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ORIGINAL ARTICLE

Pro-neural miR-128 is a glioma tumor suppressor that targets mitogenic kinases

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MicroRNAs (miRNAs) carry out post-transcriptional control of a multitude of cellular processes. Aberrant expression of miRNA can lead to diseases, including cancer. Gliomas are aggressive brain tumors that are thought to arise from transformed glioma-initiating neural stem cells (giNSCs). With the use of giNSCs and human glioblastoma cells, we investigated the function of miRNAs in gliomas. We identified pro-neuronal miR-128 as a candidate glioma tumor suppressor miRNA. Decreased expression of miR-128 correlates with aggressive human glioma subtypes. With a combination of molecular, cellular and *in vivo* approaches, we characterize miR-128's tumor suppressive role. miR-128 represses giNSC growth by enhancing neuronal differentiation. miR-128 represses growth and mediates differentiation by targeting oncogenic receptor tyrosine kinases (RTKs) epithelial growth factor receptor and platelet-derived growth factor receptor-α. Using an autochthonous glioma mouse model, we demonstrated that miR-128 repressed gliomagenesis. We identified miR-128 as a glioma tumor suppressor that targets RTK signaling to repress giNSC self-renewal and enhance differentiation.
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Introduction

In settings as diverse as malignant transformation to stem cell differentiation, when cells change their identities, epigenetic mechanisms mediate and finalize these new cellular identities. Among these epigenetic mechanisms are micro RNAs (miRNAs), small non-coding RNAs, which bind to target mRNAs to repress translation (Filipowicz *et al*., 2008). The partial complementarity of the miRNA/mRNA duplex allows for miRNAs to target multiple mRNAs of genes that may share in the regulation of common cellular processes (Lewis *et al*., 2003; Papagiannakopoulos *et al*., 2008).

Gliomas are the most common primary tumors in the central nervous system. They arise *de novo* or progress from lower grade to higher grade over time (Furnari *et al*., 2007; Stiles and Rowitch, 2008). The treatment strategies for this disease have not changed appreciably and are based on a limited understanding of the basic biology. Recent work has assisted in classification of glioma tumors (Network TCGA, 2008; Verhaak *et al*., 2010). Among the key classifiers of aggressive glioma subtypes are mitogenic receptor tyrosine kinases (RTKs), epithelial growth factor receptor (EGFR) and platelet-derived growth factor receptor alpha (PDGFRα; Verhaak *et al*., 2010).

Amplification and/or activating mutations in *EGFR* and *PDGFRα* are found in aggressive gliomas (Furnari *et al*., 2007). PDGF signaling regulates survival and mitogenic pathways in gliomas and contributes to brain tumor development (Dai *et al*., 2001; Shih *et al*., 2004; Jackson *et al*., 2006). Mutant *EGFRviii* (deletion of exons 2–7) increases cell survival and proliferation (Bachoo *et al*., 2002).

Gliomas are tumors with high cellular complexity. Work on cancer stem cells has shed light on the origin and complexity of gliomas (Singh *et al*., 2004; Furnari *et al*., 2007). According to the cancer stem cell hypothesis, neural stem cells (NSCs) can acquire oncogenic lesions that transform the cells to glioma-initiating NSCs (giNSCs; Stiles and Rowitch, 2008). EGFRviii and PDGFRα are candidate oncogenic lesions in NSCs (Dai *et al*., 2001; Bachoo *et al*., 2002; Jackson *et al*., 2006). Transformation of NSCs comes with a cost to their normal asymmetric division, which is balanced between self-renewal (proliferation) and

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differentiation. Upon NSC transformation, the balance tips from differentiation to self-renewal/proliferation (Furnari *et al*., 2007; Stiles and Rowitch, 2008). Targeting cancer stem cells by restoring the balance towards differentiation is considered a potential therapeutic approach in gliomas (Piccirillo *et al*., 2006).

miRNAs are emerging as major regulators of gene networks, which are dysregulated in diseases, including cancer (Ciafrè *et al*., 2005; Lu *et al*., 2005; Conti *et al*., 2009; Huse *et al*., 2009; Hatley *et al*., 2010). They can mediate either proliferation or differentiation of stem cells (Cheng *et al*., 2009; Gangaraju and Lin, 2009; Xu *et al*., 2009; Neveu *et al*., 2010), and are candidates for maintaining the normal balance of asymmetric stem cell divisions (Gangaraju and Lin, 2009; Xu *et al*., 2009; Neveu *et al*., 2010). In NSCs, they can implement a neuronal differentiation program (Krichevsky *et al*., 2003, 2006; Conaco *et al*., 2006; Visvanathan *et al*., 2007; Cheng *et al*., 2009). miR-128 is a neuronal-enriched miRNA whose expression correlates with central nervous system development (Krichevsky *et al*., 2003, 2006) and is repressed in gliomas (Ciafrè *et al*., 2005; Godlewski *et al*., 2008; Conti *et al*., 2009; Zhang *et al*., 2009). However, apart from the study of miR-128 in human glioblastoma cell lines (Godlewski *et al*., 2008; Zhang *et al*., 2009; Wuchty *et al*., 2011), little is known about miR-128 mechanism of function in giNSCs in culture and *in vivo*.

In this study, we identify miR-128 as a tumor suppressive miRNA that is globally downregulated in human glioma and mouse model tumors. miR-128 classifies aggressive/dedifferentiated glioma subtypes. Additionally, we validated the tumor suppressive function of miR-128 by using several genetically defined giNSC experimental systems. We establish the pro-neuronal function of miR-128 that can divert giNSCs from proliferation to differentiation by directly targeting oncogenic kinases EGFR and PDGFRα. Finally, using a robust glioma mouse model, we demonstrate the tumor suppressive potential of miR-128.

Results

miR-128 is repressed in gliomas

To identify candidate tumor suppressive miRNAs that are dysregulated in gliomas, we analyzed our previously published miRNA expression data from human glioma tumors (Liu *et al*., 2007). We were able to identify a set of miRNAs whose levels were significantly decreased in tumors (data not shown). Among these miRNAs, the expression of miR-128 was dramatically decreased in glioma tumors, which is in agreement with previous studies (Ciafrè *et al*., 2005; Godlewski *et al*., 2008). Additionally, miR-128 expression was lower in advanced high-grade compared with low-grade gliomas (Figure 1a).

We then analyzed human glioma tumor miRNA data from TCGA(Network TCGA, 2008). Interestingly, when classifying glioma tumors using a previously published approach (Verhaak *et al*., 2010), we were able to obtain sets of miRNAs that defined tumor subtypes (Figure 1b). miR-128-1 was one of the top miRNAs that could classify the least aggressive 'Pro-Neural'/'Neural' from the aggressive 'Classical' and 'Mesenchymal' glioma subtypes. Specifically, miR-128-1 expression decreased in aggressive classical tumor subtypes, compared with the more differentiated and less aggressive 'Neural' tumor subtypes (Figure 1c). The decreased levels of miR-128 in 'Classical' tumor subtypes relative to 'Neural' subtypes suggested a pro-neuronal differentiation and anti-proliferative role of miR-128.

miR-128 represses giNSC growth

giNSC present a good model for studying glioma initiation and progression events in culture. To study the potential role of miR-128 as a tumor suppressor in gliomas, we used NSCs lacking tumor suppressor genes, *p16*/*p19* that are transformed to giNSCs by oncogenic *EGFRviii* (Figure 2a and Supplementary Figure S1; Bachoo *et al*., 2002). Loss of *p16*/*p19* and amplification and/or mutation of *EGFR* are lesions commonly found in the aggressive 'classical' tumor subtypes that express low miR-128 (Figure 1b and c). This model offers the potential to study the impact of miR-128 on growth and differentiation of genetically defined primary giNSCs.

We initially assayed the levels of miR-128 in non-tumorigenic NSCs (*p16*/*p19*−/−) and tumorigenic giNSCs (*p16*/*p19*−/−; + *EGFRviii*; Figure 2a). Interestingly, miR-128 was significantly repressed in tumorigenic giNSCs, suggesting a potential function of miR-128 loss in the transformation of NSCs (*p19*/*p19*−/−) by *EGFRviii* (Figure 2b). In addition, we assayed the levels of miR-128 in glioma tumors generated from giNSCs and found that miR-128 was significantly decreased (Figure 2c). Our results identify miR-128 as a putative tumor suppressor miRNA that is repressed by oncogenic EGFRviii signaling in giNSCs.

To study the role of miR-128 in the transformation of EGFRviii-positive giNSCs, we introduced increasing amounts of miR-128 and assayed the growth of the cells over 6 days (Figure 2d). miR-128 overexpression levels were close to those observed in non-transformed NSCs (Supplementary Figure S1b). We observed a significant dose-dependent repression in giNSC growth. giNSCs contain high S-phase (DNA replication) content, which is critical for their self-renewal. Therefore, to test the effect of miR-128 on giNSC replication, we assayed BrdU incorporation in the control and miR-128 expressing cells. We observed a 25% decrease in BrdU-positive cells in miR-128-expressing giNSCs (Figure 2e).

We next tested miR-128 tumor suppressive function in a different set of glioma-initiating stem cells from a *p53*−/−; *NF1*−/− mouse genetic mosaic model that matches the pro-neural subtype of human glioblastoma multiformes (Liu *et al*., 2011). giNSCs in this model manifest salient features of oligodendrocyte precursor cells and can effectively initiate secondary glioma tumors upon orthotopic injections. When we introduced miR-128 in these cells, we observed significant repression in growth, assayed by EdU incorporation in S-phase. Furthermore, miR-128 repressed the growth of these

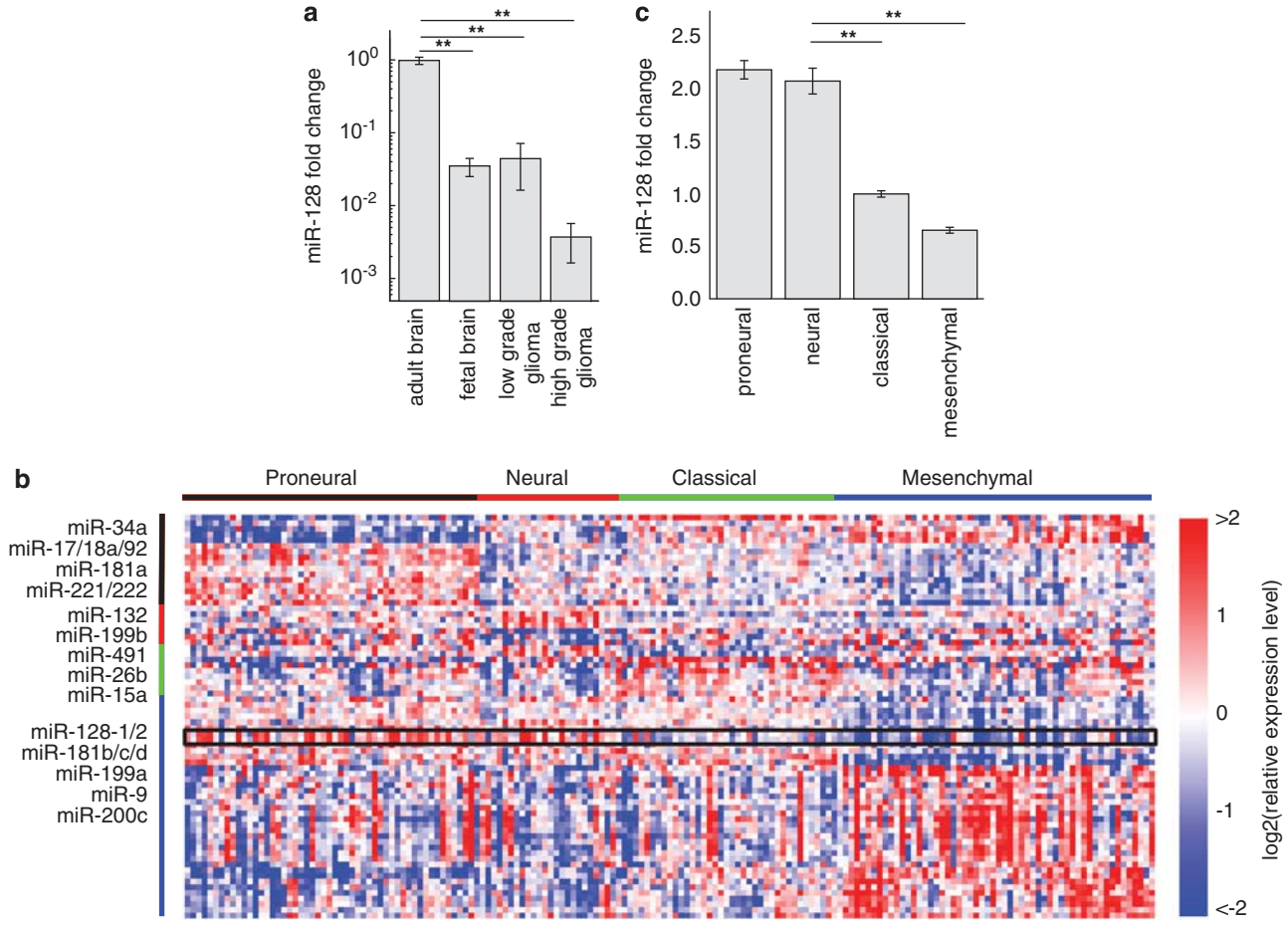


Figure 1 miR-128 expression in gliomas. (**a**) Relative expression levels of miR-128 expression in fetal brain, high-grade and low-grade glioma tumors compared with adult brain. Assayed by Taqman Realtime PCR (Applied Biosystems, Foster City, CA, USA). (**b**) Cluster analysis of miRNA expression in glioma subtypes from TCGA database. Proneural (black), Neural (red), Classical (green) and Mesenchymal (blue) subdivisions of glioma tumor samples are on the horizontal axis. Classifying miRNAs are along the vertical axis, color bars indicate the subtype that each cluster of miRNAs classifies. A subset of representative miRNAs is listed by each classifying miRNA cluster. Black box indicates the expression levels of miR-128-1/128-2 in all glioma subtypes. (**c**) Expression of miR-128 in Pro-Neural, Neural and Mesenchymal glioma subtypes relative to classical glioma subtypes. Student *t*-test, $*P<0.05$, $**P<0.01$. The error bar represents the s.d.

cells under different growth factor (PDGF or EGF) conditions (Figure 2f).

These functional studies performed by overexpression of miR-128 in two different sets of genetically defined primary giNSCs, produced by manipulating two different oncogenic systems, demonstrate that the tumor suppressive potential of miR-128 is a common feature. We observed less growth repression in the pro-neuronal *p53−/−;NF1−/−* cells, which likely reflects the expression levels of miR-128 in pro-neural compared with the classical glioma subtypes (Figure 1c).

miR-128 represses human glioblastoma cell growth

In addition to giNSCs, we further examined the effect of miR-128 on the growth of human glioblastoma cell line U251. Upon stable overexpression of miR-128, we observed a significant disruption in the growth of U251 cells over 6 days (Figure 3a). To determine the mechanism of miR-128 growth repression, we assayed cell cycle content, as well as apoptosis. We observed a significant increase in U251 cells arrested in G0/G1 phase of the cell cycle upon miR-128 overexpression (Figure 3b). A significant increase in apoptosis and death in U251 cells after 24 and 48 h also occurred (Figure 3c).

Our results demonstrate the ability of miR-128 to suppress U251 cell growth. To test whether miR-128 was able to suppress the growth of U251 cells injected subcutaneously in immunodeficient mice, we compared the tumor growth of U251 expressing miR-128 or control (mock). By monitoring tumor volume and weight, we observed a significant repression in the growth of miR-128 expressing U251 cells compared with the control (Supplementary Figures S1b and c). These results, in addition to the giNSC data, further support the conserved tumor suppressive role of miR-128 in glioma cells.

miR-128 target mitogenic kinase signaling

Our data strongly suggests that miR-128 has tumor suppressor properties. To determine the mechanism of action of miR-128, we compiled a list of predicted targets

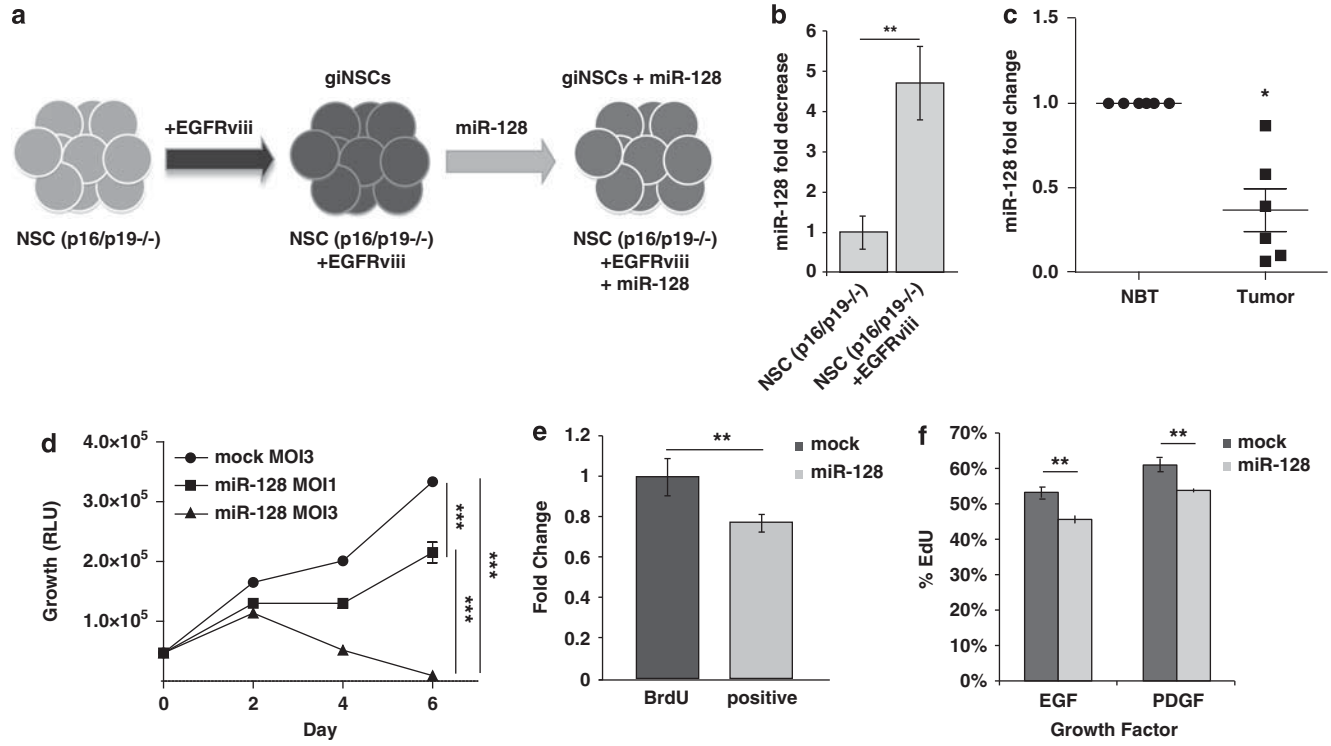


Figure 2 Assessing the function of miR-128 in giNSCs. (**a**) Schematic of experimental manipulations of NSCs used to study miR-128 function. (**b**) Taqman RealTime PCR data showing the relative decrease of miR-128 in *EGFRviii*-expressing NSCs (*p16/p19−/−*) (giNSCs). Data normalized using U6 expression. Student's *t*-test compared two data sets marked by brackets in the panel. $^{**}P<0.01$. The error bar represents the s.e.m. from three independent experiments, using different *EGFRviii*-positive clones. (**c**) Relative expression change of miR-128 in tumors generated by NSCs *(p16/p19−/−) + EGFRviii* compared with normal brain tissue (NBT). Tumors and NBT collected 60–80 days after intracranial injection. Data normalized using U6 expression. Student's *t*-test compared two data sets marked by brackets in the panel. $^{**}P<0.01$. The error bar represents the s.e.m. from six animals/group. (**d**) Six-day growth assay of NSCs *(p16/p19−/−) + EGFRviii* cells. Cells expressing increasing amounts of miR-128 by lentivirus as indicated by multiplicity of infection (MOI) and cells infected with control (mock) virus. A two-way analysis of variance compared two data sets marked by brackets in the panel. $^{***}P<0.001$. (**e**) Proliferation of NSCs (*p16/p19−/−) + EGFRviii* tested by BrdU incorporation. (**f**) Proliferation of NSCs (*p53−/−;NF1−/−*) assayed by EdU incorporation under different growth factor conditions (PDGF, EGF). Student *t*-test. $^{**}P<0.01$ The error bar represents the s.d. from three independent experiments.

that contain putative miR-128 binding sites in their 3′-UTRs (TargetScan, http://www.targetscan.org/). We then looked for gene ontology (GO)-term enrichment among the predicted targets. Among the topmost statistically significant GO terms was tyrosine kinase activity ($P<10^{-10}$) and RTK activity (Figure 4a). miR-128 is one of the top five miRNAs predicted to target the tyrosine kinase activity GO term (data not shown). Within the list of predicted targets with tyrosine kinase activity are some well-studied oncogenes involved in mitogenic tyrosine kinase signaling in many different cancers including gliomas (Supplementary Tables 1a and b). RTKs, which are essential for glioma growth, were a significant subset of the predicted targets (Figure 4a), including EGFR and PDGFRα. Furthermore, in addition to the unbiased GO analysis, we found that oncogenic tyrosine kinases MET and ABL1, and genes involved in relaying RTK signaling, such as *GRB2* and *SOS1*, also predicted targets of miR-128. Thus, our target prediction GO analysis suggests that miR-128 is a putative regulator of mitogenic tyrosine kinase signaling.

Therefore, we assessed whether global tyrosine phosphorylation is modulated by miR-128. giNSCs have high levels of phospho-tyrosine (p-Tyr), as they express EGFRviii, a constitutively active form of EGFR (Supplementary Figure S2a). Intriguingly, addition of miR-128 in giNSCs and U251 cells led to a global decrease in the overall p-Tyr content (Figure 4b and Supplementary Figure S2b). These data suggested that miR-128 is a robust negative regulator of mitogenic p-Tyr signaling.

We then validated a subset of the predicted targets (EGFR, PDGFRα, ABL1, SOS1, GRB2 and MAPK14) that we identified from our GO enrichment analysis. In particular, we focused on PDGFRα and EGFR, two RTKs with a well-established oncogenic role in gliomas. To determine that these are *bona fide* targets of miR-128, we assayed the levels of the target 3′-UTR reporters upon upregulation (pre-miR-128) or downregulation (LNA-miR-128) of miR-128. The appropriate decrease and increase in the levels of all four 3′-UTR reporters was statistically significant with overexpression and downregulation of miR-128, respectively (Figures 4d and e, and Supplementary Figures S3a and b). Furthermore, mutation of the miRNA binding sites in the target 3′-UTR led to non-responsive 3′-UTRs upon dysregulation of miR-128 (Figures 4d and e).

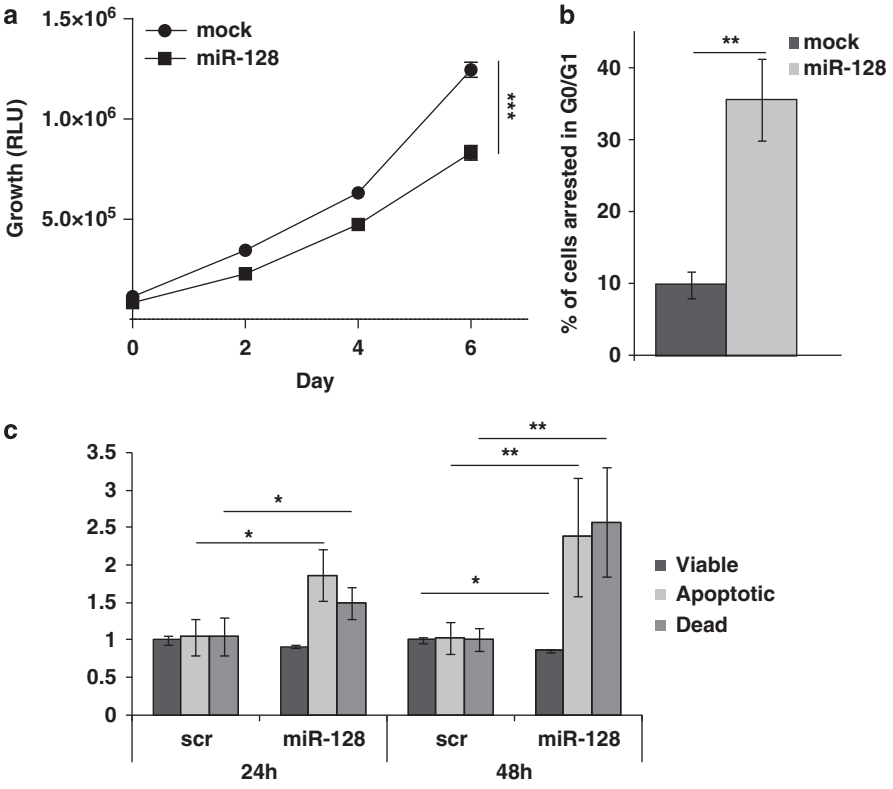


Figure 3 Assessing the function of miR-128 in human glioblastoma U251 cells. (**a**) Six-day growth assay of U251 cells. Cells expressing miR-128 or control (mock) by lentivirus. A two-way ANOVA compared two data sets marked by brackets in the panel. $***P<0.0001$. The error bar represents the s.d. from three independent experiments. (**b**) Percentage of mock or miR-128-infected U251 cells arrested in G0/G1 after synchronization with Nocodazole (200 ng/ml). Student's *t*-test compared two data sets marked by brackets in the panel. $**P<0.01$. The error bar represents the s.d. from three independent experiments. (**c**) Assaying Viable, Apoptotic and Dead cell populations assayed 24 h and 48 h post-transfection, with control oligo (scr) or miR-128 oligos. Student's *t*-test compared two data sets marked by brackets in the panel. $*P<0.05$, $**P<0.01$. The error bar represents the s.d. from three independent experiments.

To assay the regulation of the 3′-UTRs by endogenous miR-128, we introduced the 3′-UTRs of PDGFRα and EGFR in the giNSCs. We observed a derepression of the 3′-UTRs, containing the mutated miR-128 sites. These results clearly demonstrate that miR-128 can directly target EGFR and PDGFRα by binding to their 3′-UTRs and repressing translation (Figure 4e).

To test the effect of miR-128 on the endogenous levels of its targets, we introduced miR-128 in giNSCs and U251 cells. We observed a significant repression of EGFR and PDGFRα targets in the presence of miR-128 in both U251 and giNSCs (Figure 4g). In addition, we assayed for active Akt, an oncogenic serine/threonine kinase that is activated by RTK signaling in gliomas. Active p-Akt (S438), but not total Akt, decreased in the presence of miR-128 (Figure 4g).

miR-128 enhances neuronal differentiation of giNSCs

NSCs have the capacity to differentiate into glia and neurons. NSC differentiation in culture can be mediated by withdrawal of growth factors (fibroblast growth factor (FGF)/epithelial growth factor (EGF), which are required for NSC self-renewal and growth. Therefore, differentiation of NSCs is coupled with loss of mitogenic growth factor signaling. It has been previously demonstrated that EGFRviii-positive NSCs are less efficient at differentiating when compared with wild-type NSCs (Boockvar *et al*., 2003). PDGFRα and EGFR, which are involved in the growth and maintenance of NSCs, are also known to repress neuronal differentiation of NSCs (Doetsch *et al*., 2002; Boockvar *et al*., 2003; Jackson *et al*., 2006).

Our results have established that miR-128 can repress mitogenic signaling mediated by EGFRviii in giNSCs, by targeting EGFR and PDGFRα. We hypothesized that in addition to repressing the growth of giNSCs, miR-128 may also enhance giNSCs differentiation.

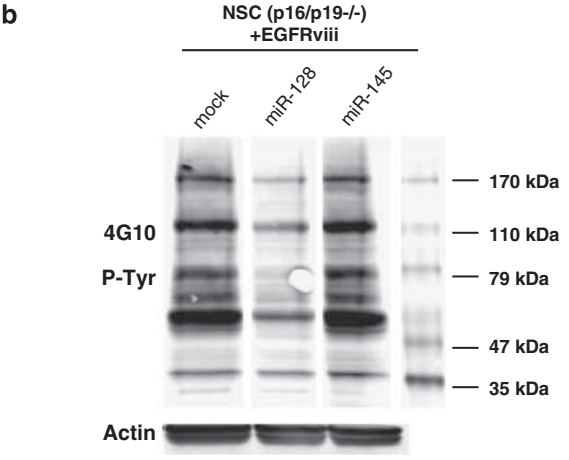
giNSCs, differentiated (3 days) in the absence of FGF/EGF, gave rise to neurons and astrocytes as expected (Supplementary Figure S4a). During the differentiation process, we observed an increase in miR-128 (data not shown), associated with a decrease in total p-Tyr levels (Supplementary Figure S4b). Therefore, the reverse correlation of miR-128 and tyrosine kinase signaling during differentiation, suggests that miR-128 may be assisting in differentiation by repressing mitogenic tyrosine signaling.

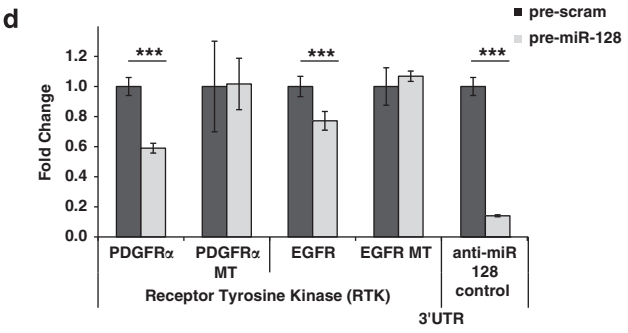
a

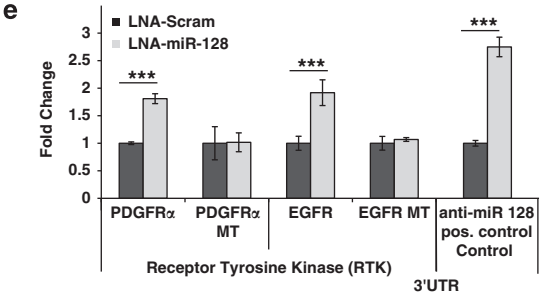
Gene Ontology (GO)	P-value
Protein tyrosine kinase activity	$<10^{-10}$
Protein serine/threonine kinase activity	$<10^{-9}$
Transmembrane receptor protein tyrosine kinase (RTK) activity	<0.0006

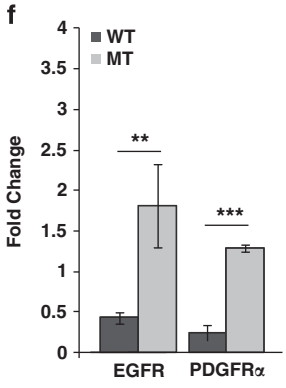
c

Predicted Target	Pairing of miRNA/Target
Position 2724-2730 of **PDGFRA** 3' UTR	5' . . . UUUAUAUUUUUUUUAACUGUGAU . . .
miR-128	3' UUUCUCUGGCCAAGUGACACU
Position 200-206 of **EGFR**3' UTR	5' . . UCAAUUGAUCUUCGAACUGUGAA . . .
miR-128	3' UUUCUCUGGCCAAGUGACACU









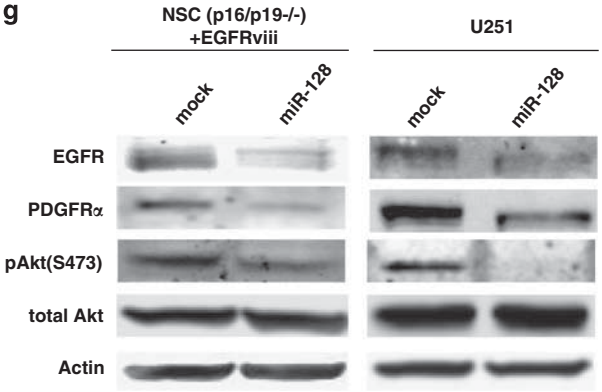


Figure 4 miR-128 targets mitogenic tyrosine kinases. (**a**) miR-128-predicted targets show an enrichment in tyrosine kinase and RTK GO terms. (**b**) Western blot indicating p-Tyr levels detected by 4G10, NSCs (p16/p19−/−) + EGFRviii expressing a mock, miR-128 or miR-145 lentivirus. (**c**) Summary of miR-128 predicted targets, including the conserved miR-128 predicted sites in the 3′-UTRs EGFR and PDGFRα. The underlined gray box nucleotides (target sites) were deleted in the mutant 3′-UTR constructs. Sites conserved in mouse and human 3′-UTRs. (**d**) miR-128 specifically represses its targets in the luciferase assay in HEK cells. Fold change = $(S_{luc}/S_{renilla})/(C_{luc}/C_{renilla})$. Luc, raw firefly luciferase activity; Renilla, internal transfection control renilla activity, MT, mutant UTRs. (**e**) miR-128 repression (LNA-128) specifically derepresses its targets in the luciferase assay in HEK cells. (**f**) Effects of endogenous miR-128 on targets. Wildtype (WT) and mutant (MT) target 3′-UTRs were introduced in giNSCs. Student's *t*-test compared two data sets marked by brackets in the panel. $*P<0.05$, $**P<0.01$, $***P<0.001$. The error bar represents the s.d. from three independent experiments. (**g**) Western Blot indicating protein levels of EGFR, PDGFR, pAkt (S473), total Akt and actin in mock or miR-128-expressing giNSCs and U251 cells.

To test the potential of miR-128 to induce a differentiation program in giNSCs as a result of mitogenic signaling repression, we initially overexpressed miR-128 in giNSCs, and monitored the changes in differentiation markers under self-renewal conditions (FGF/EGF present). We assayed the protein levels of well-established NSC and differentiation markers. The levels of both NSC markers, Nestin and Sox2 decreased significantly upon addition of miR-128 (Figure 5a). In addition, the astrocytic marker GFAP decreased significantly and the

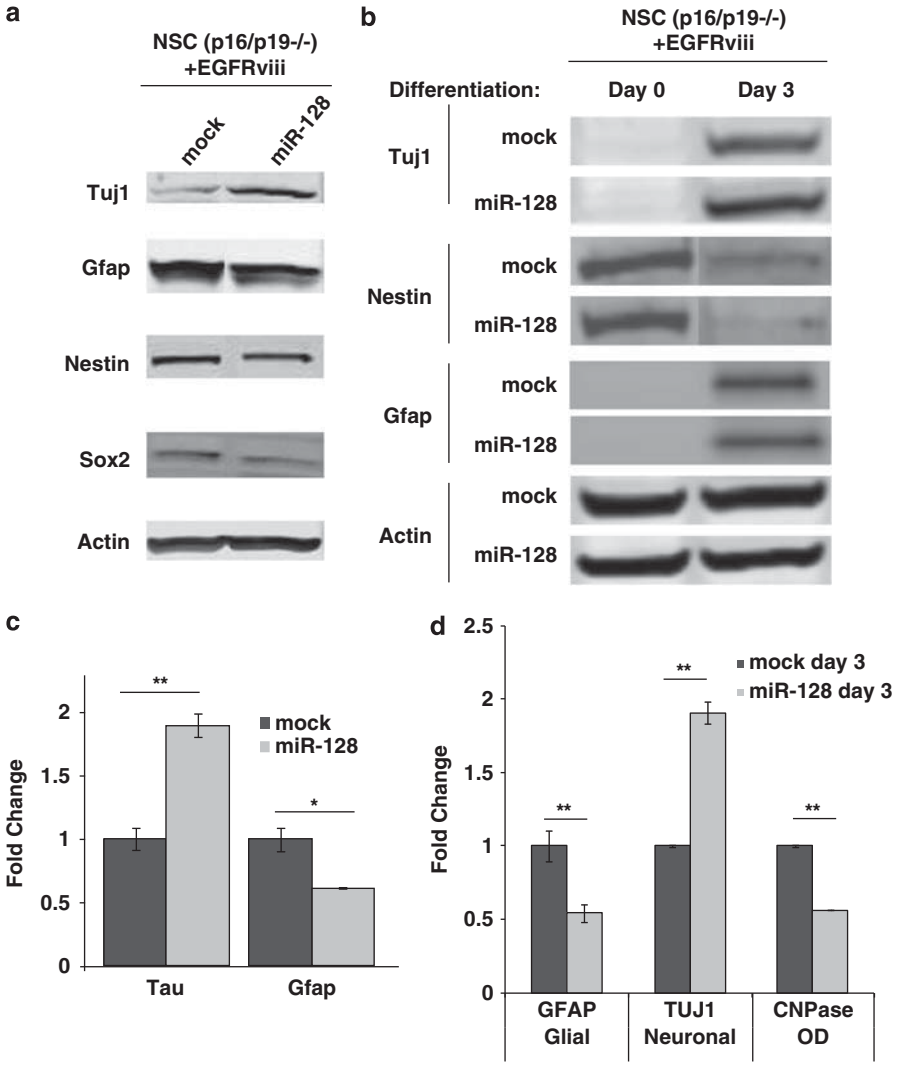


Figure 5 miR-128 enhances differentiation of giNSCs. (**a**) NSC (Nestin/SOX2), neuronal (Tuj1) and glial (GFAP) markers assayed by western blotting in giNSCs-expressing control (mock) or miR-128. Cells maintained in self-renewal conditions with FGF/EGF. (**b**) Markers assayed by western blotting in mock or miR-128 expressing giNSCs differentiated over 3 days in the absence of growth factors. (**c**) Neuronal marker Tau and glial marker GFAP assayed by flow cytometry in mock and miR-128 giNSCs differentiated for 3 days. (**d**) Astrocytic (GFAP), Neuronal (Tuj1) and Oligodendrocyte (ON, CNPase) markers assayed by RealTime PCR in mock and miR-128 giNSCs after 3 days of differentiation. Student's *t*-test compared two data sets marked by brackets in the panel. $*P<0.05$, $**P<0.01$. The error bar represents the s.d. from three independent experiments.

neuronal marker Tuj1 increased significantly (Figure 5a). These data support the hypothesis that miR-128 may be exerting its anti-proliferative role by promoting the differentiation of the cells.

To further elucidate the potential role of miR-128 in diverting tumorigenic giNSCs towards differentiation, we tested whether miR-128 enhances the differentiation of giNSCs under differentiation conditions, that is, absence of FGF/EGF. We differentiated the cells and checked the expression of NSC and differentiation markers after 3 days in cells expressing miR-128 and control (mock). giNSC expressing miR-128 contained less Nestin and GFAP after 3 days of differentiation, but expressed higher levels of the neuronal marker Tuj1 (Figure 5b). Furthermore, by flow cytometry, we confirmed this pro-neuronal effect of miR-128 by assaying the percentage of glial and neuronal cells upon differentiation. We observed a significant increase in the neuronal marker microtubule-associated protein tau (Tau) and a decrease in GFAP in miR-128 expressing giNSCs (Figure 5c). We further validated this result by analyzing mRNA expression levels of differentiation markers. As expected, we observed higher levels of neuronal markers Tuj1 and less expression of GFAP and oligodendrocyte marker 2′, 3′-cyclic nucleotide 3′-phospho-diesterase (CNPase) (Figure 5d).

These data indicate that miR-128 enhances differentiation of giNSCs and tends to direct the cells toward a neuronal fate. Overall, these data support the idea that miR-128 is a pro-neuronal miRNA, which enhances neuronal differentiation.

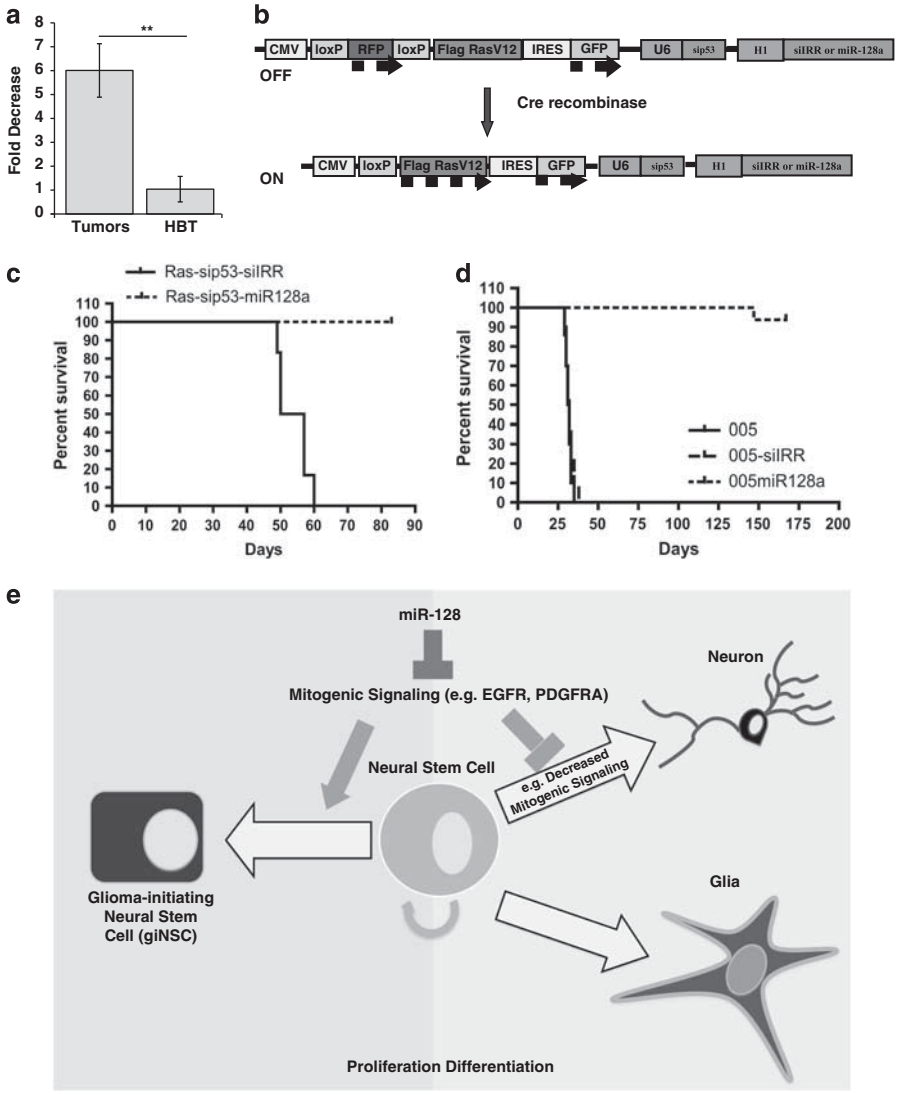


Figure 6 miR-128 tumor suppression in a glioma mouse model. (**a**) Taqman RealTime PCR of miR-128 levels assayed in normal brain tissue (NBT) and glioma tumors from H-RasV12/sip53 animals ($n=6$, error bar represents the s.e.m.), $^{**}P<0.01$. (**b**) Schematic representation of glioma-initiating system with co-expression of control shRNA (siIRR) or miR-128 from an H1 promoter. A cassette containing H-RasV12 and shRNA against p53 (sip53) is activated (ON) in cre-expressing GFAP-positive cells. (**c**) Kaplan–Meier survival curve (90 days) of animal expressing the tumor initiating cassette in addition to a control shRNA (siIRR; $n=8$) or mmu-miR-128a ($n=6$). (**d**) Kaplan–Meier survival curve (175 days) of non-obese diabetic–severe combined immunodeficient (NOD-SCID) mice injected with 005 giNSCs ($n=15$), 005 cells that express a control shRNA (siIRR; $n=15$) or mmu-miR-128a ($n=15$). Gehan–Breslow–Wilcoxon and Log-rank (Mantel–Cox) test, $P<0.0001$. (**e**) Model of miR-128 tumor suppressive action. miR-128 promoted a shift towards a differentiation fate by repressing the self-renewal/proliferation of giNSCs.

miR-128 suppresses glioma formation in a glioma mouse model

To further assess the tumor suppressive function of miR-128 *in vivo*, we utilized an autochthonous glioma mouse model that arises from activation of oncogenic Harvey-RasV12 (H-RasV12) and loss of p53 (Marumoto *et al*., 2009). Glioma tumors were initiated by lentiviral transduction of cells in the hippocampus of GFAP-cre mice. The lentiviral construct becomes active in GFAP-cre cells, driving the expression of H-RasV12 in a p53 loss-of-function background (p53+/− or shRNA-p53 (sip53)). All transduced animals developed glioma tumors, which resembled human gliomas histopathologically (Marumoto *et al*., 2009).

Initially, we determined the levels of miR-128 in the glioma model tumors. We observed a significant decrease in the levels of miR-128 in the tumors (Figure 6a). Furthermore, we observed higher levels of PDGFRα in tumors, compared with normal brain tissue (Supplementary Figure S3c). Decreased levels of miR-128 in tumors might be necessary for tumor growth. Thus, we tested the role of miR-128 in repressing glioma tumor growth in this model. We introduced miR-128 along with H-RasV12 and sip53, and assayed for survival of transduced animals (Figure 6b). We observed a striking extension of survival in animals expressing miR-128, as compared with our control (siIRR; Figure 6c). Histologically, we did not observe any

tumors in animals treated with miR-128 (data not shown).

giNSCs (005 cells) isolated from glioma tumors from this mouse model are able to form tumors when injected in the hippocampus of non-obese diabetic–severe combined immunodeficient mice (Marumoto *et al*., 2009). Addition of miR-128 to 005 giNSCs represses the levels of miR-128 target PDGFRα (Supplementary Figure S3d). We then tested whether miR-128 can repress the tumor-initiating potential of 005 cells, and observed that miR-128 suppressed glioma formation by 005 cells *in vivo* as compared with control 005-siRR cells (Figure 6d).

This *in vivo* data validates the tumor suppressive potential of miR-128 in gliomas. miR-128 repressed tumor growth in a robust model of gliomagenesis. Furthermore, miR-128 was able to repress the tumor-initiating potential of 005 giNSCs derived from the glioma mouse model.

Discussion

Among a set of clinical samples, a correlation exists between decreased expression of miR-128 and glioma tumors; furthermore, miR-128 expression was lower in advanced high-grade than low-grade gliomas among the TCGA samples. On the basis of these correlational data, we have linked miR-128 to an extensive control system, involving RTKs and their downstream effectors, and demonstrated salutary effects of raising miR-128 levels on tumor models. As miR-128 decreases in gliomas, they increasingly lose features of differentiation and concomitantly become more aggressive. Populations of cells within tumors have been compared with stem cells, with regard to their twin capacity to both differentiate and self renew (Jackson *et al*., 2006; Piccirillo *et al*., 2006; Stiles and Rowitch, 2008). Interestingly, neuronal differentiation through stages that include neural precursors and ultimately terminally differentiated neurons is accompanied by an increase in miR-128 (Krichevsky *et al*., 2003, 2006), a finding that extends the comparison of anti-parallel tracks leading in one direction to poorly differentiated gliomas, and in the other direction, to differentiated neurons. These findings lead to the hypothesis that driving glioma cells toward differentiation with miR-128 represents a possible therapeutic approach.

The results described here support the potential role of miR-128 as a pro-differentiation/anti-proliferative miRNA. miR-128 repressed the proliferative and tumorigenic potential of genetically defined primary giNSCs, containing well-characterized human glioma-like features (NF1−/−; p53−/− and EGFRviii; p16/p19−/−; Figures 2 and 6d; Verhaak *et al*., 2010). In addition to its role in repressing giNSC growth, miR-128 enhanced neuronal differentiation of malignant giNSCs (Figure 5).

Unlike healthy NSCs, EGFRviii-expressing giNSCs are unable to differentiate properly (Boockvar *et al*., 2003); therefore they lose asymmetric division balance and primarily proliferate. Differentiation factors such as miR-128 are decreased in giNSCs as part of an EGFRviii oncogenic program to suppress differentiation and promote proliferation. By introducing pro-neuronal miR-128 or other pro-differentiation genes back into giNSCs, it is possible to suppress proliferation and divert cells towards a differentiation path (Figure 6e). A conserved tumor suppressive role of miR-128 was validated in human glioblastoma cells (Figure 3 and Supplementary Figure S1).

Mitogenic tyrosine kinase signaling is essential for the proliferation of giNSCs and is thought to repress neuronal differentiation. miR-128 appears to have some switch-like properties in its targeting of the oncogenic kinases, EGFR and PDGFRα (Figures 4c–g). Indeed, similarly to miR-128 gain-of-function, EGFR or PDGFR loss-of-function leads to glioma tumor suppression (Shih *et al*., 2004; Zhang *et al*., 2004; Mukasa *et al*., 2010). Furthermore, miR-128 represses Akt activation in giNSCs and human glioblastoma cells (Figure 4g). Akt is an important downstream target of PDGFRα and EGFR signaling, which was recently shown to inhibit the differentiation of giNSCs (Zheng *et al*., 2008). EGFR and PDGFRα have been clearly implicated in glioma initiation and progression (Dai *et al*., 2001; Jackson *et al*., 2006; Furnari *et al*., 2007; Stiles and Rowitch, 2008), and classify the aggressive/dedifferentiated tumor subtypes in which miR-128 is repressed (Figure 1; Verhaak *et al*., 2010). By repressing these oncogenic tyrosine kinases, miR-128 is able to exert significant repression on the global phosphotyrosine profile of cells (Figure 4b). By repressing mitogenic EGFR and PDGFRα signaling in addition to other known oncogenic targets, such as Bmi1, E2F3 and Wee1, miR-128 is able to exert its tumor suppressive function at multiple levels (Godlewski *et al*., 2008; Zhang *et al*., 2009; Wuchty *et al*., 2011). Furthermore, our data identifies a feedback relationship between miR-128 and EGFR signaling. EGFRviii signaling is responsible for repressing an endogenous repressor of EGFR, miR-128. The mechanism of EGFR-mediated miR-128 repression requires further investigation to determine whether miR-128 is repressed at the transcriptional or post-transcriptional level.

In support of the therapeutic potential of miR-128 *in vivo*, we used several *in vivo* models. miR-128 was completely able to repress the growth of glioma tumors in an autochthonous glioma mouse model. Gliomas present a therapeutic challenge because of their very complex cellular nature, which is likely due to the fact that they originate from giNSCs. Pro-neural miR-128 very likely achieves its therapeutic effect by suppressing proliferation and enhancing differentiation of the tumor-initiating cells (Figure 6e). The attractiveness of this therapeutic approach is that miR-128 downregulates a network of functionally related genes that work together to promote the oncogenic state. This miRNA approach, in which many genes undergo modest changes, may prove more robust than the currently employed strategies that use highly specific 'warheads' to target a single gene within an oncogenic pathway. The flaw of

this later strategy is the uncanny tendency of cancer to circumvent the disabling of nearly any single gene.

Materials and methods

Analysis of miR-128 expression by RT–PCR
We used data from previous publications on miRNA expression in glioblastoma biopsies and neural samples (Liu *et al*., 2007; Neveu *et al*., 2010). We used two-tailed *t*-test to compare expression levels. Significance level was set at 0.05. Data shown as mean ± s.d.

Analysis of TCGA miRNA data
To identify miRNAs associated with glioblastoma subtypes, we used The Cancer Genome Atlas (TCGA) glioblastoma multiforme miRNA datasets. Probes were collapsed by taking the mean of probes corresponding to the same miRNA, and expression levels were globally normalized. Samples were classified as previously published (Verhaak *et al*., 2010). miRNAs associated with glioblastoma subtypes were identified using a similar procedure as described before (Verhaak *et al*., 2010). Each subtype was compared with the other three subtypes, both individually and combined by *t*-test statistics using the logarithm of the expression levels. The significance level was set at 0.01. Data shown as median ± s.d.

Mouse procedures
Immunocompromised Icr-severe combined immunodeficient and NCr nude homozygous mice were obtained from Taconic Inc. (Hudson, NY, USA, model #ICRSC-M and NCRNU-M). Intracranial injections of giNSC were performed as previously described (Bachoo *et al*., 2002). For the viral glioma model, hGFAP-Cre transgenic mice (The Jackson Laboratories, Bar Harbor, ME, USA) were stereotactically injected with 1 μl of virus in the right hippocampus (Marumoto *et al*., 2009). For subcutaneous injections, U251 cells were re-suspended in phosphate-buffered saline at a concentration of 10 000 viable cells/μl and 100 μl were injected in NCr nude mice. No effects of passage number were noted for all cell lines injected. Intracranial injected animals were placed into survival or time point cohorts, and were killed at the onset of neurological symptoms or once moribund. Subcutaneous NCr nude mouse U251 tumor volume was calculated at indicated time points as previously described (Sugahara *et al*., 2010). All animal experimentation was performed according to procedures approved by the Animal Research Committee at the University of California, Santa Barbara.

Cell cycle, BrdU and EdU analysis
For cell cycle analysis, U251 cells were infected with mock or miR-128 virus. Experiment was performed as previously described (Papagiannakopoulos *et al*., 2008). To determine the percentage of proliferating cells, neurospheres were pulsed with 5-bromo-2′-deoxyuridine (BrdU; 1 uM) during the last 2 h of culturing, dissociated and processed using the BrdU flow Kit (BD Biosciences, San Jose, CA, USA) solutions according to the manufacturer's instructions. PE-conjugated anti-BrdU antibody was used for detection using Guava EasyCyte Flow Cytometer (Guava Technologies, Billerica, MA, USA).

For the 5-ethynyl-2′-deoxyuridine (EdU) assay, transfected glioma cells were cultured for 2–3 days on glass coverslips in growth media, plus trophic factors, and then, EdU incorporation for a 24-h period at 37 °C was measured with the ClickIT EdU Alexa Fluor 594 Imaging Kit (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. Images were collected on a standard Nikon fluorescence microscope (Nikon Instruments, Melville, NY, USA) and analyzed by MetaMorph software (Molecular Devices, Sunnyvale, CA, USA) to quantify the percentages of cells (marked by 4,6-diamidino-2-phenylindole staining) that had incorporated EdU over a 24-h period.

LNA and pre-miR transfections in NSCs and glioblastoma cells lines
p16/p19−/− EGFRviii giNSCs were grown on fibronectin/poly-L-ornithine-coated six-well plates. Pre-scr (control) or pre-miR-128 (Ambion, Austin, TX, USA) of 50 nM and 14 μl transfection reagent Lullaby (OZbiosciences, Marseille, France) were used to transfect each well, according to the manufacturer's instructions.

Mouse genetic mosaic model (MADM)-giNSCs were lifted from flasks as described above. Glioma cells of $2–3 \times 10^6$ aliquots were resuspended in 100 μl Lonza Astrocyte nucleofection reagent (Lonza VPG-1007, Basel, Switzerland), plus either 0.4 nmol miR-128 mimic (Dharmacon, Lafayette, CO, USA, C-310398-07) or control miRNA mimic #1 (CN-001000-01). Glioma/miRNA mixes were then electroporated with the Amaxa nucleofection apparatus, T-20 program (Lonza, Basel, Switzerland). For protein collection, 1.5 M post-transfection cells were plated in appropriate growth factor-containing media into 10 cm tissue culture plates. For proliferation assays, 10K cells were plated onto pDL-coated 12-mm glass coverslips in 24-well tissue culture plates.

Western blots, immunofluorescece and antibodies
The following antibodies were used for immunoblotting: rabbit EGFR (sc-03, Santa Cruz Biotechnology, Santa Cruz, CA, USA), beta-actin (Sigma, St Louis, MO, USA), mouse glial fibrillary acidic protein (GFAP; C9205, Sigma) and mouse Nestin (ab6142, Abcam, Cambridge, MA, USA), rabbit pAktS473 (9271, Cell Signaling, Danvers, MA, USA), rabbit PDGFRα (3174, Cell Signaling), mouse PDGFRα (Abnova, Taipei City, Taiwan), rabbit SOX2 (Ab15830, Abcam), mouse pTyr 4G10 (05-1050, Millipore, Billerica, MA, USA), rabbit Tuj1 (MRB0435P, Covance, Princeton, NJ, USA), mouse Tau5 (AHB0042, Biosource/Invitrogen). Fluorescent-IR secondary antibodies used include, goat anti-mouse secondary Alexa Fluor 680 (A21057, Molecular Probes, Eugene, OR, USA), goat anti-rabbit IRDye 800CW, donkey anti-goat IRDye 800CW (LI-COR, Lincoln, NE, USA). HRP-conjugated goat anti-chicken or goat anti-mouse (Chemicon, Temecula, CA, USA).

Cell lysis, protein quantitation and western blotting were performed as previously described (Papagiannakopoulos *et al*., 2008; Xu *et al*., 2009). HRP-conjugated blots were developed with Amersham ECL Plus Western Blotting Detection Reagents (Piscataway, NJ, USA). Gels were imaged and analyzed on a FluorchemQ digital imager (Cell Biosciences, Santa Clara, CA, USA).

Cell growth assays
Determination of NSC neurosphere and U251 growth rates were performed by assaying ATP content using the Cell Titer Glo Assay (Promega, Madison, WI, USA). NSCs were seeded at a density of 5000 cells per well in ultra-low adhesion 96-well plates (Corning, Corning, NY, USA). U251 cells were seeded in 96-well plates at 8000 cells per well. All cells were infected with mock or miR-128 expressing lentivirus before seeding. Transduction efficiency was assessed by observing green fluorescent protein percentage. Cell Titer Glo (Promega) reagent was used to determine growth at days 0, 2, 4 and 6. Results represent the means of three separate experiments.

Conflict of interest

K.S.K. owns stock in and serves on the board of Minerva Biotechnologies corporation and is on the scientific advisory board of Nocira Pharmaceuticals.

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