-GAPDH (cat no. GTX100118), anti-IGFBP2 (cat. no. GTX128977), and anti-OCT1 (cat. no. GTX105202) antibodies were purchased from GeneTex (Hsinchu City, Taiwan). The anti-FGFR1 antibody (cat. no. 9740) was purchased from Cell Signaling Technology (Danvers, MA). The anti-ki67 antibody (cat. no. AB9260) and TMZ (cat. no. T2577) were purchased from Sigma-Aldrich (St. Louis, MO). An enhanced chemiluminescence (ECL) solution (cat. no. WBKLS0500), polyvinylidene difluoride (PVDF) membranes (cat. no. IPVH00010), and RIPA Lysis Buffer (cat. no. R0278) were purchased from Millipore (Billerica, MA). Lipofectamine 3000 (cat. no. L3000015), and secondary antibodies (cat. no. A16110) were purchased from Invitrogen (Carlsbad, CA). SYBR® Green polymerase chain reaction (PCR) master mix (cat. no. 4309155) and a MultiScribe® reverse-transcriptase kit (cat. no. N8080234) were purchased from Applied Biosystems (Waltham, MA).

Cell Culture, Gene Transfection, and Transduction

U-87 MG and U-118 MG cells were maintained in DMEM containing 10% FBS, 2.5 mM GlutaMAX, 100 units/mL penicillin, and 100 μg/mL streptomycin. PDM-123 cells were maintained in NeuroCult NS-A Basal Medium with NS-A Proliferation Supplement, 20 ng/mL EGF, 20 ng/mL bFGF, and 2 μg/mL Heparin using ultra low
attachment culture dishes. To mimic a hypoxic environment in glioma tissues, glioma cells were cultured in an Eppendorf® galaxy® 48R CO\(_2\) chamber (Hamburg, Germany) with 1% O\(_2\). To conduct gene transfection experiments, Lipofectamine 3000 was used according to the manufacturer’s protocol. In brief, seeded cells with a density of around 70% in 24- or 12-well plates were transfected with the indicated dose of plasmid, including empty pCDH (cat. no. CD510B-1; System Biosciences, Palo Alto, CA), pCDH-MIR210HG, OCT1 small hairpin (sh)RNA, and MIR210HG shRNA or the indicated concentration of MIR210HG antisense oligonucleotides (ASOs). Sequences of shRNAs and ASOs are listed in Suppl. Table 1. The MIR210HG shRNA and pCDH-MIR210HG plasmids were co-transfected with the psPAX2 (cat. no. 12260) and pMD2.G (cat. no. 12259) plasmid from Addgene (Watertown, MA) into Lenti-X 293T cells. Then, lentivirus-containing supernatants were collected after 72 h. The cells were incubated with viral supernatants for 48 h to establish glioma cells with stable knockdown or overexpression of MIR210HG.

**MIR210HG Isoform Expression Analysis**

The ensemble genome browser (https://m.ensembl.org) was queried to identify isoforms of MIR210HG. Expressions of MIR210HG isoforms were obtained from TANRIC (https://ibl.mdanderson.org/tanric/design/basic/main.html), a database that can query any user-defined lncRNA or its isoform expression in TCGA. Associations of each isoform with hypoxic activity were investigated by Pearson correlation analyses. The abundance of each isoform was compared by the Kruskal-Wallis test with a post-hoc Dunn's test.

**TMZ Treatment and Cell Viability Assay**
Glioma cells with gene transfection or hypoxic exposure were seeded in 24-well plates (at 2.5×10^4 cells/well) and treated with 200 μM TMZ or dimethyl sulfoxide (DMSO) for 3 days. Cell viability was measured by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. After the end of TMZ treatment, glioma cells were treated with 0.5 mg/mL MTT in each well for 30 min. Formazan products were collected and dissolved in DMSO. The absorbance was measured at 570 nm on a Thermo Varioskan Flash reader (Carlsbad, CA).

**Immunoblot Assays**

Total cell lysates with indicated transfection were collected using RIPA buffer with a protease inhibitor cocktail (cat. no. 539131; Calbiochem, Billerica, MA). After centrifuging at 13,800 ×g and 4 °C for 10 min, cell debris was removed. Denaturing buffer consisting of 2% sodium dodecylsulfate (SDS), 10 mM dithiothreitol, 60 mM trishydrochloric acid (Tris-HCl, pH 6.8), and 0.1% bromophenol blue were added to total cell lysates. Total cell lysates with denaturing buffer were loaded into polyacrylamide-SDS gels (7.5% or 10%). After transfer, the PVDF membrane (cat. no. 539131, IPVH00010; Merck, Burlington, MA) was blocked with phosphate-buffered saline (PBS) with 2% bovine serum albumin (BSA). Subsequently, the membrane was incubated in PBS containing a primary antibody (diluted 1:1000) and was gently mixed overnight at 4 °C. Then, after washing with PBS-Tween 20, a secondary antibody conjugated to horseradish peroxidase (diluted 1:3000) was added for 1 h at 25 °C. Antibody-protein complexes were detected with an enhanced chemiluminescence (ECL) nonradioactive detection system. The quantitative result of each gene was showed below the immunoblotting image.
**RNA Extraction and Real-Time Reverse-Transcription Quantitative Polymerase Chain Reaction (RT-PRC)**

Trizol® was utilized to extract total RNA based on the manufacturer’s instructions. To isolate cytoplasmic and nuclear RNAs, a cytoplasmic and nuclear RNA Purification Kit (cat. no. 21000) purchased from NORGEN (Thorold, ON, Canada) was used. To ensure the quality of extracted RNA, the A260/A280 ratio was measured. One microgram of extracted RNA was reverse-transcribed into complementary (c)DNA using a MultiScribe® Reverse Transcriptase kit with random primers. cDNA was dissolved in nucleotide-free water at a 1:20 dilution ratio. Primers for detecting specific genes and POLR2A are listed in Suppl. Table 1. Gene expressions were quantified using an Applied Biosystems StepOnePlus™ System (ThermoFisher Scientific, Waltham, MA) with pre-optimized conditions. For each experimental condition, a biological triplicate was performed. PCR reagents, including 5 μL 2× SYBR Green PCR Master Mix, 0.2 μL primer sets, and 3.6 μL nucleotide-free H₂O, were mixed with 1 μL cDNA. The normalized CT difference was calculated as the expression rate between the control and sample after adjusting for the amplification efficiency relative to the level of the POLR2A housekeeping gene.

**Tumor Sphere-Formation Assay**

To conduct the sphere-formation assays, 5×10³ glioma cells were seeded in a Costar® ultralow attachment multiple 96-well plate (Corning) with DMEM, B27 (1:50; Invitrogen, San Diego, CA), epidermal growth factor (EGF; 20 ng/mL, Invitrogen), basic fibroblast growth factor (bFGF; 20 ng/mL, Gibco), and Glutamax (1:100, Invitrogen). Ten microliters of sphere medium were renewed every 3 days. After 1
week, spheres were centrifuged at 1000 ×g for 5 min and resuspended to generate secondary spheres. After 2 weeks, each diameter from 20 tumor spheres per well were counted for the quantitation. Scale bar means 100 μm.

**Soft Agar Colony-Formation Assay**

Two layers of soft agar were produced in order to perform colony-formation assays. The bottom and top layers respectively contained 0.5% and 0.3% agar mixed with complete medium. After culturing for 2 weeks, 1 mg/1 mL nitrotetrazolium blue chloride (Sigma-Aldrich) was used to stain the colonies.

**In Vivo Xenograft Study**

The in vivo experiments including drug treatment, glioma xenograft establishment, and animal care were approved by the Taipei Medical University Laboratory Animal Care and Use Committee (permit no.: LAC-2019-0114). The procedures strictly followed recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH Publications no. 8023, revised 1978) and ARRIVE guidelines. Six-week-old NOD-SCID mice (NOD.CB17-Prkdscid/NcrCrl; BioLASCO Taiwan, Nankang, Taipei, Taiwan) were fed in a pathogen-free room with a 12/12-h light/dark cycle. To establish the glioma xenograft model, MIR210HG-depleted (n=10) or control (n=9) U-87MG cells (2×10⁶) in 0.1 mL DMEM containing diluted 1:1 Corning® Matrigel® Basement Membrane Matrix were subcutaneously (s.c.) injected into the right hind flank of mice. The tumor volume was obtained by measuring the length and width, and these parameters were applied to the following formula: 1/2 × length × width² (mm³). After the tumor had reached about 100 mm³, mice bearing MIR210HG-depleted or empty vector U-87MG cells were randomly
separated into two groups \((n=5 \text{ mice/group})\), including a vehicle control \((n=4; \ 20\% \text{ DMSO, i.p. injections, every 3 days})\) and TMZ treatment group (i.p. injections, 50 mg/kg body weight for 3 days). At the end of the experiment, all mice were sacrificed. Tumor tissues were excised for immunohistochemical (IHC) staining and homogenized to extract proteins or RNA for further investigation.

**Immunohistochemistry (IHC)**

The 5-μm paraffin-embedded sections mounted on poly-L-lysine-coated slides from tumor-bearing mice were used to conduct IHC staining. Tissue slide were dewaxed with xylene and rehydrated with decreasing concentrations of ethanol (95%, 85%, and 75%). Then, tissue slides were soaked in 10 mmol/L sodium citrate buffer (pH 6) and heated in a water bath at 95 °C for 30 min. After blocking with 10% BSA in PBS for 30 min, sections were incubated overnight at 4 °C with primary antibodies at a dilution of 1:100. Next, 1:1000-diluted biotinylated goat anti-rabbit immunoglobulin G (IgG) was applied for 30 min, followed by detection of immunoreactivity with an avidin-biotin system using 3,3′-diaminobenzidine tetrahydrochloride as a chromogen. Sections were lightly counterstained with Mayer’s hematoxylin (Richard-Allan Scientific, Kalamazoo, MI). Scale bar means 100 μm.

**Invasion assay**

Glioma cells \((5 \times 10^4)\) were seeded in 8.0-μm-pore transwell inserts (SPLInsert™, Naechon-myeon, Gyeonggi-do, Korea) coated with Matrigel. There was 800 μl of complete DMEM placed beneath the transwell inserts, and 200 μl DMEM without FBS was added to the transwell inserts. After overnight incubation, DMEM was aspirated, and cells were washed with PBS. Then, 1% crystal violet containing 70% ethanol was
used to fix and stain cells for 20 min. Cells in the upper layer of inserts were removed with a cotton swab, and cells in bottom layer of inserts were captured at 100× magnification in four different fields. Scale bar means 100 μm.

Next Generation Sequencing

Total RNAs harvested using the Trizol® reagent (Invitrogen) according to the manufacturer’s instructions were collected from biological replicates of empty vector- and MIR210HG-transfected U-87 MG cells. RNA-Seq analyses were conducted by Welgene Biotechnology (Taipei, Taiwan). Raw counts were utilized to perform DEG analyses. Gene candidates with a multiple of change (FC) of >1.5 or an FC of <0.7 with a false discovery rate (FDR) of <0.01 were considered to have significant alterations. UCSC_TFBS and KEGG_PATHWAY modules from the DAVID functional annotation tool (https://david.ncifcrf.gov/) were utilized to identify MIR210HG-regulated gene-enriched transcription factors and pathways.

Gene Set Enrichment Analysis (GSEA)

Predicted OCT1 targets from the UCSC_TFBS module of the DAVID functional annotation tool and multiples of change of MIR210HG-regulated genes were respectively used as a gene set and a ranked gene list. A GSEA algorithm with 1000 permutations was performed in the fgsea package.

RNA Immunoprecipitation (RIP)

RIP was conducted using an EZ-Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (cat. no. 17-701) according to the manufacturer’s protocol. An anti-OCT1 antibody was used to precipitate associated RNAs from hypoxia- or
normoxia-cultured glioma cells. Enriched MIR210HG levels were measured by a quantitative (q)PCR. An anti-mouse IgG antibody was used as a negative control.

**RNA Pull-Down Assay**

The RNA pull-down assay was performed following procedures described in Tsai et al.’s study [14]. Briefly, using the Roche Biotin RNA Labeling Mix Kit (cat. no. 11685597910) and T7 RNA polymerase (cat. no. 10881775001), transcripts of MIR210HG-203 and GAPDH, a negative control, were biotin-labeled in vitro. To degrade unwanted DNA templates, PCR products were treated with RNase-free DNase I (cat. no. M198A; Promega, Madison, WI). Subsequently, an RNA structure buffer (10 mM Tris-HCl at pH 7.0, 0.1 M KCl, and 10 mM MgCl₂) was used to fold the biotinylated RNA at 90 °C for 2 min. Then, biotinylated RNA was transferred to ice for another 2 min and room temperature for 20 min, which caused the biotinylated RNA to form a secondary structure. Nuclear proteins from glioma cells were isolated using nuclear isolation buffer (1.28 M sucrose, 40 mM Tris-HCl at pH 7.5, 20 mM MgCl₂, and 4% Triton X-100). One milligram of nuclear proteins was mixed and incubated for 1 h with biotinylated MIR210HG or GAPDH. Streptavidin Magnetic Beads (cat. no. LSKMAGT02; Millipore, Burlington, MA) were used to pull down the biotinylated RNAs and protein complexes. After washing with PBS five times, the mixture was boiled with 1× SDS loading buffer. Finally, retrieved proteins were analyzed using immunoblot assays.

**Construction of the IGFBP2 and FGFR1 Promoter Reporter Plasmids**

Reporter plasmids consisting of the IGFBP2 or FGFR1 promoter regions were constructed to evaluate transcription activities of hypoxia, MIR210HG, or OCT1 on
gene regulation. Specific primers to amplify fragments of the IGFBP2 or FGFR1 promoter are listed in Suppl. Table 1. Subsequently, the amplified fragments were cloned into pGL3 reporter vectors (cat. no. E1751; Promega) after digestion with XhoI and HindIII, creating the pGL3-IGFBP2-prom and pGL3-FGFR1-prom expression vectors. These promoters were co-transfected with indicated plasmids by Lipofectamine 3000 (Invitrogen), and pNL1.1-TK plasmids were co-transfected as an internal control. Luciferase activities were quantified using the NanoGlo dual luciferase assay system (Promega).

**Chromatin Immunoprecipitation (ChIP) Assay**

Glioma cells (10^6) transfected with indicated plasmids in normoxic and hypoxic conditions were used to perform ChIP assays according to the manufacturer’s instructions (EZ-ChIP™, cat. no. 17-295; Millipore). One microgram of the OCT1 antibody cross-linked with protein G beads was used to precipitate the protein-DNA complexes overnight. Oct1-associated DNA fragments were eluted with 1% SDS/0.1 M NaHCO₃ and reverse cross-linked at 65 °C. Mouse IgG was used as a negative control. The predicted OCT1-binding sites in the IGFBP2 and FGFR1 promoters were queried from JASPAR (http://jaspar.genereg.net/). Enrichment effects were measured by qPCR analyses with specific primers listed in Suppl. Table 1.

**Constructions of deleted mutants of MIR210HG**

To further confirm the interaction between MIR210HG and OCT1, two OCT1-binding site-deleted MIR210HG mutants were constructed. The nucleotides of MIR210HG-203 between 479 to 492 and 233 to 246 were respectively deleted and named as DM-1 and DM-2. The nucleotides of deleted mutants containing 5’-XbaI and
3’-EcoRI sites were synthesized by GenScript (Piscataway, NJ 08854, USA). Then, after restriction enzymes digesting, these DNA fragments were respectively cloned into pCDH vectors. After DNA sequencing, these DM plasmids were used for further experiments.

**RNA Pull-Down Assay with deleted mutants**

By using the Roche Biotin RNA Labeling Mix Kit (cat. no. 11685597910) with T7 RNA polymerase (cat. no. 10881775001), transcripts of MIR210HG-203, DM-1, DM-2, and GAPDH, a negative control, were biotin-labeled *in vitro*. After RNase-free DNase I (cat. no. M198A; Promega, Madison, WI) treatment, the biotinylated RNAs were treated with RNA structure buffers at 90 °C for 2 min and transferred to ice for another 2 min and room temperature for 20 min. Nuclear proteins from glioma cells were isolated using nuclear isolation buffer (1.28 M sucrose, 40 mM Tris-HCl at pH 7.5, 20 mM MgCl₂, and 4% Triton X-100). One milligram of nuclear proteins was mixed and incubated for 1 h with biotinylated RNAs. Streptavidin Magnetic Beads (cat. no. LSKMAGT02; Millipore, Burlington, MA) were used to pull down the biotinylated RNAs and protein complexes. After washing with PBS five times, the mixture was boiled with 1× SDS loading buffer. Finally, retrieved proteins were analyzed using immunoblot assays.

**Invasion assay and tumor sphere-formation with deleted mutants**

After transfection with 2 μg MIR210HG and DM-1 plasmids for 24 h, 5×10⁴ cells were seeded in 8.0-μm-pore transwell inserts (SPLInsert™, Naechon-myeon, Gyeonggi-do, Korea) coated with Matrigel. There was 800 μl of complete DMEM placed beneath the transwell inserts, and 200 μl DMEM without FBS was added to the transwell inserts. After overnight incubation, cells were washed with PBS. Then, 1%
crystal violet containing 70% ethanol was used to fix and stain cells for 20 min. Cells in the upper layer of inserts were removed with a cotton swab, and cells in bottom layer of inserts were captured at 100× magnification in four different fields. Scale bar means 100 μm.

To conduct the sphere-formation assays, 5×10³ transfected cells were seeded in a Costar® ultralow attachment multiple 96-well plate (Corning) with DMEM, B27 (1:50; Invitrogen, San Diego, CA), epidermal growth factor (EGF; 20 ng/mL, Invitrogen), basic fibroblast growth factor (bFGF; 20 ng/mL, Gibco), and Glutamax (1:100, Invitrogen). Ten microliters of sphere medium were renewed every 3 days. After 1 week, spheres were centrifuged at 1000 ×g for 5 min and resuspended to generate secondary spheres. After 2 weeks, each diameter from 20 tumor spheres per well were counted for the quantitation. Scale bar means 100 μm.

**TMZ treatment and cell viability assay with deleted mutants**

After transfection with 2 μg MIR210HG and DM-1 plasmids for 24 h, cells were seeded in 24-well plates (at 2.5×10⁴ cells/well) and treated with 200 μM TMZ or dimethyl sulfoxide (DMSO) for 3 days. Cell viability was measured by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. After the end of TMZ treatment, glioma cells were treated with 0.5 mg/mL MTT in each well for 30 min. Formazan products were collected and dissolved in DMSO. The absorbance was measured at 570 nm on a Thermo Varioskan Flash reader (Carlsbad, CA).