Interfering with long non-coding RNA MIR22HG processing inhibits glioblastoma progression through suppression of Wnt/β-catenin signalling

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Long non-coding RNAs play critical roles in tumour progression. Through analysis of publicly available genomic datasets, we found that MIR22HG, the host gene of microRNAs miR-22-3p and miR-22-5p, is ranked among the most dysregulated long non-coding RNAs in glioblastoma. The main purpose of this work was to determine the impact of MIR22HG on glioblastoma growth and invasion and to elucidate its mechanistic function. The MIR22HG/miR-22 axis was highly expressed in glioblastoma as well as in glioma stem-like cells compared to normal neural stem cells. In glioblastoma, increased expression of MIR22HG is associated with poor prognosis. Through a number of functional studies, we show that MIR22HG silencing inhibits the Wnt/β-catenin signalling pathway through loss of miR-22-3p and -5p. This leads to attenuated cell proliferation, invasion and in vivo tumour growth. We further show that two genes, SFRP2 and PCDH15, are direct targets of miR-22-3p and -5p and inhibit Wnt signalling in glioblastoma. Finally, based on the 3D structure of the pre-miR-22, we identified a specific small-molecule inhibitor, AC1L6JTK, that inhibits the enzyme Dicer to block processing of pre-miR-22 into mature miR-22. AC1L6JTK treatment caused an inhibition of tumour growth in vivo. Our findings show that MIR22HG is a critical inducer of the Wnt/β-catenin signalling pathway, and that its targeting may represent a novel therapeutic strategy in glioblastoma patients.
Introduction
Non-coding RNAs (ncRNAs), especially long non-coding RNAs (lncRNAs) and microRNAs (miRNAs), have important roles in a wide variety of cellular and physiological functions (Cech and Steitz, 2014; Adams et al., 2017). LncRNAs are defined as transcripts that are >200 nucleotides long without or with limited protein coding potential. LncRNAs exert their functions through a number of mechanisms. They are known to regulate gene expression either in cis (regulate neighbouring genes) or in trans (regulate long-distance genes) in the nucleus (Gupta et al., 2010; Hacisuleyman et al., 2014; Trimarchi et al., 2014). On the other hand, cytoplasmic lncRNAs have been shown to regulate cellular functions through post-transcriptional processing competing with endogenous-RNA (ceRNA) (Yoon et al., 2012; Sahu et al., 2015).

Adding to their roles in normal physiological processes, lncRNAs are novel regulators in oncogenesis, displaying both oncogenic and tumour suppressive effects in many cancers, such as in the breast, prostate, and liver (Gupta et al., 2010; Malik et al., 2014; Yuan et al., 2014). Recent studies also linked several lncRNAs to glioblastoma (GBM) development and pathogenesis (Xi et al., 2018). For instance, the interaction between NEAT1 and the histone-lysine N-methyltransferase enzyme EZH2 mediates histone H3K27 methylation in target promoters, thereby activating the Wnt/β-catenin pathway and thus promotes glioma proliferation and invasion (Chen et al., 2018). The HOTAIR transcript interacts with the polycomb repressive complex 2 (PRC2) and promotes cell cycle progression in GBM (Zhang et al., 2015). Finally, MIR155HG generates miR-155, which enhances proliferative and invasive capacities of glioma cells (Wu et al., 2017). Although a large number of lncRNAs have been identified in GBMs by RNA sequencing (Reon et al., 2016; Paul et al., 2018), their regulatory mechanisms and biological functions are still not fully understood. Thus, studying GBM-associated lncRNAs is important for the discovery of novel prognostic markers and therapeutic targets.

Here, we first performed analysis on publicly available genomic databases and identified the MIR22 host gene (MIR22HG) as a putative molecule involved in glioma tumorigenesis and cancer stem cell function. We subsequently conducted functional analyses of MIR22HG in vitro and in vivo to evaluate its value as a novel molecular therapeutic target. Our results show that MIR22HG promotes glioma progression and self-renewal by producing miR-22-3p and miR-22-5p. Our findings uncover a new mechanism for dysregulation of canonical Wnt/β-catenin signalling, suggesting MIR22HG as a key therapeutic target in GBMs.

Materials and methods

Ethics statement
The research strategy was approved by the Research Ethics Committee of Shandong University and the Ethics Committee of Qilu. All experiments were performed in accordance with relevant guidelines and regulations, and written informed consent was obtained from all patients. Institutional Animal Care and Use Committee (IACUC) of Shandong University approved all surgical interventions and postoperative animal care.

Cell culture
Patient-derived glioblastoma stem-like cells (GSCs) GBM#P3, GBM#BG7, GBM#BG5 and GBM#06 were isolated and functionally characterized from GBM surgical specimens as previously described (Joseph et al., 2014; Fack et al., 2015). These cells were validated by a series of functional assays and express GSC markers such as SOX2 and OLIG2 etc. The DNA fingerprinting of the GSCs was performed by short tandem repeat (STR) profiling (Supplementary Table 1). The human neural stem cells were a kind gift from Prof. Alberto Martinez-Serrano (University of Madrid–CSIC, Spain). Cells were cultured in serum-free Neurobasaltm medium (Gibco/Thermo Fisher Scientific) supplemented with 2% B-27™ Neuro Mix (Thermo Fisher Scientific), 20 ng/ml epidermal growth factor (EGF; Thermo Fisher Scientific), and 10 ng/ml basic fibroblast growth factor (bFGF; PeproTech). Tumourspheres were split using Accutase™ (Thermo Fisher Scientific) to expand GSCs. The U87MG, LN229, and LN18 cell lines were purchased from the Culture Collection of the Chinese Academy of Sciences, and cultured in Dulbecco’s modified Eagle medium (Thermo Fisher Scientific) supplemented with 10% foetal bovine serum (FBS; Thermo Fisher Scientific). Normal human astrocytes were obtained from Lonza and cultured in the provided astrocyte growth medium supplemented with rhEGF, insulin, ascorbic acid, GA-1000, l-glutamine and 5% FBS.

Nuclear and cytoplasmic fractionation
Nuclear and cytoplasmic protein fractions were isolated using nuclear and cytoplasmic extraction reagents (Thermo Fisher
Scientific), according to the manufacturer’s instructions. Levels of GAPDH and histone H3 were used as loading controls for cytoplasmic and nuclear fractions.

**Invasion assays**

To assess how MIR22HG knockdown affected tumour cell invasion we carried out two invasion assays: a 3D tumour spheroid invasion assay into an invasion matrix and a co-culture assay where GSCs invaded into normal brain organoids. In the matrix assay, GBM#P3 or GBM#BG7 spheres were used for invasion analysis. ImageJ software (https://imagej.nih.gov/ij/, USA) was used to capture images of tumour cell invasion (Leica TCS SP8). Rainbow-GSCs (GenPharma (http://www.genepharma.com/). The 3′ untranslated region (UTR) of SFRP2 and PCDH15, containing miR-22-3p and miR-22-5p binding sequences, were cloned into the pmirGLO reporter vectors expressing firefly and Renilla luciferase. A mutation in the putative binding sequences in the 3′ UTRs was used as a control. For luciferase-based reporter assays, cells were transfected with the indicated reporter genes and plasmids using Lipofectamine (Invitrogen/Thermo Fisher Scientific) according to the manufacturer’s instructions. After 48 h, cells were harvested and luciferase activity was determined using a Promega kit (E2920).

**Reporter assay**

TOP/Flash reporter plasmids were purchased from GenePharma (http://www.genepharma.com/). The 3′ untranslated region (UTR) of SFRP2 and PCDH15, containing miR-22-3p and miR-22-5p binding sequences, respectively, were cloned into the pmirGLO reporter vectors expressing firefly and Renilla luciferase. A mutation in the putative binding sequences in the 3′ UTRs was used as a control. For luciferase-based reporter assays, cells were transfected with the indicated reporter genes and plasmids using Lipofectamine (Invitrogen/Thermo Fisher Scientific) according to the manufacturer’s instructions. After 48 h, cells were harvested and luciferase activity was determined using a Promega kit (E2920).

**Immunofluorescence**

Immunofluorescence was performed on paraffin-embedded brain slices. After deparaffinization, slides were incubated with primary antibodies against CD147 (Cell Signaling Technology; dilution 1:200) and subsequently with the appropriate 568-conjugated secondary antibodies (Thermo Fisher Scientific; dilution 1:1000). DAPI (1 μg/ml) was used to visualize nuclei.

**In vitro extreme limiting dilution assay**

GSCs were placed in a 96-well plate at a density of 1 to 50 cells/well with six replicates for each concentration. After 10 days, the numbers of tumourspheres in each well were determined, and the sphere formation efficacy was calculated using extreme limiting dilution analysis as previously described (Alvarado et al., 2016; Wang et al., 2018).

**Molecular docking-based virtual high-throughput screening**

Pre-miR-22 sequences were obtained from the miRBase database (http://microrna.sanger.ac.uk/sequences/). 3D structures of the pre-miR-22 hairpin loop were built using the MC-Fold/ MC-Sym pipeline. Openbabel (https://openbabel.org/docs/dev/ Installation/install.html) was used to construct the 3D structure of 4786 small molecular compounds provided by the National Cancer Institute (NCI) diversity dataset. The AutoDock program (http://autodock.scripps.edu) was used to perform high throughput molecular dockings for the Dicer enzyme binding site in the hairpin loop of pre-miR-22 against these small molecular compounds.

**Animal studies**

For intracranial xenograft studies, 4-week-old male athymic nude mice (SLAC laboratory animal Center; Shanghai, China) were bred under specific-pathogen-free conditions at 24°C on a 12-h day-night cycle. For orthotopic transplantation, mice were grouped randomly and were anaesthetized with an intraperitoneal injection of stock solution containing ketamine HCl (2.5 mg/ml), xylazine (2.5 mg/ml), and 14.25% ethanol (diluted 1:3 in 0.9% NaCl). U87MG, GBM#P3 or GBM#BG7 cells (10⁶ per mouse; five mice per group) were implanted into the right frontal lobes of each mouse by intracranial injection (1 mm anterior, 2.5 mm lateral to the bregma and at a depth of 3.5 mm). Mice were euthanized when neurological symptoms appeared. The mice were then perfused with physiological saline and 4% paraformaldehyde (PFA). The brain of each mouse was harvested, and further fixed in 4% PFA before embedding in paraffin and haematoxylin and eosin staining. For the subcutaneous GBM model, nude mice were divided into two groups (vehicle or AC1L6JTK treatment, five mice per group). GBM#P3 cells were subcutaneously inoculated into the right flanks of nude mice. The tumour tissues were isolated 14 days after injection and tumour volumes and weights were measured from sacrificed mice.

**Statistical analysis**

Kaplan-Meier survival curves were generated and compared using the log-rank test. The cut-off level was set at the median value of MIR22HG expression levels. A two-tailed χ² test was used to determine the association between MIR22HG expression and clinicopathological characteristics. Pearson correlation was applied to evaluate the linear relationship between gene expression levels. Kolmogorov-Smirnov test was used to assess the normal distribution of data. The one-way ANOVA test or two-tailed t-test was used for all other data comparisons using GraphPad Prism 7.0 (LaJolla, CA, USA). Data for each treatment group were represented as a mean ± standard error of the mean (SEM) and compared with other groups for significance by one-way ANOVA followed by Bonferroni’s post hoc test (multiple comparison tests). All tests
were two-sided, and \( P \)-values < 0.05 were considered to be statistically significant.

**Data availability**

The data that support the findings of this study are available from the corresponding author, upon reasonable request. Additional experimental details are provided in the Supplementary material.

**Results**

**MIR22HG is a highly expressed long non-coding RNA in glioblastoma**

To identify differentially expressed lncRNAs in human gliomas, we carried out genomic analysis of publicly available gene expression data collected from WHO grade II–IV tumours. The expression profiles of lncRNAs were extracted from The Cancer Genome Atlas (TCGA) RNA-seq data based on their Refseq annotation, and expression values were normalized and Log2 transformed. Through DESeq differential analysis, we uncovered a total of 456 differentially expressed lncRNAs between GBM (\( n = 174 \)) and low-grade glioma (LGG) samples (\( n = 511 \); 176 of these lncRNAs were upregulated and 279 were downregulated in GBM relative to LGG) (Supplementary Table 3). The heat map in Fig. 1A summarizes the top 100 differential lncRNAs. The MIR22 host gene emerged as one of the most differentially expressed lncRNAs (MIR22HG; log\(_2\) fold-change = 3.43, adjusted \( P = 1.40 \times 10^{-41} \)) with increased expression among other previously characterized pro-oncogenic lncRNAs in GBM, such as HOTAIRM1 (Zhang et al., 2013), CRNDE (Zheng et al., 2016), FOXD3-AS1 (Chen et al., 2016), and NEAT1 (Chen et al., 2018; Zhou et al., 2018). These results were further corroborated using the Chinese Glioma Genome Atlas (CGGA), where MIR22HG appeared among the top two highly expressed lncRNAs (log\(_2\) fold-change = 1.88, adjusted \( P = 1.01 \times 10^{-15} \)) when overlapped with the top 50 candidates from TCGA data (Fig. 1B). As the function of MIR22HG has not been investigated in GBM, we decided to focus on this lncRNA in the subsequent analyses. Next, we evaluated, using the TCGA and CGGA cohorts, MIR22HG expression level taking into account the 2016 WHO classification of CNS tumours. MIR22HG was lower in LGG-Oligo (IDH\(_{\text{mut}}\), 1p/19q co-deletion), LGG-Astro (IDH\(_{\text{mut}}\), 1p/19q non-co-deletion) while higher in LGG-IDH\(_{\text{wt}}\) subtype in TCGA (Fig. 1C and Supplementary Fig. 1A). The GBM-IDH wild-type subtype, which is associated with worse clinical outcomes, expressed MIR22HG at high levels (Fig. 1C and Supplementary Fig. 1A). In addition, data from the Cancer Cell Line Encyclopedia demonstrated that glioma cell lines exhibited higher expression of MIR22HG than most other cancer cell lines (Supplementary Fig. 1B). As there are limited expression data available from human brain tissue, the above comparisons were done between LGGs and GBMs. However, to confirm the increased expression of MIR22HG compared to normal brain, we performed \textit{in situ} hybridization on an independent cohort of gliomas (\( n = 18 \)) and normal brain tissue (\( n = 5 \)) from Qilu Hospital. MIR22HG was consistently higher expressed in GBM samples compared to LGG (\( P < 0.05 \)) and normal brain tissue (\( P < 0.001 \)) (Fig. 1D and E).

Moreover, MIR22HG was found to be preferentially expressed in glioblastoma stem-like cells compared to normal neural stem cells (NSCs) based on microarray data described by Pollard et al. (2009) (\( P < 0.01 \); Fig. 1F) and Liu et al. (2014) (\( P < 0.001 \); Supplementary Fig. 1C). Finally, we interrogated the chromatin landscape of MIR22HG in GBM by using the ChiP-seq data derived from GEO and ENCODE databases. We found enrichment of H3K27ac peaks (marker of enhancer) at the promoter region of MIR22HG in GBM compared to normal brain tissue, along with an upregulation of MIR22HG enhancers in GSC versus differentiated glioma cells (Supplementary Fig. 1D). These results confirm an active MIR22HG transcriptional activity in GBM.

In summary, these data show that MIR22HG expression is elevated in GBM and could serve as a novel diagnostic marker.

**Increased expression of MIR22HG is associated with poor survival in glioma patients**

Clinicopathological and genetic characteristics have been associated with overall survival in glioma patients. Patient age and genetic features, including O-6-methylguanine-DNA methyltransferase (MGMT) promoter methylation, codeletion of 1p/19q, telomerase reverse transcriptase (TERT) loss, and IDH (Ceccarelli et al., 2016; Louis et al., 2016) and ATRX chromatin remodeler (ATRX) mutations, have been reported to be associated with a favourable prognosis (Yan et al., 2009; Jiang et al., 2016; Louis et al., 2016). We therefore analysed whether high or low expression of MIR22HG correlated with any of these characteristics. High expression of MIR22HG was statistically associated with patient age (\( > 45 \) years; \( P < 0.001 \)) and Karnofsky Performance Score (\( < 80 \); \( P < 0.001 \)), but did not correlate with gender (\( P = 0.913 \); Supplementary Table 4). Low MIR22HG was found to be associated with methylated MGMT, 1p/19q codeletion, loss of TERT and mutated ATRX in tumours (\( P < 0.001 \); Supplementary Table 4).

Kaplan-Meier analysis was performed to examine the relationship between patient survival and MIR22HG expression. Over 1500 cases were enrolled from three independent databases that were then assigned an MIR22HG\(_{\text{high}}\) or MIR22HG\(_{\text{low}}\) expression status determined by the median expression levels. The MIR22HG\(_{\text{high}}\)
Figure 1 MIR22HG expression is elevated in GBM. (A) Heat map of the top 100 differentially expressed lncRNAs between LGG and GBM from TCGA dataset; gene expression values are z-transformed and are coloured red for high expression and blue for low expression; red arrow indicates MIR22HG. (B) Venn plot displaying the significantly upregulated lncRNAs in GBM in both TCGA (top 50 upregulated lncRNAs) and CGGA (top 50 upregulated lncRNAs). (C) MIR22HG RNA expression (log2) based on 2016 WHO classification from TCGA. (D) Representative images of RNA in situ hybridization staining for MIR22HG in normal brain (n = 5) and different pathological grades of gliomas (n = 18). Scale bar = 100 μm. (E) Graphic representation of the quantification of MIR22HG in situ hybridization staining in normal brain and different pathological grades of gliomas. (F) MIR22HG RNA expression (log2) in NSCs and GSCs from GSE15209. (G) Kaplan-Meier analysis of patient overall survival data based on high versus low expression of MIR22HG in gliomas, grades II–IV, from the TCGA dataset. P-values were obtained from the log-rank test. Data are shown as mean ± the standard error of the mean (SEM) for each group. *P < 0.05, **P < 0.01, ***P < 0.001.
group patients exhibited significantly shorter overall survival as well as progression-free survival compared to the MIR22HG slow group in all cohorts (Fig. 1G and Supplementary Fig. 1E–G). In addition, MIR22HG levels significantly associated with poor survival in cases with wild-type IDH (Supplementary Fig. 1H). Finally, MIR22HG was validated as an independent prognostic indicator in univariate and multivariate Cox regression analysis of overall survival [hazard ratio (HR) = 1.177, 95% confidence interval (CI) = 1.034 to 1.339, \( P = 0.009\); Supplementary Table 5] and progression-free survival (HR = 1.617, 95% CI = 1.617 to 2.499, \( P = 0.030\); Supplementary Table 6) in glioma patients.

**Silencing MIR22HG inhibits glioblastoma malignant phenotypes and GSC maintenance**

To predict the MIR22HG-associated biological functions in gliomas, we performed Gene Set Enrichment Analysis (GSEA) using the TCGA GBM dataset. Biological processes, including proliferation, apoptosis, stem cell function, and tumour invasion exhibited the strongest association with high MIR22HG levels (Fig. 2A).

We first detected the expression levels of MIR22HG in a panel of GBM cell lines including three GBM cell lines (U87MG, LN229, LN18) and four patient-derived primary GSCs (GBM#P3, GBM#BG7, GBM#BG5 and GBM#06). In addition, two non-cancer cell lines, human NSCs, and normal human astrocytes were also included as normal controls. At the expression level, both human NSCs and normal human astrocytes displayed lower MIR22HG expression levels compared to all GBM cells by quantitative reverse-transcription PCR (qRT-PCR) (Fig. 2B). Similar results were observed based on microarray data where multiple GBM cell lines possess higher expression than human NSCs (Supplementary Fig. 2A). Between the GBM cells, U87MG and GBM#P3 displayed the highest MIR22HG expression among all cell lines (Fig. 2B).

To determine its biological function, MIR22HG was targeted in seven GBM cell lines (three cell lines and four patient-derived primary GSCs) and two normal controls (normal human astrocytes and normal NSC) by using two independent siRNAs compared to a control non-targeted-siRNA (si-Ctrl) (Supplementary Fig. 2B). We validated the MIR22HG knockdown efficacy by qRT-PCR (Fig. 2C and Supplementary Fig. 2C). Targeting MIR22HG potently impaired proliferation in GBM cell lines and GSCs derived from multiple patients (\( P < 0.01\), respectively; Fig. 2D). In contrast, targeting MIR22HG minimally reduced cell proliferation in human NSCs and normal human astrocytes.

Flow cytometry apoptosis assays showed that inhibition of cell growth was mediated both by an increased apoptotic cell death (~2-fold compared to controls in U87MG, GBM#P3 and GBM#BG7) (Fig. 2E and Supplementary Fig. 2D) and an induction of cell cycle arrest in the GO/G1 phase (Supplementary Fig. 2E). An increase in proteins involved in these biological processes, including the apoptotic indicator cleaved-PARP and cell cycle arrest markers such as p21 and p27, were correspondingly induced (Fig. 2F).

Given that MIR22HG was highly expressed in GSCs compared to NSCs (Fig. 1F and Supplementary Fig. 1C), we determined the impact of MIR22HG knockdown on stem cell-associated properties. In tumoursphere formation and extreme limiting dilution assays (ELDA), MIR22HG knockdown resulted in a remarkable decrease in sphere formation in GBM#P3, GBM#BG7, GBM#BG5 and GBM#06 cells (Fig. 2G, H and Supplementary Fig. 2F and G).

**MIR22HG knockdown inhibits tumour invasion and growth in vivo**

Next, we examined the influence of MIR22HG knockdown on glioma cell invasion. Analysis of intra-tumoural transcriptional heterogeneity based on the IVY project (http://glioblastoma.alleninstitute.org) (Puchalski et al., 2018) demonstrated that MIR22HG was expressed higher in the infiltrating region of tumours compared to the tumour core (Supplementary Fig. 3A and B). In a 3D spheroid invasion assay, knockdown of MIR22HG attenuated the invaded distance of GBM#P3 cells by ~50% (\( P < 0.01\); Fig. 3A). To confirm these results, we also established a novel co-culture invasion model where brain organoids were co-cultured with GBM spheroids based on our previous work (Bjerkvig et al., 1986), thus mimicking the physiologically invasive brain microenvironment of glioma cells (Fig. 3B). Using this ex vivo model, rainbow-si-Ctrl or -si-MIR22HG cells were cultured for 4 days to generate tumour aggregates and then co-cultured with mature rat brain organoids. After 72 h, the number of cells invading the brain organoids as well as the area invaded by tumour cells was significantly decreased in the si-MIR22HG group compared to the si-Ctrl group in both GBM#BG7 (\( P < 0.05\); Fig. 3C and D) and GBM#P3 cells (\( P < 0.001\); Supplementary Fig. 3D).

To validate our in vitro observations, tumour growth was assessed in orthotopic xenograft models (U87MG, GBM#P3 and GBM#BG7) transduced with a lentivirus expressing sh-Ctrl \((n = 5)\) or sh-MIR22HG \((n = 5)\). Knockdown of MIR22HG significantly reduced tumour growth and prolonged overall survival of tumour-bearing mice (\( P < 0.001\), \( P < 0.05\) and \( P < 0.05\), respectively) (Fig. 3E, F and Supplementary Fig. 4A–C). The proliferation index marker Ki-67 was decreased in sh-MIR22HG xenografts (Fig. 3G and Supplementary Fig. 4D), and importantly, these tumours displayed more circumscribed borders relative to the sh-Ctrl xenografts (Fig. 3F). Immunofluorescence staining for a human specific antigen (CD147) revealed a decrease in satellite lesions and invasive
tumour cells in peritumoural areas (Fig. 3H and Supplementary Fig. 4E). Finally, several classical invasive markers, including ZEB1, MMP2 and MMP7, were found to be dramatically decreased in U87MG-sh-MIR22HG xenografts (Supplementary Fig. 4F); while only ZEB1 and MMP2 decreased in GBM#P3 xenografts,
Figure 3 MIR22HG knockdown decreases invasive ability of GBM cells and impairs tumour growth and invasion in vivo.

(A) Representative images of spheroids in 3D invasion assays for GBM#P3 GSCs transfected with si-Ctrl, si-MIR22HG#1 or si-MIR22HG#2, and evaluated at 0, 48 and 96 h. Scale bar = 200 μm. Graphic representation of the quantification of the distance of invading cells from the tumourspheres determined after 96 h (right). (B and C) Representative images of co-culture invasion assays for GBM#GB7 GSCs transfected with LncRNA MIR22HG in GBM BRAIN 2020: 143; 512–530 |

(continued)
The results revealed MIR22HG gene of miR-22 (Fig. 5A) (Wang et al., 2012). MIR22HG has previously been demonstrated to be a host progression gene. MIR22HG miR-22-3p and miR-22-5p mediate Wnt/β-catenin signaling. To investigate the potential mechanisms of MIR22HG to be significantly associated with the Wnt signalling pathway (adjusted P < 0.05; Fig. 4B). Knockdown studies were performed in U87MG and GBM#P3 cells in vitro to determine whether MIR22HG was involved in the regulation of the canonical Wnt/β-catenin pathway. MIR22HG silencing led to a downregulation of β-catenin, a key transcriptional regulator of Wnt, along with the suppression of several Wnt downstream targets, including c-Myc, cyclin D1, and LEF1 (Fig. 4C). Western blot analysis also revealed an increase in phospho-β-catenin levels, which represents the inactivating of β-catenin (Li et al., 2012). Moreover, a decrease in phospho-GSK3β (Ser9) levels was observed indicating Wnt/β-catenin pathway inactivation (Hui et al., 2018) (Fig. 4C). In addition, knockdown of MIR22HG using a TOP/FOP luciferase reporter assay also revealed changes in β-catenin dependent transcriptional activity in U87MG and GBM#P3 cells (P < 0.05; Fig. 4D and E). After MIR22HG knockdown, levels of both cytoplasmic and nuclear β-catenin were reduced (Fig. 4F and G).

In summary, these data show that alterations in MIR22HG levels substantially affects β-catenin protein levels and Wnt signalling activity.

MIR22HG modulates the Wnt/β-catenin pathway in glioblastomas

To investigate the potential mechanisms of MIR22HG action, genome-wide transcriptomic correlation analysis was performed using the TCGA GBM data (Fig. 4A), where the genes associated with MIR22HG (Pearson analysis, P < 0.01) were subjected to GO and KEGG analysis. The results revealed MIR22HG to be significantly associated with the Wnt signalling pathway (adjusted P < 0.05; Fig. 4B). Knockdown studies were performed in U87MG and GBM#P3 cells in vitro to determine whether MIR22HG was involved in the regulation of the canonical Wnt/β-catenin pathway. MIR22HG silencing led to a downregulation of β-catenin, a key transcriptional regulator of Wnt, along with the suppression of several Wnt downstream targets, including c-Myc, cyclin D1, and LEF1 (Fig. 4C). Western blot analysis also revealed an increase in phospho-β-catenin levels, which represents the inactivating of β-catenin (Li et al., 2012). Moreover, a decrease in phospho-GSK3β (Ser9) levels was observed indicating Wnt/β-catenin pathway inactivation (Hui et al., 2018) (Fig. 4C). In addition, knockdown of MIR22HG using a TOP/FOP luciferase reporter assay also revealed changes in β-catenin dependent transcriptional activity in U87MG and GBM#P3 cells (P < 0.05; Fig. 4D and E). After MIR22HG knockdown, levels of both cytoplasmic and nuclear β-catenin were reduced (Fig. 4F and G).

In summary, these data show that alterations in MIR22HG levels substantially affects β-catenin protein levels and Wnt signalling activity.

miR-22-3p and miR-22-5p mediate MIR22HG-induced glioblastoma progression

MIR22HG has previously been demonstrated to be a host gene of miR-22 (Fig. 5A) (Wang et al., 2012). Given the fact that a major function of certain lncRNAs is the production of embedded miRNAs, we asked if MIR22HG-induced glioma progression was mediated by miR-22-3p and miR-22-5p. In culture, we observed, by qRT-PCR, a corresponding decrease of miR-22-3p and miR-22-5p, in U87MG and GBM#P3 MIR22HG-depleted cells (>80%; P < 0.01; Fig. 5B). Meanwhile, based on TCGA data, miR-22 was significantly associated with MIR22HG expression status in both LGG (Pearson = 0.4903, P < 0.001) and GBM samples (Pearson = 0.4399, P < 0.001; Supplementary Fig. 5A). Moreover, miR-22-3p and -5p expression was found to be strongly associated with MIR22HG expression (P < 0.001; Supplementary Fig. 5B).

In primary tumour samples, both miR-22-3p and -5p were highly expressed in high grade gliomas (P < 0.001, respectively; Supplementary Fig. 5C). Moreover, survival analysis demonstrated that higher expression levels of miR-22-3p and -5p were associated with poor overall survival and progression-free survival in glioma patients (Supplementary Fig. 6A–D and Supplementary Tables 7–10). GO analysis using the GBM expression profiles revealed an enrichment of miR-22-3p and -5p negatively-associated genes in the canonical Wnt pathway (Supplementary Fig. 7A). U87MG and GBM#P3 cells were then transfected with miR-22 mimics or anti-miR-22, and overexpression as well as knockdown were confirmed by qRT-PCR (Supplementary Fig. 7B). In functional studies, knockdown of miR-22-3p or -5p resulted in an inhibition of tumoursphere formation (P < 0.01 and P < 0.05; Supplementary Fig. 7C) and cell invasion capacity (P < 0.01 and P < 0.05; Supplementary Fig. 7D), along with an induction of apoptosis (Supplementary Fig. 7E) and cell cycle arrest at G0/G1 phase (Supplementary Fig. 7F). These data substantiate oncogenic roles of miR-22s in GBM progression.

To examine whether overexpression of the miR-22s are sufficient to overcome the depletion of their host gene, we transfected miR-22-3p and -5p mimics into U87MG-, GBM#P3- and GBM#BG7-si-MIR22HG cells. Ectopic expression of the two miR-22s partially restored tumour cell proliferation (Fig. 5C and Supplementary Fig. 8A and B), but inhibited apoptosis (Fig. 5D and Supplementary Fig. 8C) and cell cycle arrest (Supplementary Fig. 8D); however, miR-22-5p mimics did not significantly reverse cell cycle arrest in U87MG-si-MIR22HG cells (Supplementary Fig. 8D), which might be due to the cellular heterogeneity between primary GSCs and adherent serum-culture cell lines. Moreover, cell invasion (Fig. 5E and Supplementary Figure 3 Continued)
Figure 4  

**MIR22HG activates the Wnt/β-catenin pathway in gliomas.** (A) Whole-transcriptome hierarchical cluster analysis was performed on MIR22HG-associated genes based on MIR22HG expression from TCGA GBM data. The resultant heat map shows relative expression levels of MIR22HG-associated genes in individual GBM cases where red is higher expression and blue is lower expression. (B) Biological processes and pathway analysis performed using the set of MIR22HG-associated genes. Results are based on GO and KEGG databases with adjusted P-values. (C) Western blot for protein levels of Wnt/β-catenin indicators in U87MG and GBM#P3 cells at 48 h after transfection with si-Ctrl, si-MIR22HG#1 or si-MIR22HG#2. GAPDH was used as a loading control. (D) Schematic representation of the TOP-Luc and TCF site mutant reporter (FOP-Luc) construct to assess β-catenin activity. (E) Graphic representation of relative levels of TOP flash/FOP flash luciferase activity in U87MG and GBM#P3 cells 48 h after transfection with si-Ctrl, si-MIR22HG#1 or si-MIR22HG#2. (F) Western blot for levels of β-catenin in cytoplasmic and nuclear lysates prepared from U87MG 48 h after transfection with si-Ctrl, si-MIR22HG#1 or si-MIR22HG#2. (G) Immunofluorescence images for β-catenin (red) and nuclear (blue) staining in U87MG at 48 h after transfection with si-Ctrl, si-MIR22HG#1 or si-MIR22HG#2. Scale bar = 30 μm. Data are shown as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001.
Figure 5 miR-22-3p and miR-22-5p mediate MIR22HG-induced GBM progression. (A) Schematic model of miR-22 maturation process. (B) qRT-PCR analysis of miR-22-3p and -5p expression in U87MG and GBM#P3 cells after transfection with si-Ctrl or si-MIR22HG. (C) Relative cell growth for rescue experiments as determined using OD values from the CCK-8 assay at 96 h in U87MG and GBM#P3 cells transfected with si-Ctrl, siMIR22HG, siMIR22HG + miR-Ctrl, siMIR22HG + miR-22-3p, or siMIR22HG + miR-22-5p. (D) Annexin V-FITC and PI staining to assess apoptosis and DNA content using flow cytometry in U87MG and GBM#P3 cells transfected with si-Ctrl, si-MIR22HG, siMIR22HG + miR-22-3p, or siMIR22HG + miR-22-5p. (E) Representative images of 3D invasion assay and quantification in miR rescue experiments. GBM#P3 GSCs were transfected with si-Ctrl, siMIR22HG + miR-Ctrl, siMIR22HG + miR-22-3p, or siMIR22HG + miR-22-5p, allowed to form spheres, embedded in the invasion matrix, and evaluated thereafter at the hours indicated. Scale bar = 200 μm. (F) Representative images from
targets of miR-22-3p and -5p

MIR22HG expression in U87MG-si-MIR22HG cells reversed suppression and activated Wnt signalling (Fig. 5G and H). Immunofluorescence confirmed the restoration of β-catenin expression in U87MG-si-MIR22HG (Supplementary Fig. 8F). U87MG cells transduced with lent-sh-MIR22HG and lenti-miR-22-3p or -5p, -partially restored tumour growth and reduced overall survival of tumour-bearing mice compared to the U87-sh-MIR22HG group (P < 0.01 and P < 0.05, respectively) (Fig. S1 and Supplementary Fig. 8G). These results were further validated in GBM#P3-derived xenografts (Fig. S1 and Supplementary Fig. 8G).

Finally, to confirm that MIR22HG functions as the host gene of miR-22 in GBM progression and to exclude the effect of the other regions of the full-length 2.6 kb MIR22HG transcript, we performed ectopic expression experiments with a MIR22HG full-length construct (wild-type, WT), as well as a MIR22HG mutant construct, MIR22HG-Dexon2 (deletion of miR-22 region in exon 2) (Supplementary Fig. 8H). In a functional assay, we found that overexpression (OE) of MIR22HG-WT significantly promoted U87MG cell growth in vitro compared to the control group and MIR22HG-Dexon2-OE group (P < 0.001); while MIR22HG-Dexon2-OE minimally promoted cell proliferation in GBM. These data strongly support an oncogenic role of MIR22HG as a precursor of miR-22s in GBM.

Taken together, we demonstrate that miR-22s are indispensable in MIR22HG-induced GBM growth.

**SFRP2 and PCDH15 are downstream targets of miR-22-3p and -5p**

We then sought to identify genes that might be directly targeted by miR-22-3p or -5p. Using computational target prediction based algorithms (TargetScan v7.1 and miRanda), several genes were found to have 3’ UTR putative binding sites for miR-22-3p and miR-22-5p (Fig. 6A). Among them, SFRP2 was further identified to be strongly negatively associated with miR-22-3p (Pearson_{miR-22-3p} = –0.479, P < 0.001; Fig. 6B); a similar relationship was revealed between miR-22-5p and PCDH15 (Pearson_{miR-22-5p} = –0.351, P < 0.001; Fig. 6C). However, other candidates such as TET2 (Pearson_{miR-22-3p} = –0.201), CLIC4 (Pearson_{miR-22-3p} = 0.163), DUSP1 (Pearson_{miR-22-5p} = –0.096) or TXN2 (Pearson_{miR-22-5p} = –0.101), showed no significant association with the miR-22s in gliomas. Therefore, we identified SFRP2 and PCDH15 as the most attractive candidates.

The clinical relevance of SFRP2 and PCDH15 was also verified by the TCGA dataset where a lower expression of SFRP2 and PCDH15 was associated with a shorter overall survival and progression-free survival in patients (P < 0.001; Supplementary Fig. 9A and Supplementary Tables 11 and 12). In addition, the combined expression levels of the miR-22-3p$^{high}$ and SFRP2$^{low}$, or miR-22-5p$^{high}$ and PCDH15$^{low}$, in glioma patients, more accurately predicted a shorter overall survival and progression-free survival (Supplementary Fig. 9B). These results were further validated in TCGA GBM patients (Supplementary Fig. 9C).

Secreted frizzled-related proteins (SFRPs) and protocadherins have been shown to be critical for the regulation of canonical Wnt signalling (Lee et al., 2016; Mah and Weiner, 2017). Consistently, GSEA analysis of TCGA data indicated that both SFRP2 and PCDH15 enrichment was associated with Wnt signalling regulation (Supplementary Fig. 10A and B). SFRPs have been shown to have tumour suppressor functions in cancers such as medulloblastoma. By immunohistochemistry staining, the expression of both SFRP2 and PCDH15 was found to be highly expressed in the normal brain and LGG, but to a lesser extent in GBMs (Supplementary Fig. 10C). In addition, these genes were found to be preferentially expressed in non-GSCs (n = 3) compared to GSCs (n = 3) based on GSE41032 (Supplementary Fig. 10D).

We therefore assessed if miR-22-3p and -5p could regulate SFRP2 and PCDH15 expression levels. Overexpression of miR-22-3p or miR-22-5p mimics in GBM cells resulted in a decrease in SFRP2 or PCDH15 protein and mRNA levels, respectively (Fig. 6D and E). To assess if there was a direct interaction between the miRs and mRNAs, we used a 3’ UTR luciferase reporter assay where the luciferase mRNA is regulated by the miR binding sequence (Fig. 6F). Luciferase activity decreased by ~50% in the
Figure 6  **SFRP2 and PCDH15 are downstream targets of miR-22 and suppress growth of GBM in vivo.** (A) Venn diagrams represent the candidate target genes of miR-22-3p and -5p identified by TargetScan and miRanda. (B and C) Correlation between SFRP2 mRNA and miR-22-3p expression ($r = -0.479, P < 0.001$), and PCDH15 mRNA and miR-22-5p expression ($r = -0.351, P < 0.001$) in WHO grade II–IV gliomas. (D) Western blot for protein levels of SFRP2 or PCDH15 from U87MG cells transfected with miR-22-3p or -5p for 48 h. GAPDH was used as a loading control. (E) qRT-PCR analysis for SFRP2 or PCDH15 mRNA levels 48 h after transfection of U87MG or GBM#P3 with miR-Ctrl, or miR-22-3p or -5p mimics. (F) Alignment of predicted binding sites in the SFRP2 3’ UTR for miR-22-3p and the PCDH15 3’ UTR for miR-22-5p, and the corresponding bases substituted for mutant binding sequences. Wild-type and modified UTRs are cloned into a reporter vector expressing luciferase, and luciferase activity is used to evaluate efficiency of miR targeting. (G) Quantitation of luciferase reporter assays in U87MG cells after (continued)
presence of the wild-type binding sequences for each of these miRs. However, mutations in the corresponding miR binding sites rendered the miR mimics ineffective in targeting the luciferase mRNA construct (Fig. 6G). In summary, these data show that SFRP2 and PCDH15 are downstream targets of miR-22.

**SFRP2 and PCDH15 repress the Wnt/β-catenin pathway**

We next assessed whether miR-22-3p and miR-22-5p interfered with the activity of Wnt signalling via SFRP2 or PCDH15 in GBM cells. The cells were transfected with SFRP2 or PCDH15. Overexpression was confirmed by western blots (Supplementary Fig. 10E). Ectopic expression of either SFRP2 or PCDH15 inhibited tumoursphere formation and invasion in GBM#P3 cells, which was partially reversed by co-transfection with miR-22-3p or miR-22-5p mimics, respectively (Fig. 6H and I). Consistently, transfection of miR-22-3p or miR-22-5p mimics partially rescued the suppression of β-catenin activity by SFRP2 or PCDH15, as assessed using TOP/FOP (Fig. 6J). Moreover, we observed that overexpression of SFRP2 or PCDH15 significantly blocked the miR-22-promoted tumoursphere formation in GBM#P3 cells (P < 0.05; Supplementary Fig. 10F). Collectively, these data suggest that miR-22-3p/PCDH15 as well as miR-22-5p/SFRP2 signalling is involved in regulating GBM development through the Wnt/β-catenin pathway. Finally, tumour growth was evaluated in a GBM orthotopic xenograft model. Overexpression of SFRP2 or PCDH15 in U87MG and GBM#P3 cells suppressed tumour growth in vivo and prolonged the overall survival of tumour-bearing mice compared to controls (n = 4 per group; Fig. 6K and L).

**AC1L6JTK blocks the MIR22HG/miR-22 axis**

Molecular docking-based virtual high-throughput screening (vHTS) is an effective *in silico* drug design method (Detering and Varani, 2004; Parisien and Major, 2008; Shi et al., 2013). We therefore used vHTS to identify novel compounds that might competitively combine with the Dicer binding site in the pre-miR-22 hairpin loop.

First, the 3D structure of pre-miR-22 and its hairpin loop was constructed using the MC-Fold/MC-Sym pipeline and AutoDock Vina tool (Supplementary Fig. 11A and B). Second, we performed high-throughput molecular dockings for pre-miR-22 against 4786 compounds from the National Cancer Institute (NCI) diversity set using the AutoDock Vina tool and the Lamarckian Genetic Algorithm (LGA) (Fig. 7A). Based on the virtual dockings, we discovered 50 compounds with high-binding affinity (Supplementary Table 13). Of these, NSC61610 (AC1L6JTK) had the highest affinity score (−10.7 kcal/mol; Fig. 7B) displaying half maximal inhibitory concentration (IC50) around 150 μM in GBM cells by CCK8 assay (Supplementary Fig. 11C). Based on these results, we therefore queried if AC1L6JTK could suppress the production of miR-22 in GBM cells. qRT-PCR indicated that after treatment of U87MG and GBM#P3 cells with AC1L6JTK (50 and 100 μM) for 24 h, the expression levels of both miR-22-3p and -5p were significantly decreased (P < 0.001, Fig. 7C).

In functional assays, the frequency of sphere formation was decreased (Fig. 7D and Supplementary Fig. 11D and E) whereas cell apoptosis was conversely increased following AC1L6JTK treatment for 48 h (Fig. 7E). AC1L6JTK treatment also attenuated transcriptional activity of β-catenin in GBM#P3 cells, similar to MIR22HG knockdown, implicating the Wnt signalling pathway as a potential target (Fig. 7F).

To assess the selectivity and specificity of AC1L6JTK on seven unrelated miRNAs: miR-23a-3p, miR-155-3p, miR-720, miR-149-5p, miR-612 and miR-24-3p. After 24 h of treatment, qRT-PCR showed that the expression levels of most miRNAs were unchanged in U87MG cells, except a decrease of miR-149-5p (P = 0.048; Supplementary Fig. 11F). In a rescue study, AC1L6JTK treatment (100 μM) for 48 h led to an inhibition of cell growth, while this effect was restored when U87MG cells were transfected with miR-22-3p (P < 0.01) or miR-22-5p mimics (P < 0.05; Supplementary Fig. 11G). Moreover, transfection with miR-149-5p mimics could not rescue the inhibitory effect of AC1L6JTK (P > 0.05), indicating that...
miR-149-5p might not mediate MIR22HG-induced GBM progression.

Based on the docking results (Fig. 7B and Supplementary Fig. 12A and B), we observed three ribonucleotides in the binding pocket, C47, A48 and A52, which could form hydrogen bonds with AC1KL6JTK molecule structure. Consequently, to verify the specificity of AC1KL6JTK to the hairpin loop region of pre-miR-22 RNA sequence, we constructed new 3D structures of pre-miR-22 hairpin loop with point mutations at these binding sites, including MUT-1 (A52G), MUT-2 (C47T), MUT-3 (A48C) and MUT-4 (A52G, C47T, A48C) (Supplementary Fig. 12C). When AC1KL6JTK docked with these mutant 3D structures as well as the wild-type structure, the estimated free energy of binding was −7.5 kcal/mol, −8.3 kcal/mol, −7.9 kcal/mol and −7.2 kcal/mol, which were much weaker compared to the binding affinity with wild-type structure (−10.7 kcal/mol) (Supplementary Fig. 12D). These results strongly suggest that point mutations within the pre-miR-22 hairpin loop region attenuates AC1KL6JTK binding affinity and thus weaken its activity.

Molinspiration Cheminformatics software (http://www.molinspiration.com) predicted that AC1L6JTK might not efficiently penetrate the blood–brain barrier. Therefore, we evaluated the in vivo therapeutic efficacy of AC1L6JTK using a subcutaneous xenograft model. GBM#P3 tumours were established in nude mice, which were then randomized into either vehicle or AC1L6JTK (10 mg/kg/d) treatment groups, with drug or vehicle (DMSO) given via intraperitoneal injection. Treatment with AC1L6JTK led to a significant reduction in tumour size and
tumour weight at the treatment endpoint (Fig. 7G and 7H). These results were consistent with our in vitro observations.

In summary, the AC1L6JTK treatment shows that MIR22HG is druggable, which implies therapeutic development towards this target should be further exploited (Fig. 7I).

Discussion

LncRNAs have been linked to multiple physiological and pathological functions. More recently, studies have shown that lncRNAs are important in cancer initiation and progression (Xi et al., 2018) and represent therefore potential biomarkers or therapeutic targets. However, only a small proportion of lncRNAs have been well characterized, especially in human gliomas. Here, we analysed genomic datasets from glioma patients and identified MIR22HG as one of the most significantly upregulated lncRNAs in GBM. We show that MIR22HG overexpression is associated with poor prognosis and represents a novel driver of cell proliferation and invasion. Intriguingly, we found that MIR22HG triggers GBM progression by producing miR-22-3p and -5p, which are encoded in exon 2 of the gene (Li et al., 2010; Wang et al., 2012). Finally, knockdown of MIR22HG in an in vivo orthotopic human xenograft model led to improved survival. Our results indicate that MIR22HG is a GBM promoting lncRNA that might serve as a novel prognostic marker in glioma patients.

MIR22HG has been reported to be involved in the development of several cancer types (Li et al., 2016; Su et al., 2018). Furthermore, increased expression of MIR22HG was observed in omental metastases when compared to matched primary ovarian tumours. Functional studies have shown that MIR22HG knockdown represses migration, invasion and proliferation of ovarian cancer cells (Li et al., 2016). Conversely, MIR22HG has recently been described to mediate a tumour-suppressive effect in lung cancer and hepatocellular carcinoma (Su et al., 2018; Zhang et al., 2018). It has been reported that MIR22HG can serve as a competing endogenous RNA (ceRNA) to modulate the miRNA-10a-5p level and its downstream target gene, NCOR2, in hepatocellular carcinoma (Wu et al., 2019). This discrepancy may indicate that the function of MIR22HG varies between different malignancies.

Dysregulation of miRNAs also contributes to malignant progression, including GBM (Banelli et al., 2017). As previously reported, miR-22 has a multifaceted role during initiation and progression of various cancer types, including cell growth, cell cycle, epithelial to mesenchymal transition (EMT) and migration (Wang et al., 2017). For instance, miR-22 was identified as a potent proto-oncogenic miRNA promoting proliferation and invasion of prostate cancer (Budd et al., 2015). In a Cre-based mammary gland-specific transgenic mouse model, it has been demonstrated that miR-22 triggers EMT, enhances stemness, and promotes breast cancer development and metastasis (Song et al., 2013b). In haematopoietic malignancies, miR-22 enhances the repopulating capacity of haematopoietic stem progenitor cells in vivo, and miR-22 transgenic mice develop primary haematological diseases (Song et al., 2013a). Recently, miR-22-3p was observed to be upregulated in spinal diffuse astrocytoma where it promotes glioma invasion by downregulating TIMP2, which leads to MMP2 activation (Ohnishi et al., 2017). In contrast, miR-22 has also been reported to have tumour suppressive roles in some cancers. For instance, in colorectal and gastric cancers, overexpression of miR-22 significantly inhibits distant cancer metastasis by directly targeting MMP14 and Snail (Zuo et al., 2015). These contradictory results underline a complex role of miR-22 function in different cancer types. In our study, both miR-22-3p and -5p were found to be highly expressed in GBM, where their expression was associated with poor clinical outcomes. Suppression of miR-22-3p and -5p by anti-miRs led to inhibition of tumour cell invasion and stemness, along with an induction of apoptosis and cell cycle arrest. In our ‘rescue’ experiments, the miR-22 mimics partially restored these effects in the context of MIR22HG knockdown in vitro and in vivo. Notably, we found that overexpression of wild-type MIR22HG promoted cell proliferation, while mutant MIR22HG (deletion of miR-22 region in exon 2) lost the growth promoting effect. Collectively, our findings indicate that miR-22-3p and -5p mediate the pro-oncogenic functions of MIR22HG in GBM.

Based on pathway enrichment analysis, Wnt/β-catenin signalling was one of the top 10 pathways enriched in the MIR22HG/miR-22 associated gene signatures. Importantly, constitutive Wnt/β-catenin activation has been observed in GBM and is essential for GBM growth, invasion and stemness (Lee et al., 2016). Our results in vitro were consistent with the in silico analysis. Downregulation of MIR22HG led to a suppression of β-catenin transcriptional activity and thus a reduced expression of Wnt/β-catenin pathway target genes.

We also show that two genes, SFRP2 and PCDH15, are direct targets of miR-22-3p and -5p inhibiting Wnt signalling in GBM. Multiple negative regulators, through various mechanisms, are natural antagonists to Wnt/β-catenin signalling. For instance, SFRPs and the Wnt inhibitory factor-1 (WIF-1) can directly bind Wnt proteins. GSK3b, however, negatively regulates the Wnt/β-catenin pathway by directly phosphorylating β-catenin, which tags it for proteasome degradation (Kogan et al., 2012). SFRP2 is an antagonist of the Wnt pathway by competing for Wnt binding to Frizzled (Kawano and Kypta, 2003). However, SFRP2 also acts as an agonist of the Wnt pathway (Mirotosou et al., 2007; Gehmert et al., 2008; Courtwright et al., 2009). In addition to the inhibition of SFRP2 by miR22 described here, SFRP2 is also downregulated in GBM via promoter hypermethylation (Kongkham et al., 2010). Besides, several SFRP family members (SFRP1, 2 and 3) have been found to function as tumour suppressors in
medulloblastoma (Kongkham et al., 2010). In the current study, we found that expression levels of SFRP2 were significantly downregulated in GBM as compared to LGG and normal brain tissue. Lower expression levels of SFRP2 were also observed in non-GSCs compared to GSCs. In functional assays, ectopic expression of SFRP2 suppressed GBM malignant behaviours and Wnt signalling activity, thus demonstrating its tumour-suppressive role in GBM.

Protocadherins play important roles in the regulation of cell adhesion and signal transduction. Recently, multiple members of the protocadherin family were found to suppress tumour progression by antagonizing the Wnt/β-catenin pathway (Mah and Weiner, 2017). Although PCDH15 led to a significant reduction of sphere formation and invasion. This coincided with a decrease in Wnt activity. The inhibitory effect was partially restored by cotransfection of miR-22-5p (or miR-22-3p in the case of SFRP2) mimics. Although we found that miR-22-3p and miR-22-5p work together to target negative regulators of Wnt signalling, providing a previously unknown regulatory mechanism of this pathway, we cannot exclude the possibility that these microRNAs contribute to glioma progression through other mechanisms than Wnt signalling.

Exact knowledge on how MIR22HG is processed into miRs can be used for pharmacological interference based on Dicer, an enzyme that is essential for cleavage of the terminal hairpin loop of precursor microRNAs (pre-miRNAs) to produce mature miRNAs. Based on the 3D structure of pre-miR-22 and its hairpin loop (the Dicer binding site), we performed high-throughput molecular dockings for pre-miR-22 against the 4786 compounds using the NCI diversity set. We identified a small-molecule inhibitor of MIR22HG/miR-22, ACIL6JTK, which efficiently suppressed the expression of miR-22-3p and -5p in GBM cells. Interestingly, ACIL6JTK has previously been tested for therapeutic efficacy in inflammatory bowel disease (Lu et al., 2012, 2014). In the present study, we found that ACIL6JTK treatment inhibited GBM proliferation and induced apoptosis, indicating that ACIL6JTK could be used as an anticancer agent in GBM (see schematic representation Supplementary Fig. 11). However, the molecule requires further optimization in particular with regard to blood–brain barrier penetration.

In conclusion, our work not only uncovers an oncogenic role for MIR22HG in promoting GBM aggressiveness and GSC self-renewal, but also implicates MIR22HG/miR-22 as a potential target for treating gliomas through pharmacological blockade.

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Competing interests
The authors report no competing interests.

Supplementary material
Supplementary material is available at Brain online.

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