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Authors

Silber, Joachim

Hashizume, Rintaro

Felix, Tristan

et al.

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Expression of miR-124 inhibits growth of medulloblastoma cells

Joachim Silber, Rintaro Hashizume, Tristan Felix, Sujatmi Hariono, Mamie Yu, Mitchel S. Berger, Jason T. Huse, Scott R. Vandenberg, C. David James, J. Graeme Hodgson, and Nalin Gupta

Brain Tumor Research Center, Department of Neurological Surgery, University of California, San Francisco, San Francisco, California (J.S., R.H., T.F., S.H., M.Y., M.S.B., S.R.V., C.D.J., J.G.H., N.G.); Human Oncology & Pathogenesis Program, Memorial Sloan-Kettering Cancer Center, New York, New York (J.S., J.T.H.); Present affiliation: Department of Pathology, University of California, San Diego, California (S.R.V.); Department of Translational Biology, Ariad Pharmaceuticals, Inc., Cambridge, Massachusetts (J.G.H.)

Medulloblastoma is the most common malignant brain tumor in children, and a substantial number of patients die as a result of tumor progression. Overexpression of CDK6 is present in approximately one-third of medulloblastomas and is an independent poor prognostic marker for this disease. MicroRNA (miR)-124 inhibits expression of CDK6 and prevents proliferation of glioblastoma and medulloblastoma cells *in vitro*. We examined the effects of miR-124 overexpression on medulloblastoma cells both *in vitro* and *in vivo* and compared cell lines that have low and high CDK6 expression. MiR-124 overexpression inhibits the proliferation of medulloblastoma cells, and this effect is mediated mostly through the action of miR-124 upon CDK6. We further show that induced expression of miR-124 potently inhibits growth of medulloblastoma xenograft tumors in rodents. Further testing of miR-124 will help define the ultimate therapeutic potential of preclinical models of medulloblastoma in conjunction with various delivery strategies for treatment.

Keywords: CDK6, D425, medulloblastoma, microRNA, miR-124.

Medulloblastoma is the most common malignant brain tumor in children.¹ Despite treatment with surgery, radiation, and chemo, a substantial number of patients die as a result of tumor progression,² and treatment-associated toxicities severely impair quality of life for most long-term survivors.¹ The long-term goal of targeted therapeutics is to

reduce systemic toxicity and normal brain injury while still achieving durable tumor control or cure.

Overexpression of CDK6 is present in approximately one-third of medulloblastomas and is an independent poor prognostic marker for this disease.³ Development of therapeutic agents that target inhibition of CDK6 may be effective for the subset of CDK6-positive tumors. For example, PD 0332991 is a highly specific small molecule inhibitor of CDK6 and CDK4 and has antitumor activity in human tumor xenografts.⁴ However, tumors acquire resistance to small-molecule inhibitors such as imatinib through several mechanisms, such as primary and secondary mutations.⁵

RNA interference (RNAi) is a powerful cellular process initiated by short double-stranded RNAs resulting in sequence-specific posttranscriptional gene silencing. The goal of RNAi-based therapy is to mimic naturally occurring RNAi and target specific messenger (m)RNA sequences to induce selective gene silencing. The principal advantages of RNAi over small-molecule and protein therapeutics are (i) ability to inhibit all targets, including “undruggable” ones;⁶ (ii) increased potency; and (iii) high specificity.

Micro (mi)RNAs are a class of small noncoding RNAs that regulate diverse cellular processes through RNAi-based mechanisms. MiRNAs are transcribed as primary RNA transcripts, processed in the nucleus to smaller precursor hairpin structures, and then exported to the cytoplasm, where they are processed further by the dicer nuclease to become mature, functional miRNAs ~21 nucleotides in length. Mature miRNAs, the endogenous equivalent of short-interfering (si)RNAs, are then incorporated into the RNA-induced silencing complex, which facilitates their interaction with, and inhibition of, target mRNAs by translational repression or message cleavage.⁷

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Corresponding Author: Nalin Gupta UCSF Neurosurgery, 505 Parnassus Ave., Rm M778, San Francisco, CA 94143 (guptan@neurosurg.ucsf.edu).

One of the best characterized miRNAs in the central nervous system is miR-124. Expression of miR-124 is upregulated during development of the rodent nervous system^{8,9} during neuronal differentiation of embryonic stem cells¹⁰ and of mouse and human embryonal carcinoma cells.¹¹ Furthermore, neuronal differentiation is enhanced following ectopic expression of miR-124 in mouse neuroblastoma cells,¹² mouse embryonal carcinoma cells,¹⁰ and mouse embryonic stem cells.¹⁰ We have previously shown that miR-124 promotes neuronal differentiation of postnatal neural stem cells and glioma stem cells.¹³ MiR-124 is also an important regulator of adult neurogenesis in the subventricular-zone stem cell niche.¹⁴

Studies have shown that miR-124 is downregulated^{15,16} and that CDK6 is overexpressed in medulloblastoma tumors.³ MiR-124 inhibits expression of CDK6 and prevents proliferation of glioblastoma¹³ and medulloblastoma cells in vitro.^{15,16} In this report, we show that miR-124 inhibits proliferation of medulloblastoma cells in vitro and that induced expression of miR-124 potently inhibits growth of medulloblastoma xenograft tumors in rodents.

Materials and Methods

Primary Human Tissues

Human tumor samples and normal tissues were acquired from the Brain Tumor Research Center Tissue Bank at the University of California, San Francisco (UCSF). All procedures were in accordance with the Committee on Human Research. All samples were reviewed by a neuropathologist (S.V.), and medulloblastoma specimens were confirmed to contain at least 90% tumor tissue. Medulloblastoma cell lines were provided as a kind gift by Dr Darryl Bigner (Duke University).

Cell Lines, miRNA, and siRNA Oligonucleotides

Human medulloblastoma cell lines D283, D341, and D425 were maintained in Dulbecco's modified Eagle's medium (DMEM)–Ham's F-12 50:50 media supplemented with 15% fetal bovine serum (FBS) under standard conditions; 293T cells were grown in high-glucose DMEM supplemented with 10% FBS under standard conditions. MiRIDIAN miRNA hsa-miR-124 and negative control #1 (cel-miR-67) oligonucleotides (Dharmacon) were used as previously described.¹³ CDK6 targeting ON-TARGETplus SMARTpool CDK6 siRNA and control were purchased from Dharmacon. Oligonucleotides were transfected to a final concentration of 100 nM using HiPerFect transfection reagent (Qiagen) according to the manufacturer's instructions.

Modification of Tumor Cell Lines with Luciferase Reporter Gene

D425 tumor cells were transduced with lentiviral vectors containing firefly luciferase as previously described.^{17,18} Cells were screened in vitro for transduction efficiency

by treatment with luciferin (D-luciferin potassium salt, 150 mg/kg; Gold Biotechnology) and analyzed using an IVIS Lumina System (Caliper Life Sciences).

Inducible miRNA Lentiviral Expression

MiR-124 shRNA and a nonsilencing (NS) control shRNA were subcloned from pLemiR vectors (Open Biosystems) to the pTRIPZ inducible lentiviral shRNAmir vector (Open Biosystems) using *MluI* and *XhoI* restriction sites in both vectors. To generate lentivirus, 293T cells were transfected with pTRIPZ constructs using the Trans-Lentiviral Packaging System (Open Biosystems) according to the manufacturer's instructions. D425 cells were infected with lentivirus, and transduced cells were selected with 1 μ g/mL puromycin. Two independent inducible cell lines expressing miR-124 and one NS control cell line (pTRIPZ-miRNS) were obtained. pTRIPZ is a tetracycline (Tet)-On system, and miRNA and turbo red fluorescent protein (tRFP) are expressed from the same Tet-inducible promoter. MiRNA and tRFP expression was induced by addition of 1 μ g/mL doxycycline. Expression of tRFP was observed within 24 h post-induction (data not shown).

Quantitative Reverse Transcriptase Polymerase Chain Reaction

Total RNA was extracted using the miR-Vana RNA isolation system (Ambion). MiRNA expression was quantitated using individual TaqMan MicroRNA Assays (Applied Biosystems). The comparative cycle threshold ($\Delta\Delta C_t$) method was used to determine expression fold change, using U6, U38 RNA, hsa-let-7a, or hsa-miR16 as an endogenous control as previously described.¹³

Cell Cycle Analysis and Immunoblotting

Cell cycle analyses were conducted using the fluorescein isothiocyanate BrdU (5-bromo-2'-deoxyuridine) Flow Kit (BD Pharmingen) following the manufacturer's recommendations as previously described.¹³ Immunoblotting was performed using standard protocols with antibodies CDK6 (1:1000; Cell Signaling) and Hcs70 (1:4000; Santa Cruz Biotechnology) as previously described.¹³

Immunohistochemistry

After resection, mouse brains were fixed for 48 h in 4% paraformaldehyde. Brains were then paraffin embedded and sectioned (10 μ m) for staining with hematoxylin and eosin and immunohistochemical analysis using anti-Ki67 (2 μ g/mL; Ventana).¹⁸ For quantification, 2 tumors (4 fields each) in each group were used.

Animals

Six-week-old female athymic mice (nu/nu genotype, BALB/c background) were purchased from Simonsen Laboratories. Animals were housed in the UCSF animal facility and were maintained in a temperature-controlled and light-controlled environment with an alternating 12-hour light/dark cycle. All protocols were approved by the UCSF Institutional Animal Care and Use Committee.

Subcutaneous Tumors in Mice

Mice were injected s.c. in the right flanks with 4×10^6 D425 medulloblastoma cells transduced with pTRIPZ-miR-124 clone B ($n = 7$) or the control vector pTRIPZ-miRNS ($n = 8$) in 0.2 mL of cell culture media with matrigel (BD Bioscience). Prior to implantation, animals' drinking water had been supplemented with doxycycline for 1 week, and we continued adding doxycycline to the drinking water after the implantation. Animal experiments were repeated ($n = 5$ in each group) with similar results obtained. Tumors were measured every 3 days with calipers, and tumor volume was calculated according to the following formula: $(\text{width})^2 \times (\text{length})/2$. All procedures were carried out under sterile conditions.

Intracerebellar Implantation of Tumor Cells

D425 cells were transfected with hsa-miR-124 or control oligonucleotide using HiPerFect transfection reagent as described above. The transiently transfected cells ($n = 5$ for each group) were then implanted into the brains of athymic mice as previously described.¹⁸ Briefly, mice were anesthetized with an intraperitoneal injection of a mixture containing ketamine (100 mg/kg) and xylazine (10 mg/kg) in 0.9% saline. A 1-cm sagittal incision was made along the scalp, and the skull suture lines were exposed. A small hole was created by puncture with a 25-g needle at 3 mm to the right of the midline and 6.5 mm behind the bregma. With the use of a sterile Hamilton syringe (Stoelting), 3×10^5 cells in 3 μ L Hanks' Balanced Salt Solution without Ca^{2+} and Mg^{2+} were manually injected very slowly (over 1 min) into the right cerebellum at 3 mm deep from the bottom of the skull. Mice were monitored daily and euthanized at the onset of neurological symptoms or once moribund.

Bioluminescence Imaging

In vivo bioluminescence imaging was performed with the IVIS Lumina System (Caliper Life Science) coupled to LivingImage data-acquisition software. Mice were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine and imaged at 10 min after intraperitoneal injection of D-luciferin (potassium salt, 150 mg/kg; Gold Biotechnology). Signal intensity was quantified within a region of interest over the mouse head, as defined by

LivingImage software. Bioluminescence measurements for each animal were normalized against corresponding readings obtained at the beginning of therapy.

Results

MiR-124 and CDK6 Expression in Medulloblastoma Tissues and Cell Lines

Because miR-124 potently inhibits CDK6 protein expression in medulloblastoma¹⁵ and glioblastoma cell lines,¹³ we investigated whether decreased miR-124 expression resulted in increased CDK6 protein expression in primary medulloblastoma samples ($n = 8$) and medulloblastoma cell lines ($n = 3$). Consistent with previous observations, we observed that miR-124 was expressed 0.7–3.5-fold lower in 8/8 tumors and that CDK6 was overexpressed in 4/8 (50%) tumors relative to normal cerebellum (Fig. 1A and B). In medulloblastoma cell lines, miR-124 was expressed at least 6.5-fold lower compared with normal cerebellum, whereas CDK6 protein expression was detected in 1/3 lines (D425). CDK6 mRNA was detected in D283 medulloblastoma cells (Supplementary material, Fig. S1). However, we did not observe a consistent relationship between miR-124 and CDK6 protein expression levels in medulloblastoma tissue samples and cell lines. This suggests that CDK6 overexpression in medulloblastoma tumors is predominantly regulated by factors other than miR-124 expression levels, such as genomic amplification.³

MiR-124 Preferentially Inhibits Proliferation of CDK6-positive Medulloblastoma Cell Lines

Overexpression of miR-124 has been shown to inhibit proliferation of glioblastoma cells¹³ and medulloblastoma cells DAOY,^{15,16} D283,¹⁵ and ONS-76¹⁶ and to inhibit expression of the cell cycle promoter CDK6.¹⁵ Since our results revealed differential CDK6 protein expression in medulloblastoma cell lines (Fig. 1B), we examined whether ectopic expression of miR-124 inhibited medulloblastoma cell proliferation. Overexpression of miR-124 resulted in a more dramatic effect on proliferation in D425 cells compared with D283 and D341 cells (Fig. 2A). The effect on D283 cells was intermediate between those on D341 and D425 cells. These results suggest that CDK6-expressing cell lines are more responsive to proliferation inhibition than cells expressing low amounts of CDK6.

MiR-124 and CDK6 siRNAs Inhibit Proliferation of CDK6-positive Medulloblastoma Cells

To further define the inhibitory role of miR-124 on medulloblastoma cell proliferation, D425 cells were pulsed with BrdU, and analysis of cell cycle distribution was performed. Relative to nonsense control oligonucleotides, transfection of miR-124 resulted in a marked reduction in the number of cells in the S phase of cell

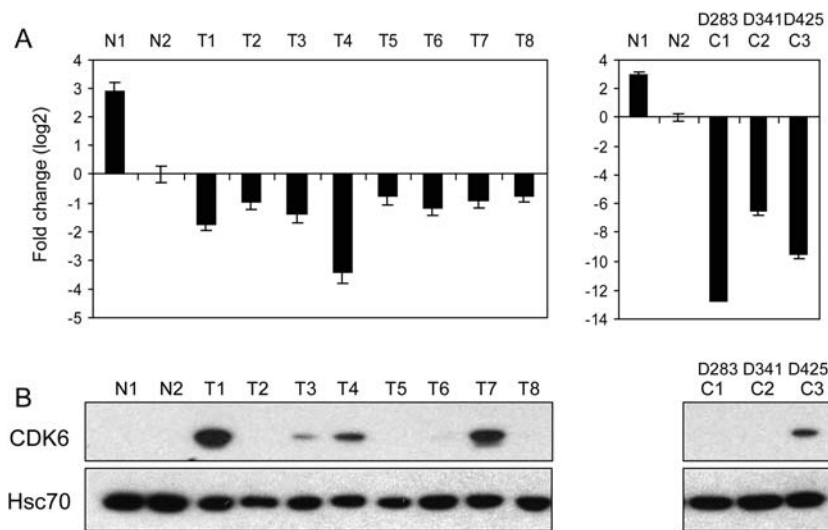


Fig. 1. MiR-124 is downregulated and CDK6 is upregulated in medulloblastoma. (A) Expression of miR-124 in individual medulloblastoma tumor samples and medulloblastoma cell lines measured relative to U38 small nucleolar RNA and normalized to expression in fetal cerebellum. N1: adult cerebellum, N2: fetal cerebellum, T1–T8: medulloblastoma tumors, C1: D283 cell line, C2: D341 cell line, C3: D425 cell line. (B) Expression of CDK6 protein in control tissues, individual tumor samples, and medulloblastoma cell lines.

cycle and a marked increase in the number of cells in G₀/G₁ in D425 medulloblastoma cells (Fig. 2B). No reproducible differences were observed for cells in the G₂/M phase of the cell cycle or in cells undergoing apoptosis (sub-G₁) in any of the cell lines examined (Fig. 2B). These results suggest that miR-124 induces a G₀/G₁ cell cycle arrest in medulloblastoma cells.

Previous studies have shown that miR-124 directly targets the cell cycle regulator CDK6.^{15,19} To further investigate the specific effect of miR-124 on cell proliferation, we transfected D425 medulloblastoma cells with siRNA specific for CDK6 and compared cell cycle distribution with cells transfected with miR-124. Transient transfection of D425 cells with siRNAs targeting CDK6 resulted in a reduction in cells in the S phase of cell cycle and an increase in cells in G₀/G₁ (Fig. 2C). No reproducible differences were observed for cells in the G₂/M phase of the cell cycle or in cells undergoing apoptosis. The effect of inhibiting CDK6 is slightly less potent than overexpression of miR-124 alone (30% vs 48%). When miR-124, or siRNAs targeting CDK6, were overexpressed in D283 cells—which have low expression of CDK6—we observed a potent effect by miR-124 alone (45% of cells in S phase; Fig. 2D) but only a small effect when inhibiting CDK6 (82% of cells in S phase; Fig. 2D). These results suggest that miR-124 can function independently of CDK6 and likely has other targets that affect cell growth.

MiR-124 Inhibits Medulloblastoma Tumor Growth In Vivo

In previous studies, transient miR-124 overexpression in oligodendroglioma and glioblastoma neurosphere cultures resulted in neuronal-like differentiation in

addition to cell cycle arrest,¹³ suggesting that transient overexpression of miR-124 may result in prolonged growth inhibition in vivo. To investigate this, we transiently transfected D425 medulloblastoma cells with control or miR-124 oligonucleotides in vitro and transplanted the transfected cells into the cerebellum of athymic BALB/c mice. The D425 cells were modified with a luciferase construct, allowing monitoring of tumor formation in vivo. Transient transfection of miR-124 delayed tumor formation by 11–14 days; however, after 14 days, the mice transfected with miR-124 formed tumors at the same growth rate as the control-transfected group (Supplementary material, Fig. S2).

We next addressed whether constitutive expression of miR-124 was sufficient to induce significant growth inhibition in vivo. We created an inducible miR-124-expressing lentiviral construct, allowing us to monitor in vivo growth of D425 medulloblastoma cells expressing miR-124. Relative to D425 cells transduced with pTRIPZ negative control (pTRIPZ-miR-neg), doxycycline stimulation of 2 independent pTRIPZ-miR-124 D425 cell lines (A and B) led to a significant increase in miR-124 expression (Fig. 3A), a decrease in CDK6 expression (Fig. 3B), and a concomitant decrease in cell numbers (Fig. 3C).

We then subcutaneously implanted D425 cells transduced with pTRIPZ-miR-124 (clone B) or pTRIPZ-miRNS (nonsilencing control) into athymic mice. Their drinking water had been supplemented with doxycycline 1 week prior to implantation and we continued adding doxycycline after implantation. Tumor growth was inhibited in mice implanted with medulloblastoma cells expressing miR-124 compared with pTRIPZ control medulloblastoma cells, as evidenced by tumor size up to 37 days post-implantation (Fig. 4). Both the

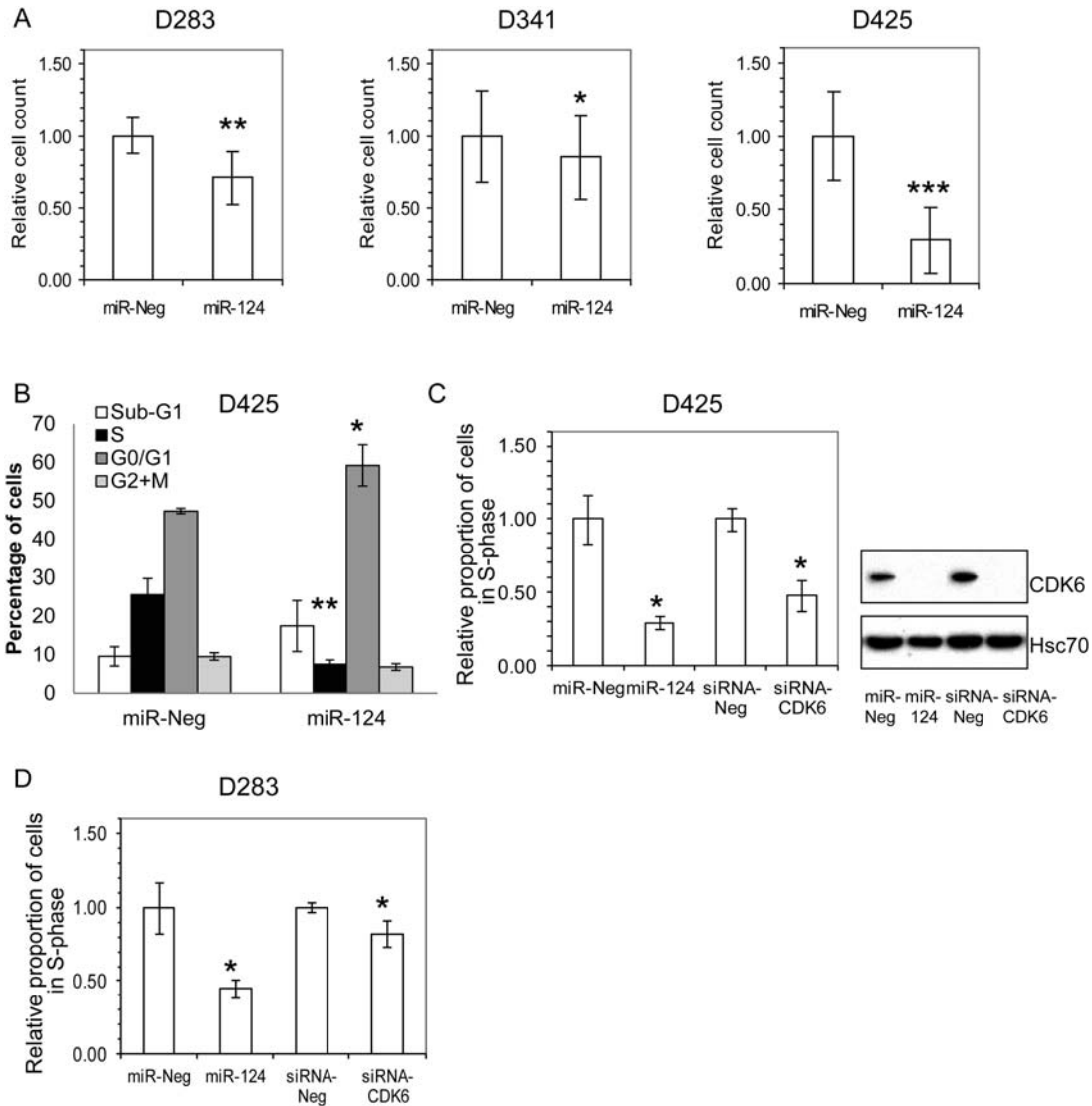


Fig. 2. MiR-124 inhibits proliferation of CDK6-expressing D425 medulloblastoma cells and induces G_0/G_1 cell cycle arrest miR-124. (A) Cell numbers were determined at 72 h after transfection of 100 nM miR-124 or negative control oligonucleotides to D283, D341, and D425 medulloblastoma cells. Values represent mean \pm SD of replicate experiments normalized to the cell count in the control samples (D283 $n = 51$; D341 $n = 34$; D425 $n = 14$). Differences between miR-124 and control transfected cells were analyzed using Student's t -test; $*P < .05$. $***P < 2e-7$. $***P < 4e-15$. (B) Cell cycle analysis conducted by fluorescence-activated cell sorter (FACS) at 48 h after transfection of 100 nM miR-124 or negative control oligonucleotides to D425 cells. Cells were treated with BrdU for 30 min, fixed, treated with fluorescein isothiocyanate (FITC)-labeled anti-BrdU antibody and the DNA stain 7-amino-actinomycin D, and subjected to flow cytometry. Values represent mean \pm SD ($n = 2$) of replicate experiments ($n = 3$). Differences between miR-124 and control transfected cells were analyzed using Student's t -test; $*P < .0005$; $**P < 2e-6$. (C) Cell cycle analysis was conducted by FACS at 48 h after transfection of 100 nM CDK6 siRNAs, miR-124, or negative control oligonucleotides to D425 cells. Cells were treated with BrdU for 30 min, fixed, treated with FITC-labeled anti-BrdU antibody and the DNA stain 7-amino-actinomycin D, and subjected to flow cytometry. Values represent mean \pm SD ($n = 2$) of replicate experiments ($n = 3$). Differences between the different transfections were analyzed using Student's t -test; $*P < 3e-6$. (D) Cell cycle analysis was conducted by FACS at 48 h after transfection of 100 nM CDK6 siRNAs, miR-124, or negative control oligonucleotides to D283 cells. Values represent mean \pm SD ($n = 2$) of replicate experiments ($n = 3$). Differences between the different transfections were analyzed using Student's t -test; $*P < .0001$.

pTRIPZ-miR-124 and NS control-transduced D425 xenografts displayed a prominent large cell cytoarchitecture with conspicuous nucleoli and characteristic arrangement in large sheets. Histologic evidence of apoptosis (apoptotic bodies) was present to the same

extent in both groups (data not shown). The effect on proliferation was examined by measuring Ki67-positive cells in the flank tumors. The percentage of Ki67-positive cells was reduced from 29% to 4% in the tumors from animals given doxycycline.

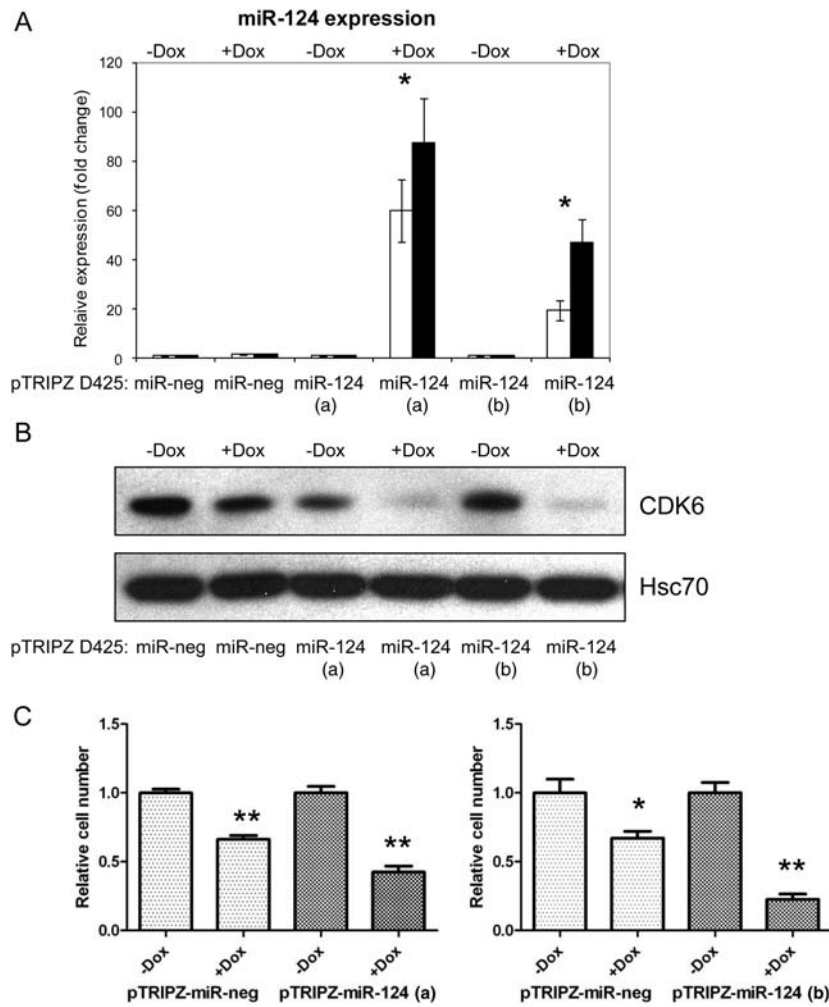


Fig. 3. Inducible expression of miR-124 in D425 medulloblastoma cells. (A) Doxycycline stimulation of lentiviral transduced D425 cells leads to increased expression of miR-124. Expression of miR-124 is measured relative to let-7a (white) and miR-16 (black). Two independently transduced D425-miR-124 cell lines (a and b) were tested. Expression differences following doxycycline stimulation were analyzed using Student's *t*-test; **P* < .005. (B) Doxycycline stimulation leads to decreased levels of CDK6 in pTRIPZ-miR-124 lentiviral transduced D425 cells. (C) Cell numbers were determined at 96 h after doxycycline stimulation. Values represent mean ± SE (*n* ≥ 3) from replicate experiments (*n* = 2) normalized to the cell count in non-doxycycline-stimulated samples. Differences in cell numbers were analyzed using Student's *t*-test; **P* < .05; ***P* < .0001.

Discussion

Overexpression of CDK6 promotes cell cycle progression,²⁰ has been observed in approximately one-third of medulloblastomas, and is an independent poor prognostic marker for this disease.³ It is reasonable to assume that therapeutic inhibition of CDK6, at least in a subset of patients, may be effective in achieving better tumor control. Similar to what we observed in mouse postnatal neural stem cells and in glioblastoma cells and stem cells,¹³ miR-124 inhibits proliferation of medulloblastoma cells, leading to a G₁ cell cycle arrest. Interestingly, we observe that the inhibitory effect on cell cycle progression mediated by miR-124 seems to be more pronounced in medulloblastoma cells with higher expression of CDK6. Our results suggest that cell

proliferation inhibited by miR-124 in CDK6-expressing medulloblastoma can for a large part be replicated by directly targeting CDK6. Although the majority of our results suggests that miR-124 overexpression requires CDK6, the effect on the S-phase population in D283 cells suggests that miR-124 can also function through alternative downstream targets. Despite this effect on cell cycle distribution, we did observe that overall effects on cell proliferation were more pronounced in cells that expressed higher levels of CDK6. The effect on cell proliferation was confirmed in our *in vivo* results, where we observed a marked reduction in Ki67-positive cells in subcutaneous tumors.

MiRNAs with therapeutic potential have been identified in several other tumors, including pancreatic,²¹ prostate,²² and lung cancers.²³ Indeed, RNAi-based

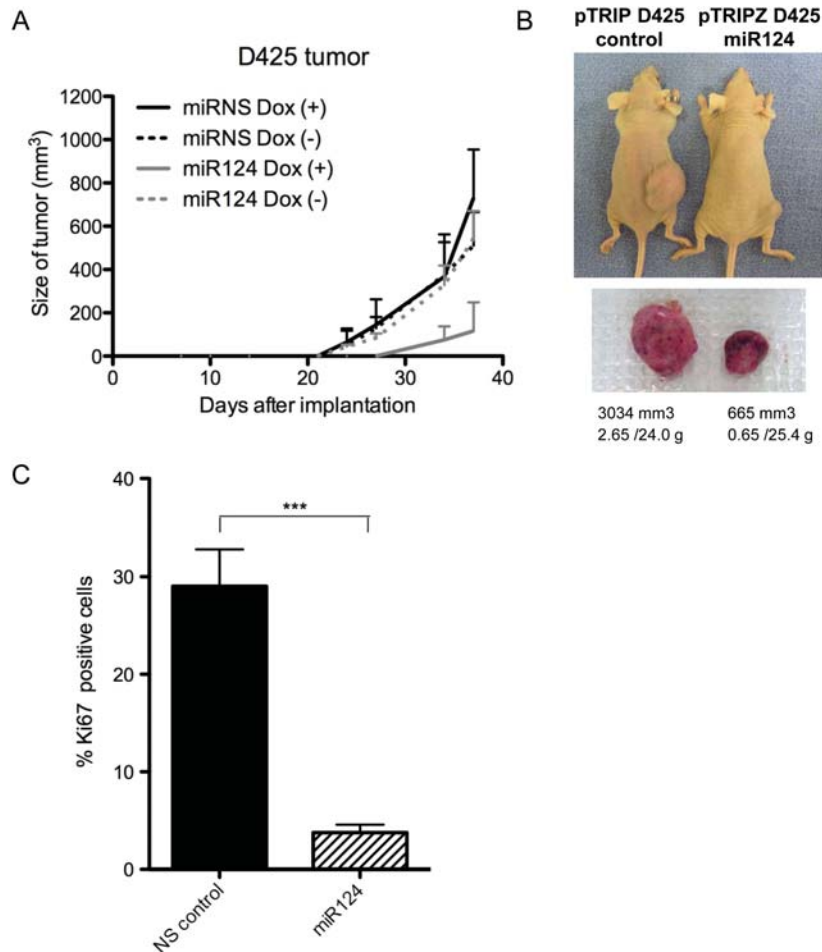


Fig. 4. MiR-124 inhibits tumor growth in vivo. (A) Subcutaneous growth of D425 tumors expressing inducible miR-124 clone B is inhibited in animals provided doxycycline. (B) Tumors were visibly smaller at the time animals were sacrificed. (C) Ki67 staining of tumors demonstrated a marked reduction in the percentage of Ki67-positive cells (2 tumors from each group, 4 fields each; SE of the mean; $P = .0006$). Unpaired *t*-test was used to determine differences between the 2 groups.

agents represent an entirely new class of antitumor treatment.²⁴ One limitation to the delivery of siRNAs or miRNAs to the central nervous system is the presence of the blood–brain barrier. It is unclear whether gene expression can be adequately reduced for a meaningful period of time using conventional systemic delivery strategies. There are, however, a number of promising strategies that may circumvent this problem. These include intranasal delivery of oligonucleotides,²⁵ lipid encapsulation and targeted delivery of nucleic acids,^{26,27} and direct administration of therapeutic agents to brain tumor tissues by convection-enhanced delivery.^{28,29} Recently, direct CNS delivery of siRNA using convection-enhanced delivery demonstrated robust silencing of an endogenous oligodendrocyte-specific gene in nonhuman primates.³⁰

In addition to inhibiting CDK6 expression, miR-124 has been shown to directly inhibit expression of multiple genes that abrogate neurogenesis. MiR-124 thus appears

to be critical for the homeostasis of differentiation versus proliferation.³¹ For example, miR-124 repression of the sex-determining region Y–box (SRY) transcription factor Sox9 is important for progression of stem cells in the subventricular zone into neurons.¹⁴ We recently speculated that loss of miR-124 expression in neural stem cells may result in a pool of premalignant, differentiation-impaired, hyperproliferative stem cells that may progress to brain tumors upon acquisition of additional oncogenic mutations.³² It is possible that expression of miR-124 may be more effective than pure inhibition of CDK6 because of this additional effect on stem cell populations.

Further testing of miR-124 in preclinical models of medulloblastoma in conjunction with various delivery strategies will help define its ultimate therapeutic potential for treatment of medulloblastoma. In particular, combined miR-124 therapy in conjunction with other therapeutics may enhance the therapeutic outcome.

Supplementary Material

Supplementary material is available at *Neuro-Oncology Journal* online (<http://neuro-oncology.oxfordjournals.org/>).

Conflict of interest statement. None declared.

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