Inhibition of GPR158 by microRNA-449a suppresses neural lineage of glioma stem/progenitor cells and correlates with higher glioma grades

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Abstract
To identify biomarkers for glioma growth, invasion and progression, we used a candidate gene approach in mouse models with two complementary brain tumour phenotypes, developing either slow-growing, diffusely infiltrating gliomas or highly proliferative, non-invasive primitive neural tumours. In a microRNA screen we first identified microRNA-449a as most significantly differentially expressed between these two tumour types. miR-449a has a target dependent effect, inhibiting cell growth and migration by downregulation of CCND1 and suppressing neural phenotypes by inhibition of G protein coupled-receptor (GPR) 158. GPR158 promotes glioma stem cell differentiation and induces apoptosis and is highest expressed in the cerebral cortex and in oligodendrogliomas, lower in IDH mutant astrocytomas and lowest in the most malignant form of glioma, IDH wild-type glioblastoma. The correlation of GPR158 expression with molecular subtypes, patient survival and therapy response suggests a possible role of GPR158 as prognostic biomarker in human gliomas.

Introduction
The prognostication of human gliomas has seen significant changes over the last 10 years. The identification of mutations in two isocitrate dehydrogenase genes, IDH1 and IDH2, in gliomas [1] was a major discovery, leading to a biomarker-defined glioma classification, IDH and ATRX-mutant astrocytomas and glioblastomas and IDH-mutant 1p/19q codeleted oligodendrogliomas [2]. The clinical value of molecular subtyping of IDH wild-type glioblastoma instead had limited clinical impact [3, 4]. The only prognostic biomarker in GBM is the methylation of MGMT but has no diagnostic value [5].

To identify additional biomarkers of diagnostic and/or prognostic value, we used a mouse model of intrinsic brain tumours generated by Cre-mediated inactivation of Ptenlox/lox and p53lox/lox genes or of Rblox/lox and p53lox/lox genes in the neurogenic cell population of the subventricular zone (SVZ) of the brain, previously in-depth molecularly characterized [6, 7]. Mice with tumours mutant for Pten and p53 (in short Pten/p53) develop diffusely infiltrative high grade gliomas (Fig. 1a) [8–10] with an expression profile of the TCGA classical GBM [4] or Phillips proneural [11]. Cre-mediated recombination of the Rb and p53 genes (in short Rb/p53) in the SVZ, gives rise to poorly differentiated, well-
demarcated tumours with a primitive neural phenotype and less frequently also to gliomas (Fig. 1a) [6–8]. These tumours, previously described as primitive neuroectodermal tumours (PNET), have an expression profile corresponding to a malignant childhood tumour, atypical teratoid/rhabdoid tumour (AT/RT) [6, 12]. We chose to compare these two models of intrinsic brain tumours with their distinctive morphology, survival rates and transcriptomic profiles, to

![Fig. 1](image_url)

**Fig. 1** Identification and validation of miRNAs, differentially expressed (DEmiRs) between glioma and PNET. 

**a** Representative histology of Rb/p53-mutant glioma (left) and PNET (right), and Pten/p53-mutant glioma (centre). Scale bar corresponds to 1 mm in the upper row (overview) and 50 µm in the lower row (detail). It shows the infiltrative growth of gliomas (columns 1&2) and the demarcated margins of the hyper-cellular PNET (column 3).

**b** Heat map of DEmiRs between Pten/p53-glioma, Rb/p53-glioma, and Rb/p53-PNET (one-way ANOVA, p < 0.05). Unsupervised clustering analysis was performed on R. 

**c** Venn diagram of DEmiRs between Rb/p53 and Pten/p53-mutant gliomas. Upregulation indicated with red, and down regulation with blue arrows next to the miRNAs.

**d** Validation of DEmiRs by RT-qPCR. In each panel, Rb/p53-PNETs are set as ‘calibrator’ sample (orange error bars), and the y-axis represents log2 fold change (−ΔΔCp) with samples as indicated in the figure legend. The bars are means ± s.e.m. (Student’s t-test; *FDR < 0.05; **FDR < 0.01)
identify mechanisms controlling growth, differentiation and tumour invasion, and corresponding biomarkers in human gliomas.

First we set out to discover differentially expressed micro-RNAs (miRNAs, miR), small non-coding RNAs regulating gene expression at post-transcriptional level by binding to the 3′ untranslated region (UTR) of the targeted mRNA for degradation [13]. miRNAs participate in controlling many biological processes, such as cell cycle, apoptosis, stem cell differentiation, and immune responses [14], and they also play a key role in the differentiation and maintenance of tissue identity [15]. In our screen, miR-449a was amongst the most significantly differentially regulated. It was selected for subsequent analysis as a tumour suppressive role of miR-449a has been suggested in a number of malignancies [16–19], most notably in prostate cancer by targeting classical proto-oncogenes CCND1 [20], c-Myc [21] and HDAC-1 [22]. miR-449a is a direct transcriptional...
target of E2F1, negatively regulating pRb-E2F1 activity by CDK6 and CDC25A [23]. A role of miR-449a targeting MYC-associated zinc finger proteins has been suggested in glioblastoma [17]. In contrast, during neural development miR-449a has a different regulatory role. It is expressed highest during the proliferative phase of embryonic neurogenesis [24] and is essential for the production of inter-mEDIATE progenitors during cortical development [25, 26]. Amongst the established targets of the miR-124 family are E2F1, CDK6, CCND1 and BCL2 [20, 23, 27]. miR-449a targets a site in the 3′ UTR of the CCND1 transcript, and miR-449a significantly reduces Cyclin D1 protein in PC-3 cells [20]. Here, we identify a new target of miR-449a, the G-protein coupled receptor 158 (GPR158), a member of a large group of cell surface proteins exerting a range of diverse cellular functions. GPR158 and two others, GPR156 and GPR179 belong into the gamma-aminobutyric acid receptor branch of the GPCR glutamate family (Group III), containing 7 orphan receptors [28]. The first identified roles of GPR158 were those of a plasma membrane scaffold protein in retinal bipolar neurons and [29], and the expression in trabecular meshwork cells in the eye’s aqueous outflow pathways, contributing to the pathophysiology of steroid-induced ocular hypertension and glaucoma [30]. It has relevance to prostate cancer growth and progression [31], and a role in lung cancer outcome was identified [32], thus presenting a potential relevant link to our findings in brain tumours. Here we show a target dependent effect of miR-449a, inhibiting growth and migration by down-regulating CCND1 and suppressing neural differentiation by inhibiting GPR158. In human gliomas, high levels of miR-449a and low expression of GPR158 are associated with higher malignancy and poorer survival.

**Results**

**miR-449a is significantly differentially expressed between experimental gliomas and primitive neural tumours**

To identify genes that are differentially expressed (DE) between gliomas and PNET (Fig. 1a) we performed miRNA microarrays. Unsupervised hierarchical clustering identified 89 differentially expressed miRNAs (DE-miRs) between gliomas of both genotypes (Pten/p53 and Rbh/p53), and PNET’s (Rbh/p53) (Fig. 1b, Supplementary Table 1). Twenty miRs were differentially expressed between gliomas (Pten/p53) and PNETs (Rbh/p53) (Supplementary Table 1). Twenty-six significantly DE-miRs were identified between gliomas (Pten/p53) and PNETs (Rbh/p53), and 21 top DE-miRs between Rbh/p53 glioma and PNETs (Fig. 1c). We found a high degree of overlap with 16 miRNAs co-existing in both DE-miR groups. Reverse transcription (RT)-quantitative PCR (RT-qPCR) reduced the group to 9 DE-miRs between gliomas and PNETs (Fig. 1d; Supplementary Table 1), and of those, miR-449a was most significantly differentially expressed (Fig. 1d). Gene ontology analysis of these nine miRNAs showed an association with neurogenesis and cell migration (Supplementary Table 2), miR-449a is enriched in astrocytes [33], whereas miR-219 and miR-338 are essential for oligodendrocyte differentiation [15]. Considering that miR-449a is involved in the regulatory network of RB and P53 [23, 34], it was a promising candidate and most likely relevant to the brain tumour phenotype.

**miR-449a directly targets Ccnd1 and Gpr158**

miR-449a targets were identified with TargetScan 7.1, resulting in a list of 101 putative targets with conserved binding sites (Fig. 2a; Supplementary Table 1). To identify DE genes between the two tumour types, we retrieved the top 1000 DE-genes ranked by logarithmic fold change (glioma/PNET) from our published Exon Microarray dataset (GSE42515), and matched them against the 101 putative
miR-449a: cell migration, proliferation and invasion

**Fig. 3** miR-449a inhibits cell proliferation and migration of BTSC in vitro. 

**A** Analysis of the stem cell frequency of Pten/p53 and Rb/p53 BTSCs using the extreme limiting dilution assay. Negative wells were counted 7 days post-seeding. Frequency of sphere-forming cells: Pten/p53: 1/4.96; Rb/p53: 1/8.32, n = 12, p = 0.065, indicating lower rate of self-renewal of Rb/p53 cells. **B** High levels of miR-449a in Rb/p53 mBTSC compared to Pten/p53 BTSC (p < 0.001), normalized for Pten/p53. **C** miR-449a reduces cell proliferation (confluence assay): Pten/p53 (miR-449alow) BTSC grow faster than Rb/p53 (miR-449a high) mBTSC. Treatment of Pten/p53 BTSCs with miR-449a mimic reduces their proliferation, whilst antagomir-treated Rb/p53 (Rb/p53 ant) BTSC show increased proliferation, compared to scrambled-treated controls, respectively. **D** The gap closure assay shows the cell migration over 12 h into an artificially generated cell-free space. miR-449alow cells (i.e., Rb/p53 ant and Pten/p53 scramble) migrate faster and almost close the gap after 12 h; whereas miR-449ahigh cells (i.e., Rb/p53 scramble and Pten/p53 mimic) hardly advance into the gap. Recording of multiple time points during the 12 h period visualizes the dynamics of migration and confirms a statistically significant difference in the live cell imaging assay (right figure part). **E** Quantification of the invasion of a mass of 5000 cells into 3D collagen. The left figure part shows an example of the outgrowth of processes from spheres. miR-449alow cells (i.e., Rb/p53 ant (top right) and Pten/p53 scramble (bottom left)) show substantial outgrowth into the matrix. In contrast, miR-449ahigh cells (i.e., Rb/p53 scramble (top left) and Pten/p53 mimics (bottom right)) hardly advance into the matrix (left panel). The right figure part shows quantification with Image J, confirming the statistically significant difference (n = 12). **F** RT-qPCR analysis of differentiation markers in BTSC shows a proneural gene expression pattern in Pten/p53 and in antagomir-treated Rb/p53 BTSCs compared to the baseline of untreated Rb/p53 BTSC (orange error bars). Inhibition of miR-449a (i.e., Rb/p53 ant, red bars) resulted in the shift of basal expression profile of Rb/p53 cells towards that of Pten/p53 BTSCs (grey bars).
targets, following permissive filtration criteria: (i) miR-449a is highly expressed in PNETs, expecting downregulated targets; (ii) DE-genes with \( p > 0.05 \) were also considered to minimize false negative calls. Eight genes were selected (Notch1, Met, Gnao1, Meto1, Gpr158, Parp8, Ccnd1 and St8sia3) (Fig. 2a,b). To identify candidates regulated by miR-449a, we analysed \( Rb/p53 \) (miR-449a\textsuperscript{high}), \( Pten/p53 \) (miR-449a\textsuperscript{low}), and \( Rb/p53 \) anti-

agent mBtScs (miR-449a\textsuperscript{low}).
miR-449a reduces cell proliferation and migration by suppressing Ccnd1 levels in mBTSC. a MiR-449a reduces Ccnd1 levels in mBTSC, miR-449a antagonism (ant) treatment of miR-449a-blow Rb/p53 mBTSC restores Ccnd1 expression, and conversely miR-449a mimic (mim) treatment of Pten/p53 miR-449a-mbBTSC reduces Ccnd1 expression. Scr = scramble. (b) Transfection of Rb/p53 mBTSC with a Ccnd1 expression vector results in twofold overexpression of Ccnd1 and increased cell proliferation, and knockdown decreases it. c Forced Ccnd1 overexpression (transfection) antagonises miR-449a-mediated inhibition of cell proliferation. Top curve (grey) baseline Pten/p53 (miR-449a-low), bottom curve miR-449a knockdown, and middle curve miR-449a kd + Ccnd1 restore. d Ccnd1 accelerates cell proliferation in a confluence assay: Pten/p53 cells (grey) grow faster than Rb/p53 cells (orange). E Ccnd1 overexpression increases proliferation of Rb/p53 cells (red) which now proliferate faster than Pten/p53 cells. These grow slower than untransfected cells Rb/p53 cells (orange) when Ccnd1 is inhibited (black). f Inhibition of Ccnd1 reduces outgrowth of tumour sphere processes, demonstrating the role of Ccnd1 in cell proliferation and migration. G-S, effects of overexpression or inhibition of Gpr158 in Rb/p53 or Pten/p53 mBTSC (g) Gpr158 levels in naïve and Gpr158 transfected Rb/p53 or Pten/p53 mBTSC. h Gpr158 downregulates cell proliferation, i cell migration and j self-renewal proportionally in Rb/p53 or Pten/p53 mBTSC. A 2-fold decrease of tumour sphere forming cells was observed upon Gpr158 overexpression. BTSCs stably expressing Gpr158 = 1/6; BTSCs control = 1/3; i.e., requiring the presence of 3 cells to form 1 neurosphere in controls, vs. 6 cells to form a sphere in Gpr158 overexpressors (p = 0.02) n = 12; p = 0.02. k Suspension culture of mBTSCs in serum-free medium. Upon stable expression of Gpr158, mBTSC attach to the surface of the cell culture well, change morphology and involute/grow slower. The Caspase-3/7 activity assay indicates that Gpr158 significantly increases apoptosis in mBTSC. m Knock-down of Gpr158 using siRNA in mouse BTSCs, confirmation of abolition of Gpr158 mRNA expression. Down-regulation of Gpr158 promotes cell proliferation (n), migration (o) and tumour sphere forming ability (p). q Stable expression of Gpr158 significantly upregulates expression of neural genes, assessed in a mouse neurogenesis qPCR profiler array, while siRNA knock-down of Gpr158 significantly reduces the expression of Map2, Sox2 and Pdgfra. r Stable knock-down of Gpr158 in three human GBM primary cell lines cultured in serum-free medium, containing hBTSC reduces BTSC apoptosis. s Overexpression of Gpr158 significantly increases apoptosis of human GBM primary cells (hBTSC). All figures: *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001; ****p ≤ 0.0001 (Student’s t-test). Each assay was performed at least twice and selected those targets which were upregulated in miR-449a-blow mBTSCs. Quantification confirmed that only Gpr158 and Ccnd1, but not the other six genes, were correspondingly regulated in both Pten/p53 and Rb/p53 antagonimir cells, compared to the baseline of Rb/p53 cells (Fig. 2b). Ccnd1 and Gpr158 carry conserved miR-449a binding sites within their 3′ UTR [20], (Fig. 2f). In keeping, primary Rb/p53 brain tumours (PNET) express low, and Pten/p53 gliomas high Gpr158 levels (Fig. 2c).

We then confirmed a functional link between miR-449a and its target Gpr158 by two functionally independent approaches: a modified hybrid Argonaute 2 (AGO2) pull-down assay and a luciferase reporter assay. The AGO2 assay [35, 36] (Fig. 2d) uses an established miR-449a target, CCND1, as positive control [20]. After double pull-downs of AGO2 and biotin-labelled miR-449a mimics in tandem, the relative enrichment of each fragment compared to input RNA was measured by RT-qPCR (Fig. 2e, number corresponds to the fraction in Fig. 2d). We confirmed that the degradation of Gpr158 mRNA by miR-449a was dependent of RNA-induced silencing complex [37] (Fig. 2e), demonstrating direct regulation of Gpr158 expression by miR-449a. The luciferase assay confirmed physical binding of miR-449a to the Gpr158 3′ UTR and to locate the conserved binding sequence within the 3′ UTR of Gpr158. Luciferase reporter plasmids containing wild-type (pMIR-Gpr158-3′ UTR-wt) and mutant (pMIR-Gpr158-3′ UTR-mut) sequence complementary to the seed region of miR-449a were generated (Fig. 2f). MiR-449a mimics significantly reduced luciferase activity under a wild-type 3′ UTR (p < 0.001), which was rescued by substituting the reporter plasmid with a mutant seed sequence (Fig. 2g), indicating that the mutated 3′ UTR of Gpr158 reduced miR-449a binding.

miR-449a inhibits brain tumour stem cell self-renewal, proliferation and migration in vitro

To characterize the role of miR-449a in brain tumour stem cells (BTSC), murine (m)BTSC were derived from SVZ stem/progenitor cells of naïve Pten/p53 or Rb/p53 mice, recombined in vitro [7] and expanded in EGF and FGF-enriched serum-free stem cell medium [38]. An in vitro extreme limiting dilution assay [39] showed a tendency (p = 0.065) of reduced self-renewal of Rb/p53 BTSC (miR-449a-high), compared to Pten/p53 cells (miR-449a-low) (Figs. 3a, b). In a proliferation assay, Pten/p53 cells grew faster (Fig. 3c) and in a gap closure assay they moved faster than Rb/p53 cells into an artificially introduced gap (Fig. 3d). To test if these were miR-449a-mediated effects, miR-449a antagonir or mimics were introduced into mBTSCs. Transient inhibition of miR-449a with antagonir in Rb/p53 mBTSCs significantly increased their proliferation and migration, consistent with previous reports in cell lines derived from hepatocellular carcinoma [16], colon cancer [27], or prostate cancer [22], whilst the opposite effect was seen when a miR-449a mimic was introduced into Pten/p53 cells (Figs. 3c, d). In a 3D collagen matrix tumour invasion assay, inhibition of miR-449a expression in Rb/p53 cells increased cell migration, whereas miR-449a mimics in Pten/p53 cells slowed down migration (Fig. 3e), consistent with findings in hepatocellular carcinoma cells [16]. In conclusion, in stem cell medium, miR-449a-high mBTSC grow slower than miR-449a-low mBTSC, suggesting a suppressive role of miR-449a on proliferation, migration and invasion.
miR-449a targets Ccdn1, inhibiting proliferation and migration of BTSC in vitro

First we confirmed the known effect of miR-449a to downregulate Ccdn1. In keeping, treatment of Rbp/p53 mBTSC (miR-449a$_{\text{high}}$) with miR-449a antagonir rescued (i.e., increased) Ccdn1 expression, whilst treatment of Pten/ p53 mBTSC (miR-449a$_{\text{low}}$) with miR-449a mimic reduced Ccdn1 expression (Fig. 4a). This effect can be antagonised by overexpressing Ccdn1 in Rbp/p53 mBTSC, increasing proliferation (Fig. 4b), or by Ccdn1 siRNA treatment of Pten/p53 mBTSC, reducing proliferation (Fig. 4c), consistent with previous reports [27]. Co-transfection of Pten/ p53 mBTSC with mir-449a mimic and Ccdn1 expression vector restored the reduced proliferation of cells treated with mir-449a mimic alone (Fig. 4d). Likewise Ccdn1 overexpression increases migration of Rbp/p53 cells, and inversely inhibition in Pten/p53 cells inhibits cell migration in a gap closure assay (Fig. 4e). In keeping the ability of Pten/ p53 (miR-449a$_{\text{low}}$; Ccdn1$_{\text{high}}$) mBTSC to invade a matrigel matrix is antagonised by Ccdn1 shRNA (Fig. 4f).

The miR-449a target Gpr158 promotes differentiation and apoptosis, and inhibits proliferation and migration of BTSC in vitro

Next we characterized the effects of miR-449a action on Gpr158 in vitro (Fig. 4). Expression levels of GPR158 are highest in murine neural stem/precursors cells (mNSPC), lower in Pten/p53 and virtually undetectable in Rbp/p53 mBTSC (Supplementary figure 1A). Unexpectedly, overexpression of Gpr158 in Rbp/p53 and Pten/p53 mBTSC, resulted in slower growth of both cell lines, whereby Pten/ p53 cells always proliferated and migrated faster than Rbp/ p53 cells (Figs. 4g-i), i.e., Gpr158 further reduces proliferation and migration. This is seemingly incompatible with the finding that miR-449a inhibits Gpr158, as both miR-449a$_{\text{high}}$ Rbp/p53 and miR-449a$_{\text{low}}$ Pten/p53 cells show further reduction of proliferation, i.e., in the same direction as miR-449a treatment. However, when we explored if Gpr158 regulates the ability of BTSC to form tumour spheres, we found in an extreme limiting dilution assay [39] a significant reduction of sphere forming cells upon Gpr158 overexpression (Fig. 4j). Gpr158 overexpressing BTSC were smaller and tended to attach and differentiate (Fig. 4k), suggesting a role of Gpr158 to induce neural differentiation, and the potential to override miR-449a effects. Indeed siRNA knock-down of Gpr158 in naïve, adherently growing murine neural stem/progenitor cells (mNSPC) (Supplementary Figure 1B) showed global reduction of expression levels of the majority of genes associated with neural differentiation/neurogenesis (Supplementary Figure 1C), of which Nrp1, S100a6 and Tnr were significant.

Knockdown of Gpr158 in Pten/p53 mBTSCs (Fig. 4m) increases cell migration (Fig. 4o), and sphere formation (Fig. 4p). The role of GPR158 to induce neural marker expression, was quantified in a qRT-PCR profiler assay. There is significant up-regulation of the proneural markers Map2, Sox2, and Pdgfra [4], and of three extracellular matrix-associated genes Filamin A (Flna), Netrin 1 (Ntn1), and Pleiotrophin (Ptn), and downregulation upon Gpr158 knock-down (Fig. 4q).

Neural differentiation of stem and progenitor cells is associated with apoptotic cell death [40], and in keeping, overexpression of Gpr158 in mBTSC resulted in significant induction of caspase-3/7 activities (Fig. 4l), whilst Gpr158 siRNA knockdown increased proliferation ($p<0.05$, Figs. 4m, n), and migration (Fig. 4o), and there was increased sphere formation (Fig. 4p). Consistent with this observation, also GPR158 knockdown in three human GBM primary cultures significantly reduced apoptosis (Fig. 4r), and in keeping, overexpression of GPR158 in two human GBM primary cultures induced apoptosis (Fig. 4s). Baseline levels of a selection of glioma cell lines is shown in Supplementary Figure 3G.

In conclusion, increase of GPR158 expression in mBTSC and hBTSC reduces proliferation, migration and cancer stem cell formation, upregulation of proneural markers and induction of apoptosis whilst downregulation of GPR158 has the opposite effect. We identified that miR-449a has distinct, target-dependent (i.e., CCND1 and GPR158) effects on cellular growth, migration and differentiation.

BTSC differentiation induced by GPR158 is antagonised by miR-449a

Under growth-promoting conditions in EGF, FGF enriched serum-free medium, miR-449a suppresses Ccdn1 and inhibits migration and invasion, suggestive of a tumour-suppressive effect. This effect is seen in cell lines derived from somatic cancers, such as non-small lung cancer [18], hepatocellular carcinoma [16], colon cancer [27] or neuroblastoma [19]. A different role of miR-449a emerges from studies on CNS development where high miR-449a levels are associated with neural progenitor expansion and suppression of neural differentiation [25, 26], possibly through an inhibition of GPR158. Therefore, we first determined Gpr158 and Ccdn1 expression in Pten/p53 or Rbp/p53 mBTSC, under proliferative (serum-free, EGF, FGF enriched) and differentiating (3% FBS) conditions. Immunofluorescent labelling for Gpr158 shows virtually no labelling under proliferative conditions (Fig. 5a) and upregulation when grown in differentiating conditions, whilst density of nuclear labelling for Ccdn1 markedly decreased upon culturing under differentiation conditions.

SPRINGERNATURE
Quantification confirms upregulation of Gpr158 and downregulation of miR-449a upon induction of neural differentiation (Fig. 5a). Next we investigated the effect of miR-449a on neural differentiation on individual cellular level, again comparing serum-free, EGF and FGF-enriched growth-promoting environment with differentiation-inducing culture conditions containing foetal bovine serum (FBS) (Fig. 5c). Gpr158 overexpression in Ptten/p53
miR-449a antagonises Gpr158-induced neural differentiation. a Induction of neural differentiation with 3% FBS increases Gpr158 and reduces miR-449a expression. Ccd1 expression in EGF, FGF-enriched stem cell medium (upper part) is suppressed in miR-449a

or Rb/p53 mBtsc induces neural differentiation, demonstrated by the detection of doublecortin (DCX), a protein expressed in migrating neuroblasts and immature neurons [41]. Immunolabelling for DCX in lentivirus-transduced Rb/p53 mBtsc confirms expression (Fig. 5b) and qPCR profiler expression analysis of these cells shows upregulation of genes associated with pronetal signature Rb/p53 mBtsc (miR-449a

We further confirmed the interaction of miR-449a with Ccd1 and Gpr158 in vivo. Allografts of Rb/p53 (miR-449a

or Rb/p53 mBtsc with fewer nuclei labelled, and the overall expression is strongly reduced upon growth in 3% FBS supplemented medium. Quantification of miR-449a and Gpr158 expression in these cells on the right. b Stable overexpression of Gpr158 antagonises miR-449a and promotes neural differentiation in miR-449a

or Rb/p53 mBtsc (miR-449a

Fig. 5 miR-449a antagonises Gpr158-induced neural differentiation. a Induction of neural differentiation with 3% FBS increases Gpr158 and reduces miR-449a expression. Ccd1 expression in EGF, FGF-enriched stem cell medium (upper part) is suppressed in miR-449a

or Rb/p53 mBtsc (miR-449a

バイオロジーの日本語翻訳者として、この文脈をより自然に理解できるように翻訳を行います。
Fig. 6 Expression of neural markers, Gpr158 and Ccnd1 in allografts of miR-449a<sup>high</sup> and miR-449a<sup>low</sup> tumours. Phenotype of tumours generated from allografted Rb/p53 (left column), miR-449a antagonomir-treated Rb/p53<sup>antag</sup> (centre column) and Pten/p53 cells (right column) into NOD-SCID mice. a Morphology (H&E) shows a well demarcated tumour border in Rb/p53 PNET (single line of arrowheads), and an infiltrative margin in both, antagonomir-treated Rb/p53 grafts (b) and Pten/p53 grafts (c). Image analysis of regions of interest on whole slide digitized images of immunostained histological sections with Definiens Developer shows a reversal of Rb/p53<sup>antag</sup> grafts to levels found in Pten/p53 grafts (markers Olig2 (d–g), Pdgfra (l–o), and Sox2 (p–s)), whilst only DCX it is unaltered (h–k). Expression of these markers is reduced in Rb/p53; miR-449a<sup>high</sup> tumours, high in Pten/p53 (miR-449a<sup>low</sup>) grafts, and restored upon suppression of miR-449a in antagonomir-treated Rb/p53<sup>antag</sup>; miR-449a<sup>low</sup> grafts. (t–ae) miR-449a regulates Ccnd1 and GPR158 in tumours in vivo: (t–w), Ccnd1 expression is low in Rb/p53 (miR-449a<sup>high</sup>) tumours (t), and high in Pten/ p53 (miR-449a<sup>low</sup>) tumours (u) as well in antagonist-treated Rb/ p53; miR-449a<sup>antag</sup> tumours (v, w). Gpr158 expression is regulated correspondingly (x–aa). In keeping with the shorter survival of mice with Rb/p53; miR-449a<sup>high</sup> tumours, proliferation is high in these tumours (ab). Scale bar 50 µm. All figures: *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001 (Student’s t-test). Each value represents the mean ± s.d.
**A**

- GPR158 expression levels across different tumor types.
- CNS, GBM, and GBM-IDH early stages.
- Bar graph showing expression levels with error bars.
- n.s indicates non-significant differences.

**B**

- GPR158 fold change across CNS, A, and GBM-IDH.
- Significant fold changes indicated by asterisks.

**C**

- miR-449a expression levels across GBM-IDH.
- Significant fold changes indicated by asterisks.

**D**

- Spearman correlation between GPR158 and miR-449a.
- Correlation coefficient: -0.75.
- p-value: 0.002.

**E**

- CCND1 expression levels across CNS, A, and GBM-IDH early stages.
- Significant fold changes indicated by asterisks.

**F**

- miR-449a fold change across GBM-IDH.
- Significant fold changes indicated by asterisks.

**G**

- GPR158, IDH1, ATRX expression in different tumor stages.
- H&E staining images for CNS, O, A, GBM-early, GBM.

**H**

- GPR158 staining levels across CNS, O, A, GBM-early, GBM.
- p-value: 0.037.

**I**

- Integrated diagnosis table for different tumor types.
- Age, Gender, Grade, GPR158, Integrated diagnosis, IDH1, ATRX, TERT, MGMT, EGFR.
- Data representation with color codes for different statuses.
- Spearman correlation: -0.75.
- p-value: 0.002.
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Fig. 7 Analysis of GPR158 expression in tumour and control brain samples. Expression of GPR158 in CNS, and the five glioma subgroups oligodendroglioma, astrocytoma, GBM-IDH, early GBM and GBM, as defined by the integrated (morphological and molecular) diagnosis. a TCGA RNA sequencing (rsem) data indicate highest GPR158 expression in CNS (n = 5) and oligodendrogliomas (O, n = 84), a reduction in astrocytomas (A, n = 138), GBM-IDH (n = 9), and further down-regulation in 'early GBMs' (n = 54) and GBMs (n = 148). The differences between O, A/GBM-IDH, early-GBM and GBM (both IDH+) are highly significant. (b) GPR158 RNA expression in frozen CNS and glioma tissue from our institution. Relative GPR158 expression levels measured by RT-qPCR is consistent with TCGA rsem data. (c) Expression levels of miR-449a in the same samples, and (d) plot of inverse correlation of miR-449a and GPR158 RNA expression levels. e CCND1 expression is higher in gliomas than in CNS but not significantly differentially expressed across all glioma groups in TCGA samples. f In our samples, CCND1 expression is lower in GBM than in oligodendrogliomas, in keeping with the observation in our brain tumour allografts (Figs. 6t, u, v) that the proliferative Rb/p53 tumours downregulate Ccdn1. g Representative histology and immunostaining patterns in tumours (n = 93) from our institution. GPR158 immunoreactivity is strong in CNS and oligodendroglioma, much weaker in astrocytoma, and nearly negative in IDH wild-type 'early' GBM and GBM. Mutant IDH1 is expressed in oligodendrogliomas and astrocytomas, but not in CNS, and glioblastomas. ATRX is lost only in IDH mutant astrocytomas. All other tumours and the CNS maintain ATRX expression. Scale bar corresponds to 100 µm. h Quantification of protein expression by whole slide imaging and image analysis of tissue sections immunostained for GPR158. CNS tissue shows the highest expression, followed by oligodendrogliomas and astrocytomas, whilst there is a significantly lower expression in IDH wild-type early-GBM and GBM, consistent with the RNA expression data shown in (a) and (b). Oligodendroglioma (n = 17), astrocytoma (n = 16), early-GBM (n = 12) and GBM (n = 34). CNS tissue data were obtained from tissue fragments within some of the resection specimens containing normal CNS. i Overview and summary of demographic parameters, tumour grade, integrated diagnosis and molecular profile of the tumours analysed in (e). There are two types of IDH mutant gliomas, oligodendrogliomas, defined by a loss of chromosomal arms 1p and 19q (1p/19q codeleted) and typically with a mutation in the telomerase reverse transcriptase (TERT) promoter, and astrocytomas or glioblastomas (GBM) which carry a mutation of alpha thalassemia/mental retardation syndrome X-linked (ATRX) resulting in functional loss of the protein. Patients with IDH mutant tumours are younger than those with IDH wild-type GBM. GPR158 levels are highest in oligodendrogliomas, lower in astrocytomas and lowest in GBM, as described above.

GPR158 is highly expressed in the human CNS, and differentially expressed in human oligodendrogliomas, astrocytomas and glioblastomas

To establish the roles of miR-449a and GPR158 in human gliomas, we first analysed 'RNA-Seq by Expectation-Maximization' (rsem) data of 431 gliomas (155 GBM and 276 low-grade gliomas (LGG), Supplementary table 3) from the TCGA database, and complemented this dataset with mRNA expression analysis and immunohistochemical detection of GPR158 protein in human gliomas from our institution (NHNN, Supplementary table 3). To correlate GPR158 expression with clinically, diagnostically and biologically relevant tumour entities [2], we defined oligodendrogliomas (O, n = 83) as IDH mutant, 1p/19q codeleted tumours, and astrocytomas (A, n = 138) as IDH mutant, ATRX mutant tumours with no 1p/19q codeletion. As the WHO grade is of disputed clinical significance [42, 43] in astrocytomas, we did not further stratify these tumours by WHO grades II or III. IDH mutant GBM (GBM-IDH, n = 9) which carry a poorer prognosis than IDH mutant astrocytomas, were grouped separately from astrocytomas. IDH wild-type high grade gliomas with 7p gain, 10q loss, EGFR amplification and TERT promoter mutation were considered as GBM (corresponding to WHO grade IV) (n = 146). Histological lower grade IDH wild type astrocytomas with molecular profiles of glioblastoma are considered as 'early stage' GBM [44] (eGBM; n = 54). Using these criteria and TCGA RNA sequencing data, we investigated GPR158 expression in these glioma subtypes. GPR158 expression was highest in the CNS (n = 5) and in oligodendrogliomas, followed by astrocytomas (p < 0.0001), and significantly lower in eGBM and GBM (p < 0.0001; Fig. 7a, Supplementary Figure 3A-C). The difference of GPR158 expression levels was statistically significant between eGBM and GBM (p < 0.0001), suggesting that GPR158 was further down-regulated upon tumour progression to a higher (histological) grade. In 29 other TCGA tumour entities (Supplementary Figure 3D), with the exception of pheochromocytoma and paraganglioma [45], the remaining tumours types expressed very little or no GPR158. In conclusion, we show here that there is a statistically significant difference of GPR158 expression between clinically and biologically distinct glioma subgroups, and GPR158 expression was specific in nervous system-related tumours. mRNA expression data of miR-449a and GPR158 were confirmed on 25 frozen glioma samples and 5 CNS samples from our own collection (miR-449a: CNS, n = 5; O, n = 7; A, n = 7; GBM, n = 8; and GPR158 CNS, n = 5; O, n = 8; A/GBM-IDH, n = 8; GBM, n = 9) (Figs. 7b, c) by RT-qPCR analysis. miR-449a inversely correlates with GPR158 expression (Figs. 7b, c, d), consistent with previous experiments. To assess, if the expression of the miR-449a target CCND1 may also correlate with tumour grade and type, we retrieved expression data from TCGA. CCND1 rsem is low in CNS tissue, and slightly increased within a wide range of expression levels across all subgroups of gliomas, with no statistically significant difference between oligodendrogliomas, astrocytomas and IDH wild-type glioblastoma (Figs. 7e, f). Thus, the expression of CCND1 remains largely independent of the tumour subtypes, supporting the notion that GPR158 may have a role as biomarker that is independent from the miR-449a target CCND1.
Fig. 8 Patients with gliomas expressing higher level of GPR158 survive longer and show better response to chemotherapy. 

(a) Kaplan–Meier estimates of overall survival in all grades of gliomas from our institution, grouped by miR-449a expression levels or by (b) GPR158 expression measured by staining intensity. Patients with tumours expressing low levels of miR-449a or high levels of GPR158 survive significantly longer. The ‘miR-449a low’ and ‘miR-449a high’ groups, and the ‘GPR158 low’ and ‘GPR158 high’ groups were separated by the median of expression levels or staining intensity. 

(c) Stratification of survival according to GPR158 expression levels (rsem) in the TCGA cohort. The survival pattern from TCGA data confirms the finding in (a). 

(d) The cohort of patients (from TCGA) with IDH mutant tumours expressing high levels of GPR158 show a trend of longer survival than low expressors. However, in this cohort the difference does not reach significance level. 

(e) Proneural and (f) neural subtypes of GBMs (from TCGA) with higher expression of GPR158 show a significantly longer survival, whilst no difference is seen in GBM of (g) classical (h) or mesenchymal profiles. 

(i) GBM with high GPR158 expression show a significantly better response to chemotherapy. All p values were estimated using log-rank test. 

(j) Low grade or (k) high grade gliomas, stratified by CCND1 expression do not show a difference in survival, indicating a role mediated by the miR-449a target GPR.158 rather than CCND1.
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Target-dependent effects of miR-449a on proliferation, differentiation and glioma grade

Fig. 9 Target dependent effects of miR-449a on proliferation, differentiation and glioma biology (a) in stemness—maintaining growth conditions with EGF, FGF enriched serum-free medium, miR-449a—mediated effects are predominantly exerted through CCND1 inhibition, thus reducing invasion and proliferation. For example, miR-449a

To confirm GPR158 protein expression on paraffin-embedded tissue sections by immunohistochemical (IHC) detection, we tested 85 samples from our archive, all molecularly characterized (IDH1/2, Histone H.3.3, BRAF, TERT promoter and 1p/19q, 7p/EGFR, 10q copy numbers, ATRX IHC, Fig. 7g). Again, GPR158 expression strongly correlated with histological entities (Figs. 7g, h, i) being highest in CNS cortex, followed by oligodendroglomas, whilst astrocytomas stained much weaker, but had still a clearly visible fine granular cytoplasmic stain. eGBM and GBM showed no staining or only focal, patchy weak and diffuse staining (no statistically significant difference between both groups). Small fragments of CNS cortex, present in many samples, in particular of surgical aspirate, served as internal technical control and were used to generate quantitative CNS grey matter expression data by whole slide image analysis (Fig. 7h) [6], and also correlate with TCGA and our own expression data (Figs. 6a, b, c). In conclusion, we show here that GPR158 RNA and protein expression correlate with distinct diagnostic glioma entities, suggesting that GPR could represent a biomarker for stratifying these tumours (Fig. 7i).

Lower miR-449a and higher GPR158 expression correlates with longer survival of glioma patients

Finally we evaluated if miR-449a and GPR158 also have a role as potential prognostic biomarker, i.e. correlate with patient survival (Fig. 8). First we separated the 25 NHNN patients of whom we had miR-449a expression values into 2 groups (above and below median) and found significantly better survival in the miR-449a

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GPR158 in the four subtypes of GBM [4] and found significantly longer survival of GPR158<sup>high</sup> patients in the proneural and neural groups (Fig. 8c and Supplementary table 4), but not in the other two groups (Figs. 8g, h), and there is no influence of CCND1 expression on survival (Figs. 8j, k). In summary, in both the NHNN and the TCGA glioma cohorts, we confirmed that higher GPR158 transcript and protein expression levels correlate with better survival, and patients with GPR158<sup>high</sup> IDH<sup>wt</sup> GBM responded significantly better to chemotherapy compared to patients with GPR158<sup>low</sup> tumours (Fig. 6i, Supplementary table 4).

**Discussion**

To identify differentially regulated genes and their targets in gliomas, we used mouse models which develop two well-defined tumour phenotypes—gliomas (Pten/p53) or PNET (Rb/p53)—with distinctive lineage, growth rate and invasiveness [6, 7]. In a microRNA screen, we identified miR-449a as significantly differentially regulated. miR-449a belongs to the miR-34/449 family, and shares with miRNA-34, 449b, and 449c seed sequences, secondary structures [46], and downstream targets, including CCND1 and E2F transcription factor 5 (E2F5) [27, 47–49]. To understand the regulatory function of miR-449a in BTSC, we looked for targets relevant for intrinsic brain tumours (Fig. 2). We confirmed and validated the known target CCND1 [20] (encoding Cyclin D1) (Figs. 2e and 4a–f) and we identified a new target, G-protein coupled receptor 158 (GPR158) which is downregulated by miR-449a (Figs. 2c, e, f). GPR158 was considered highly relevant, as it is most closely related to GABA receptors [28], and is widely and strongly expressed in the central nervous system and has a role in cognition [50]. It is highly expressed in the CNS and low in somatic organs https://www.proteinatlas.org/search/GPR158, and it is also highly expressed in ‘low grade glioma’ and in phaeochromocytoma/paraganglioma, a neuroendocrine neoplasm. Instead it is reduced in glioblastoma and expressed at even lower levels in a malignant childhood tumour, AT/RT (Supplementary Figure 3D, E). We show here that miR-449a has target-dependent effects on cell migration, proliferation and differentiation, mediated by CCND1 or GPR158 (Fig. 9). miR-449a inhibits CCND1 under proliferative conditions in stem cell medium (serum free, EGF, FGF supplemented), reducing proliferation and migration (Figs. 3f, 4a–f and 5a). It also inhibits GPR158, suppressing neural differentiation (Fig. 3f). Under these conditions, however, GPR158 overexpression reverses miR-449a—induced phenotypes (caused by CCND1 inhibition) by promoting neural differentiation and apoptosis, thus further reducing migration and proliferation (Figs. 4g–s) and this creates a seemingly paradox result. This paradox has been addressed by further studying the effects of miR-449a and GPR158 in conditions facilitating neural differentiation in vitro and in vivo. Under these conditions, GPR158 induces neural, glial and neuronal phenotypes and apoptosis (Figs. 4k–s and 5, Supplementary Figure 2) in tumour spheres and cells and in allografts in vivo (Fig. 6). This can be antagonised by miR-449 mimics (Fig. 5, Supplementary Figure 2). GPR158 knockdown has opposite effects, reducing neural differentiation, apoptosis and increasing growth (Figs. 4k–o, and Supplementary Figure 2), whilst miR-449a antagonism restores a neural phenotype (Fig. 5c4, Supplementary Figure 2). This antagonistic effect between miR-449a and GPR158 was consistently found in conditions promoting neural (FBS, Fig. 5), glial (LIF/BMP2), and neuronal (retinoic acid/forskolin) phenotypes (Supplementary Figure 2) in vitro, and in murine allografts in vivo (Fig. 6).

In conclusion, the effects of miR-449a are modulated by growth conditions and environment of BTSC (Fig. 9). Our data raise the possibility that the effects of miR-449a may be target-dependent, acting on CCND1 or GPR158 (Fig. 9). miR-449a has an inhibitory effect on migration and invasion in vitro in some cancer types [19, 20, 23] and Figs. 3d, e). In vivo, miR-449a has a tumour suppressive effect in some cancers, such as hepatocellular carcinoma, [16], or lung cancer [18], but not in others, where an association with cancer progression was found, such as breast [51] or colorectal cancer [52]. A previous study on brain tumours shows results similar to our in vitro data [17], whilst a discrepancy of miR-449a correlation with brain tumour grade is noted. In that study tumour types were not specified and thus the results may not be directly comparable to our cohort which used molecularly characterized and stratified tumours. A yet different role of miR-449a has been identified in the developing neuroepithelium, where a transient upregulation is associated with neural progenitor expansion in the rat brain at embryonic day 10 [53] and suppression of neural cell fate activates choroid plexus.

CCND1 can have context-dependent roles in vitro and in vivo: It is one of the major regulators of the cell-cycle progression, can act as an oncogene, and aberrant expression is commonly seen in human cancers [54, 55]. In keeping, in vitro under appropriate conditions CCND1 promotes proliferation and migration of BTSC (Figs. 4a–c, 5a and 9a), and is modulated by miR-449a expression, i.e., is higher in Pten/p53 mBTSC (miR-449a<sup>low</sup>) than in Rb/p53 mBTSC (miR-449a<sup>high</sup>) (Figs. 4c and 5a) and in miR-449a<sup>high</sup> experimental tumours, Ccnd1 is downregulated (Fig. 6t). Instead, in neural differentiation conditions Ccnd1 is downregulated (Fig. 5a) in keeping with overall reduction of cell proliferation and increased neurogenesis [56, 57] and differentiation into astrocytes [58]. This is consistent with
our observation of higher expression of neural markers, and of GPR158 and CCND1 in miR-449a
low tumours developing from allografted Pten/p53 cells and from Rbb/p53 cells treated with miR-449a antagonim (Fig. 6). However, unlike in many other cancers, CCND1 is not differentially expressed in different grades and types of brain tumours (Fig. 7e), and TCGA outcome data do not show a difference in survival between patients with gliomas expressing low or high CCND1 levels (Figs. 8j, k).

An important finding of our study is the inverse correlation of miR-449a and GPR158 expression (p = 0.002, Fig. 7d). Of translational importance, miR-449a expression correlates with tumour grade (Fig. 7c) and with poorer survival (p = 0.004, Fig. 8a), and consistent with our experimental data, GPR158 expression correlates with better prognosis (Figs. 8b-i) and inversely with tumour grade (Figs. 7e, i, h and 9b). Our results are discrepant to those of a study on prostate cancer where increased expression of GPR158 correlates with poorer survival [31]. This could be related to the specific pathobiology of prostate cancer, where enrichment of GPR158 expressing neuroendocrine cells (thought to represent transdifferentiated prostate epithelial cells) show more aggressive clinical behaviour and thus are associated with poorer survival. In contrast, expression of GPR158 and (pro-) neural markers is high in low-grade gliomas and typically much lower in GBM. Correlation of GPR158 expression levels to GBM, stratified according to the molecular subtypes [4] further supports this notion, as GPR158 expression is highest in the proneural subtypes (a class that is enriched for IDH mutations and PDGFR amplifications), and decreases in classical (enriched for EGFR amplified and CDKN2A mutant tumours) and mesenchymal subtypes, which are most commonly NF1 mutated (Supplementary Figure 3c). The neural subtype surprisingly appears close to the classical and mesenchymal type, and this could be ascribed to a possible “contamination” of the individual subclasses [59], or superimposed effects of the tumour microenvironment [60]. In keeping with these observations, the expression analysis with the mouse Profiler PCR Array showed a significant up-regulation of Map2, Sox2, and Pdgfra (Fig. 4q) in Gpr158 expressing cells, and corresponding protein expression in tumours developing from allografted cells in vivo (Fig. 6), thus further strengthening the correlation of our experimental data with clinical outcome data.

A further difference between prostate cancer and brain tumours is that in the former, the C-terminal portion of GPR158 is translocated to the nucleus whilst we did not observe nuclear staining in CNS grey matter or in glioma cells using a specific anti-GPR158 C-terminal antibody. This discrepancy could be explained by different signalling pathways relevant in prostate and brain tissues. A role of GPR158 long non-coding antisense (AS) RNA in lung cancer has recently been described, where high expression of GPR158 AS1 correlates with poorer overall survival [32]. In contrast, we show that GPR158 AS1 expression level positively correlates with GPR158 mRNA level (Supplementary Figure 3F). It has been shown previously that antisense RNA can act both, enhancing and suppressing and this is possibly related to differences in tissue specificity [61, 62].

In conclusion, we identified through a phenotypic screen the highly differentially regulated miR-449a, which targets CCND1 and GPR158 and has target-dependent functions (Fig. 9). It is highly expressed in a growth-promoting environment in vitro, reducing proliferation and invasion. In a neurogenic environment in vitro and in tumours in vivo it inhibits neural phenotypes. We show that miR-449 directly targets and downregulates CCND1, resulting in reduced proliferation in vitro, and GPR158, antagonising neural differentiation and apoptosis in glioma stem cells. High miR-449a expression levels correlate with shorter survival, whilst high GPR158 expression is associated experimentally with neural phenotypes, cell differentiation and clinically with lower glioma grades and better patient survival and may serve as predictive biomarker. miR-449a could be considered as druggable target, e.g., using antagonims, and GPR158, a member of a large family of receptors may be targeted by pharmacological agents [63, 64], e.g., by stimulating the downstream pathway of GPR158, to reduce tumour growth.

Materials and methods

Animals

All procedures performed on mice were according to Institutional and UK Home Office guidelines (Project licenses 70-7428 and PA79953C0). The ARRIVE guidelines were followed as part of the institutional policy and the licensing of the experiments. Mice carrying p53loxP/loxP, RhloxPloxP [65] or PtenloxPloxP [66] transgenes were intercrossed resulting in co-deletion of Rbp53 or Ptenp53 and the ROSA26-lacZloxPloxP reporter gene upon cre-mediated recombination. Tumours were induced and BTSC derived as described [7].

Microarray preparation, hybridization and data analysis

Total RNA used for microarray was extracted from frozen tumours using TRIzol [6], miRNAs from 600ng of total RNA were labelled with Hy3 or Hy5 fluorophores according to manufacturer’s protocol (miRCURY LNA microRNA Hi-Power Labelling kit, Exiqon). Pair-wise RNA
samples labelled with Hy3 or Hy5 dye were hybridized to the miRCURY LNA microRNA Array 7 (Exiqon). Spike-ins were used for array quality control. Microarrays slides were scanned (G2565BA Agilent) and images quantified using ImaGene 9 (Exiqon, Denmark). Background signals were corrected using normexp method as described before [67] with limma package on R. Expression validation by quantitative real-time-PCR on a LightCycler 480 (Roche). Amplification curves were analysed using the Roche LC software and normalized using five normalizers (Supplementary table 1) to determine the $-\Delta\Delta \text{Cp}$ value.

**Validation of miRNA targets by Ago2 and biotin double pull-down assay and luciferase assay**

Direct miRNA-mRNA interaction was confirmed by double pull-down assay [35]. $8 \times 10^6$ mBTSC were transfected with 450 pmol of biotin-labelled miR-449a mimic (Exiqon). 10% of the lysate was used as input RNA (Fraction 1), 90% for double pull-down. The first pull-down was on Argonaute TISC-Catalytic Component 2 (Ago2) immunoprecipitation with Protein G Dynabeads (75 μl; Invitrogen). For the second pull-down, Dynabeads were incubated with Relay Samples 2 and 3. RNA was eluted (miRCURY RNA Isolation kit, Exiqon) to obtain Fractions 2 and 3. Ccnd1 and Gpr158 was quantified by qRT-PCR. For the luciferase assay, the 3′ UTR of Gpr158 (174 bp) containing wild-type or mutant miR-449a-5p binding site were synthesized commercially (GeneArt, Invitrogen). The 3′ UTR fragment was inserted to pMIR-REPORT luciferase vector and reporter assay was carried out 48 h post-transfection (Dual-Light system, Applied Biosystems).

**Cell proliferation, gap closure assay and migration assays**

Assays were performed on IncuCyte (Essen bioscience, US) in 96 well plates. Proliferation: 1500 cells per well; Gap closure assay: 75,000 cells per well, gap generation 24 h post-seeding (wound maker, Essen bioscience) and mitomycin C (0.01 mg/ml) was added. Chemotactic migration assay: 3000 cells seeded on a ClearView chemotaxis plate (Essen bioscience). The bottom was filled with attractant medium (40 ng/ml growth factor). Plates were scanned and analysed using Incucyte Chemotaxis module. Collagen-based invasion assay: 5000 cells per well in a U-bottom 96-well plate in 100 μl of 20% (v/v) methylcellulose in culture medium to form a sphere (24 h). Three hours after embedding on fibrillary bovine collagen (2.1 mg/ml), the spheres were repeatedly imaged and quantified with Image J.

**Knock-down or overexpression of miR-449a, CCND1 and GPR158**

Murine brain tumour stem-like cells (mBTSCs) were transfected with (i) LNA-miR-449a antagonor/inhibitors (Exiqon), (ii) LNA-miR-449a mimics (Exiqon), (iii) mouse Gpr158 siRNA (Life Technology Co.), (iv) CCND1 siRNA (Cell Signaling), or (v) scramble controls using Viromer Black (lipocalyx) according to the manufacturer’s protocol. After 24 h, cells were used for proliferation, gap closure, invasion, tumour-sphere, or differentiation assay; alternatively total RNA was extracted for transcript quantification after 48 h.

The lentiviral vector containing short hairpin RNA (shRNA) for stable inhibition of human GPR158 and the control vector were purchased from the UCL RNAi library. Mouse Gpr158 cDNA clone in entry vector pENTR223.1 was purchased from DNASU, and cloned into pLX301 lentiviral vector. Lentivirus was produced in HEK293T cells using Fugene according manufacturer’s protocol (Promega). The human GPR158 expression vector was a kind gift from Elizabeth Fini, University of Southern California, Los Angeles [30].

**Human tissue resources**

The use of human tissue samples was licensed by the NRES, University College London Hospitals NRES license for using human tissue samples: Project ref 08/0077 (S.B.). The storage of human tissue is licensed by the Human Tissue Authority, UK, License #12054 to SB. hGBM-IC were derived and cultured as described [68]. Glioma tissue blocks and associated clinical and molecular information were from the archives of the NHNN.

**Extreme limiting dilution assay**

Cells were plated in 96-well plates at 1, 2, 5, 10, 20, 50, 100, and 200 cells/well as described previously [69], and cultured in 100 μl of serum-free medium per well as mentioned above. The percentage of wells with neurosphere formation was determined 7 days post seeding. Stem cell frequency was estimated using software available at [http://bioinf.wehi.edu.au/software/elda/][39]. Further instructive information can be found on this informal resource [http://www.biology-pages.info/L/LimitingDilution.html].

**Differentiation assay of cells overexpressing or knockdown for GPR158 or for miR-449**

Murine brain tumour stem cells of the Rb/p53 or Pten/p53 genotypes transduced with lentivirus expressing GPR158 or GFP as control, containing puromycin a selection marker.
(4 weeks selection). 24 h after transfection/transduction growth medium was exchanged with differentiation medium containing DMEM/F12, 2% B27, 1% penicillin-streptomycin, and supplemented with 3% FBS, or 10 μM RA (Sigma, R2625) and 20 μM Forskolin (Abcam, ab120058) for neuronal differentiation, or with 50 ng/ml LIF (Santa Cruz, sc-4989) and 50 ng/ml BMP2 (Thermo Fisher, PHC7145) for astrocytic differentiation [70]. After 48 h differentiation cells were fixed and stained for doublecortin (DCX, ab18723, 1:800, Abcam) and GFAP (ab4674, 1:1000, Abcam), followed by secondary antibodies conjugated with Alexa dyes.

**RT-qPCR and mouse neurogenesis profiler array**

cDNA was synthesized using RevertAid RT kit (Thermo Fisher Scientific Inc.). RT-qPCR was performed in triplicate with SYBR Green Mastermix (Thermo Fisher Scientific Inc.). The primers are listed in Supplementary table 5. Gene expression was normalized against GAPDH levels and fold changes were calculated using the 2^ -ΔΔCt method on DataAssist 3.1 software (Thermo Fisher).

**Caspase-3/7 activity assay**

BTSCs or GBM primary cells with different expression levels of Gpr158 (or GPR158) were lysed using lysis buffer (#7018; Cell Signaling). Subsequently, 40 μg of the samples was diluted to a final volume of 150 μl with protease assay buffer (20 mM HEPES (pH 7.5), 10% glycerol, and 2 mM DTT), and supplemented with 20 μM caspase-3 preferred, fluorogenic substrate Ac-DEVD-AMC (#556449, BD Pharmingen) for a 2-h incubation 37 °C in a 96-well plate as per the manufacturer’s instruction. Fluorescence was determined (excitation, 360 nm; emission, 460 nm) with a CytoFluor series 4000 plate reader (Applied Biosystems). Background fluorescence was determined in wells containing the assay buffer only.

**Data acquisition of human gliomas**

Human gliomas used in this study were from The Cancer Genome Atlas (TCGA) or our institution as specified. The TCGA cohort contained 281 LGG and 160 GBM cases, of which the clinical information and RNA sequencing data were downloaded from the TCGA data portal (https://tcga-data.nci.nih.gov/tcga/) in July 2015. IDH mutation and 1p/19q codeletion status of LGG were taken from the dataset, which the clinical information and RNA sequencing data of this data set, and analysed then using GEO2R (https://www.ncbi.nlm.nih.gov/geo/info/geo2r.html).

**IHC staining**

All IHC stainings were carried out on immunostaining instruments (Roche Ventana Discovery or LEICA Bond-Max) following manufacturer’s guidelines. The following antibodies were used in this study: anti-GPR158 (ab121388, Abcam), anti-ATRX (HPA001906, Sigma), anti-IDH1 (ab132147, DIA H09, Dianova), anti-Olig2 (ab33427, abcam), anti-Pdgfrx (ab15501, Abcam), anti-Sox2 (AB 5603, Chemicon) and anti-DCX (ab18732, Abcam).

**Image analysis**

Histological slides were digitized on LEICA SCN400 scanner (LEICA, Milton Keynes UK) at 40× magnification. Digital image analysis was performed on Definiens Developer 2.4 (Munich, Germany). Image analysis was done as previously published [6].

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**Author contributions**

NL and SB conceived and supervised the study. NL, YZ and SB designed experiments. YZ, NL, KS, MI, JL, ARL, LG, TEH and JB performed experiments. NL, YZ, KS, ME and SB analysed data. IE and PF provided reagents and equipment. NL, YZ, KS and SB wrote the manuscript.

**Conflict of interest**

The authors declare that they have no conflict of interest.

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References

1. Parsons DW, Jones S, Zhang X, Lin JC, Leary RJ, Angenendt P, et al. An integrated genomic analysis of human glioblastoma multiforme. Science. 2008;321:1807–12.

2. Louis DN, Perry A, Reifenberger G, von Deimling A, Figarella-Branger D, Cavenee WK, et al. The 2016 World Health Organization Classification of Tumors of the Central Nervous System: a summary. Acta Neuropathol. 2016;131:203–20.

3. Ceccarelli M, Barthel FP, Malta TM, Sabedot TS, Salama SR, Murray BA, et al. Molecular profiling reveals biologically discrete subsets and pathways of progression in diffuse glioma. Cell. 2016;164:550–63.

4. Verhaak RG, Hoadley KA, Purdom E, Wang V, Qi Y, Wilkerson MD, et al. Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRα, IDH1, EGFR, and NF1. Cancer Cell. 2010;17:98–110.

5. Weller M, Pfister SM, Wick W, Hegi ME, Reifenberger G, Stupp R. Molecular neuro-ontology in clinical practice: a new horizon. Lancet Oncol. 2014;14:e370–379.

6. Henriquez NV, Forshew T, Tatevossian R, Ellis M, Richard-Loendt A, Rogers H, et al. Comparative expression analysis reveals lineage relationships between human and murine gliomas and a dominance of glial signatures during tumor propagation in vitro. Cancer Res. 2013;73:5834–44.

7. Jacques TS, Swales A, Brzozowski MJ, Henriquez NV, Linehan JM, Mirzadeh Z, et al. Combinations of genetic mutations in the adult neural stem cell compartment determine brain tumor phenotypes. EMBO J. 2010;29:222–35.

8. Chow LM, Endersby R, Zhu X, Rankin S, Qu C, Zhang J, et al. Cooperativity within and among Pten, p53, and Rb pathways interacts with high-grade astrocytoma in adult brain. Cancer Cell. 2011;19:305–16.

9. Kwon CH, Zhao D, Chen J, Alcantara S, Li Y, Burns DK, et al. Pten haploinsufficiency accelerates formation of high-grade astrocytomas. Cancer Res. 2008;68:3286–94.

10. Zheng H, Ying H, Yan H, Kimmelman AC, Hiller DJ, Chen AJ, et al. p53 and Pten control neural and glial stem/progenitor cell renewal and differentiation. Nature. 2008;455:1129–33.

11. Phillips HS, Kharbanda S, Chen R, Forrest WF, Soriano RH, Wu TD, et al. Molecular subclasses of high-grade glioma predict prognosis, delineate a pattern of disease progression, and resemble stages in neurogenesis. Cancer Cell. 2006;9:157–73.

12. Sturm D, Orr BA, Toprak UK, Hovestadt V, Jones DTW, Capper D, et al. New brain tumor entities emerge from molecular classification of CNS-PNETs. Cell. 2016;164:1060–72.

13. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004;116:281–97.

14. He L, Hannon GJ. MicroRNAs: small RNAs with a big role in gene regulation. Nat Rev Genet. 2004;5:522–31.

15. Pham JT, Gallicano GI. Specification of neural cell fate and regulation of neural stem cell proliferation by microRNAs. Am J Stem Cells. 2012;1:182–95.

16. Chen SP, Liu BX, Xu J, Pei XF, Liao YJ, Yuan F, et al. MiR-449a suppresses the epithelial-mesenchymal transition and metastasis of hepatocellular carcinoma by multiple targets. BMC Cancer. 2015;15:706.

17. Yao Y, Ma J, Xue Y, Wang P, Li Z, Li Z, et al. MiR-449a exerts tumor-suppressive functions in human glioblastoma by targeting Mbc-associated zinc-finger protein. Mol Oncol. 2015;9:640–56.
36. Tan SM, Kirchner R, Jin J, Hofmann O, McReynolds L, Hide W, et al. Sequencing of captive target transcripts identifies the network of regulated genes and functions of primate-specific miR-522. Cell Rep. 2014;8:1225–39.

37. Pratt AJ, MacRae JI. The RNA-induced silencing complex: a versatile gene-silencing machine. J Biol Chem. 2009;284:17897–901.

38. Pollard SM, Yoshikawa K, Clarke ID, Danovi D, Stricker S, Russell R, et al. Glioma stem cell lines expanded in adherent culture have tumor-specific phenotypes and are suitable for chemical and genetic screens. Cell Stem Cell. 2009;4:568–80.

39. Hu Y, Smyth GK. ELDA: extreme limiting dilution analysis for comparing depleted and enriched populations in stem cell and other assays. J Immunol Methods. 2009;347:70–78.

40. Fernando P, Brunette S, Megeney LA. Neural stem cell differentiation is dependent upon endogenous caspase 3 activity. FASEB J. 2005;19:1671–3.

41. Brown JP, Couillard-Despres S, Cooper-Kuhn CM, Winkler J, Aigner L, Kuhn HG. Transient expression of doublecortin during adult neurogenesis. J Comp Neurol. 2003;467:1–10.

42. Reuss DE, Mamattaj Y, Schirmpf D, Capper D, Hovestadt V, Kratz A, et al. IDH mutant diffuse and anaplastic astrocytomas have similar age at presentation and little difference in survival: a grading problem for WHO. Acta Neuropathol. 2015;129:867–73.

43. Vigneswaran K, Neill S, Hadjipanayis CG. Beyond the World Health Organization grading of infiltrating gliomas: advances in the molecular genetics of glioma classification. Ann Trans Med. 2015;3:95.

44. Reuss DE, Kratz A, Sahm F, Capper D, Schirmpf D, Koelsche C, et al. Adult IDH wild type astrocytomas: biological and clinical resolution into other tumor entities. Acta Neuropathol. 2015;130:407–17.

45. Dahia PL. Pheochromocytoma and paraganglioma pathogenesis: learning from genetic heterogeneity. Nat Rev Cancer. 2014;14:108–19.

46. Lize M, Klimke A, Dobbelstein M. MicroRNA-449 in cell fate determination. Cell Cycle. 2011;10:2874–82.

47. Redshaw N, Wheeler G, Hajhosseini MK, Dalmay T. microRNA-449 is a putative regulator of choroid plexus development and function. Brain Res. 2009;1250:20–26.

48. Sun F, Fu H, Liu Q, Tie Y, Zhu J, Xing R, et al. Downregulation of CCND1 and CDK6 by miR-34a induces cell cycle arrest. FEBS Lett. 2008;582:1564–8.

49. Welch C, Chen Y, Stallings RL. MicroRNA-34a functions as a potential tumor suppressor by inducing apoptosis in neuroblastoma cells. Oncogene. 2007;26:5017–22.

50. Khrimian L, Obri A, Ramos-Brossier M, Rousseaud A, Moriceau S, Nicot AS, et al. Gpr158 mediates osteocalcin secretion and overexpression of cyclin D1 in human hepatocellular carcinoma. Biochem Biophys Res Commun. 1993;196:1010–6.

51. Siddiki H, McAluliffe J, Fael Al-Mayhani TM, Ball SL, Zhao JW, Ichimura K, Fawcett J, et al. Amplification and overexpression of cyclin D1 in human hepatocellular carcinoma. Biochem Biophys Res Commun. 2007;347:459–64.

52. Jiang W, Kahn SM, Tomita N, Zhang YJ, Lu SH, Weinstein IB. Transcriptome analysis of miRNA in developing cerebral cortex of rat. BMC Genom. 2012;13:232.

53. Jiang W, Kahn SM, Tomita N, Zhang YJ, Lu SH, Weinstein IB. Amplification and expression of the human cyclin D gene in esophageal cancer. Cancer Res. 1992;52:2980–3.