

# Regulation of cyclin dependent kinase 6 by microRNA 124 in medulloblastoma

Jessica Pierson · Bruce Hostager · Rong Fan ·  
Rajeev Vibhakar

Received: 29 January 2008 / Accepted: 28 May 2008 / Published online: 8 July 2008  
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**Abstract** Despite recent advances in treatment medulloblastoma continues to remain a vexing problem. Recently increased expression of cyclin dependent kinase 6 (CDK6) was identified as an adverse prognostic marker in medulloblastoma. Genomic amplification accounts for some, but not all of the CDK6 over-expression. We hypothesized that CDK6 expression is also regulated by microRNAs in medulloblastoma. We identified putative miR sites in the CDK6 including microRNA 124a, a brain enriched microRNA. Expression of miR 124a was significantly decreased in medulloblastoma cells compared to normal adult cerebellum. Functional association between miR 124a and CDK6 in medulloblastoma was established using luciferase assays. Additionally, re-expression of miR 124a in medulloblastoma cells decreased expression of CDK6 protein. Transfection of miR 124 significantly decreases medulloblastoma cell growth but does not alter apoptosis. Furthermore, in patient samples expression of miR 124a is significantly decreased. Our data strongly indicate that CDK6 is regulated by microRNA 124 in medulloblastoma and that miR 124 modulates medulloblastoma cell growth.

**Keywords** Medulloblastoma · Cyclin Dependent Kinase 6 · MicroRNA 124

## Introduction

Medulloblastoma comprises approximately 20% of all primary pediatric brain tumors. Advances in treatment with surgery, radiation, and chemotherapy have resulted in a five-year survival rate of approximately 70% for standard risk medulloblastoma [1–3]. However, patients with high risk and infant tumors continue to do poorly [4]. Recently cyclin dependent kinase 6 (CDK6) was identified as an adverse prognostic marker in medulloblastoma [5]. CDK6 is a member of a family of serine-threonine kinases involved in the control of cell cycle progression [6]. CDK6 partners with cyclin D to phosphorylate the retinoblastoma (RB) protein during the G1-S cell cycle transition [6]. Interestingly, mice deficient in CDK6 are resistant to ErbB2 induced breast tumors indicating a role for CDK6 in tumorigenesis [7]. In medulloblastoma CDK6 is genomically amplified in some but not all of the tumors found to over-express CDK6 protein. These findings suggest a role for other regulatory mechanisms in controlling CDK6 expression in medulloblastoma.

MicroRNAs are a large family of small, non-coding RNAs with gene regulatory functions [8, 9]. They consist of 18–22 nucleotide RNA molecules with post transcriptional gene silencing activity [9]. They control gene expression through association with the 3'-untranslated region (3'UTR) of genes and inhibit protein translation [10]. MicroRNAs can also destabilize and mediate the degradation of RNA transcripts [11]. In addition to their role in normal development, microRNAs are also associated with carcinogenesis [12, 13]. For example microRNA 21 (miR 21) is over expressed in Glioblastoma and inhibition of miR 21 inhibits glioblastoma growth in vitro and in vivo. Recently miR 34 was shown to function as a p53-dependent tumor suppressor in neuroblastoma [14].

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J. Pierson · B. Hostager · R. Fan · R. Vibhakar (✉)  
Department of Pediatrics, University of Iowa Children's  
Hospital, University of Iowa, 5202 MERF, Iowa City, IA 52242,  
USA  
e-mail: Rajeev-vibhakar@uiowa.edu

Because not all CDK6 over-expression in medulloblastoma can be explained by genomic amplification and given that microRNAs are powerful regulators of gene expression, we hypothesized that CDK6 expression might be regulated by microRNAs in medulloblastoma. We found CDK6 is regulated by microRNA miR 124 and that miR 124 is significantly decreased in medulloblastoma. In addition, miR 124 decreased medulloblastoma cell proliferation. Our data suggest that miR 124 may play an important role in medulloblastoma pathogenesis, potentially through the regulation of CDK6.

## Methods

### Cell lines and primary tumor samples

Daoy, D283 Med and D341 Med were obtained from American Type Culture Collection (Rockville, MD.) and cultured in modified Eagle's medium (Gibco, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (Gibco) (20% for D341) according to the supplier's recommendations. Primary cell cultures were derived from biopsy specimens of medulloblastoma patients under a protocol approved by the institutional review board at the University of Iowa Hospitals and Clinics. To generate primary cell cultures, approximately 200–250 mg of tumor tissue was immersed and incubated in 0.05 mM EDTA solution containing 0.05% trypsin (Sigma, St. Louis, Mo.) at 4°C for 8 h. The tissue samples were minced into 0.3 mm<sup>3</sup> fragments and suspended in Hank's balanced salt solution (HBSS) containing 4 mg/ml DNase I, 40 mg/ml collagenase IV, and 100 units/ml of hyaluronidase type V (all from Sigma). Single-cell suspensions were then passed through no. 100 nylon mesh, washed twice in HBSS, and added to fibronectin-coated tissue culture flasks. Cultures were maintained at low passage numbers (p2–p4) in modified Eagle's medium supplemented with 20% fetal bovine serum as described above. Normal human cerebellum and medulloblastoma patient samples were obtained from the Pediatric Co-operative Human Tissue Network (Columbus, Ohio). All normal cerebellar samples were from non-malignant adult brain. All medulloblastoma samples were from pediatric patients (<18 years of age). Detailed data on the normal samples, primary cultures, and patient samples has been previously described by us [15].

### RNA isolation and miRNA analysis

Total RNA was isolated from medulloblastoma cell lines by Trizol (Invitrogen, San Diego, CA) extraction according to the manufacturer's instructions. Isolation of total RNA from brain tissues was performed using the mirVANA

RNA isolation kit (Ambion, Texas). MicroRNA expression was measured by quantitative PCR using a ABI 7700 thermal-cycler. MicroRNA primers and probes were purchased from Applied Biosystems (Foster City, CA) and the assays performed per the manufacturer's recommendations with several modifications to enable us to detect low abundance microRNAs. For the reverse transcription (RT) reaction 20 nanograms of total RNA was used. For the qPCR reaction the resultant cDNA was diluted 1:2. Each RT step was performed in duplicate and the qPCR in triplicate for each RT reaction. U6b and U66 small RNAs were used as endogenous controls and relative microRNA quantity calculated by the  $\Delta\Delta CT$  method. CDK6 expression was measured by qRT-PCR using Taqman reagents (ABI, Foster City, CA) and amplified on a ABI 7700 cyclor as above. GAPDH was used as an endogenous control and relative CDK6 quantity calculated by the  $\Delta\Delta CT$  method.

### Luciferase assays and western blotting

Luciferase experiments were done using the the siCHECK dual luciferase system (Promega, Madison, WI). The putative miR 124 target sites from the CDK6 3'UTR were cloned into the 3'UTR of the *Renilla* luciferase gene using the Xho and Not 1 enzyme sites (Fig. 2b) [16]. Target sites were amplified by PCR and mutations introduced by alternate primers. Cells were transfected with 0.5 ug siCHECK using LT1 (Mirus, Madison, WI) and *Renilla* luciferase activity was measured 48 h after transfection and normalized to firefly luciferase per the manufacturer's recommendations. Some cells were co-transfected with 50 or 100 nM miR 124 microRNA oligonucleotide (Ambion, Texas).

Western blotting was performed as previously described [15]. Briefly, cells were transfected with 100 nM control or miR 124 oligonucleotides (Ambion) and protein harvested in RIPA buffer 48 h later. Protein was resolved by SDS-PAGE and transferred to PVDF membrane. Blots were probed with mouse monoclonal anti-CDK6 (Cell Signaling Technology, Boston, MA), at 1:1000 dilution, striped and re-probed with mouse monoclonal anti-actin antibody (Cell Signaling Technology, Boston, MA).

### Cell proliferation and apoptosis

To assay for cell proliferation, D283 cells were transfected with control miR or miR 124 and 10,000 cells/well seeded in triplicate in to 96 well plates in 100 ul of medium. After 2 days, 100 ul of Cell Titer AQueous (Promega, Madison, WI) was added and cells incubated for 1 h and the absorbance measured at 490 nm using a microplate reader per the manufacturers recommendations. Relative cell number was calculated by normalizing the absorbance to untreated cells.

To assay for apoptosis, caspase activation was measured. The Caspase-Glo 3/7 assay kit (Promega) was used according to the manufacturer's instructions. D283 cells were treated and seeded in to 96 well plates as above. After 48 h, 100 ul/well Caspase-Glo reagent was added. Following a 1 h incubation room temperature, samples were read on a microplate luminometer and normalized to blank wells.

## Results

### CDK6 is over-expressed in medulloblastoma cells

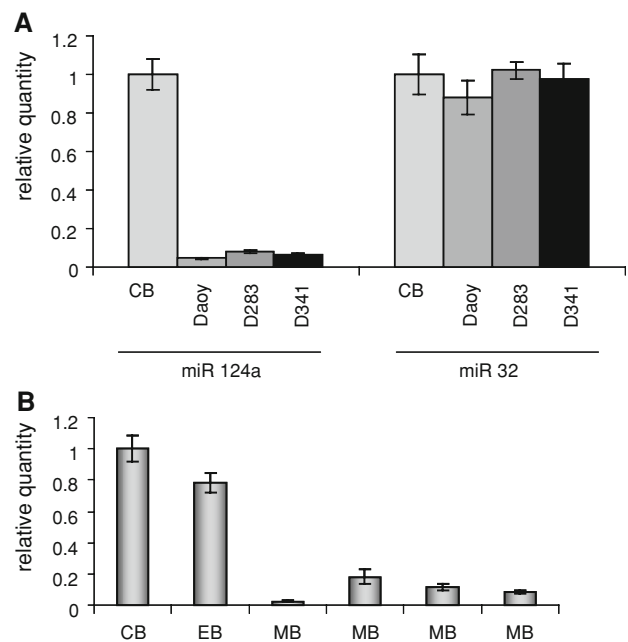
We first evaluated the expression of CDK6 in a panel of medulloblastoma cell lines and primary cells using quantitative RT-PCR. Compared to normal adult cerebellum, several common medulloblastoma cell lines (D283, Daoy, D341) demonstrated increased expression of CDK6 mRNA (data not shown). This is consistent with previous studies showing over-expression of CDK6 in medulloblastoma [5]. Next we identified putative miR sites in the CDK6 UTR by using three bio-informatic algorithms (TargetScan v4.0, PICTAR, and miRBase) [17–19]. Among the potential microRNA (miR) sites were two sites for microRNA 124 (Fig. 2a). We chose to further examine microRNA 124 since it is known to be associated with a differentiated neurogenic phenotype [20, 21].

### MicroRNA 124 expression is decreased in medulloblastoma

First we examined the expression of miR 124 expression in three commonly used medulloblastoma cell lines. Expression of miR 124 was significantly decreased in Daoy, D283 Med and D341 Med compared to normal adult cerebellar RNA (Fig. 1a). As expected, expression of miR 32, a non-brain enriched microRNA, was not significantly different in the medulloblastoma cell lines compared to normal cerebellum. To further evaluate miR 124 in medulloblastoma, we also examined expression in a panel of primary cell explants we have established from patient tissue and previously described [15]. MiR 124 expression was indeed decreased in the primary cells, although not to the same extent as the cell lines (Fig. 1b).

### MicroRNA 124 targets CDK6 in medulloblastoma

Next we further investigated putative miR 124 targets in CDK6. We identified 2 putative miR 124 binding sites in the CDK6 3'UTR, one which is well conserved (seed position 1648–1654 of CDK6 3'UTR) and the other less so (seed position 7788–7794 of CDK6 3'UTR) (Fig. 2a). To establish a functional association between miR 124 and CDK6 in



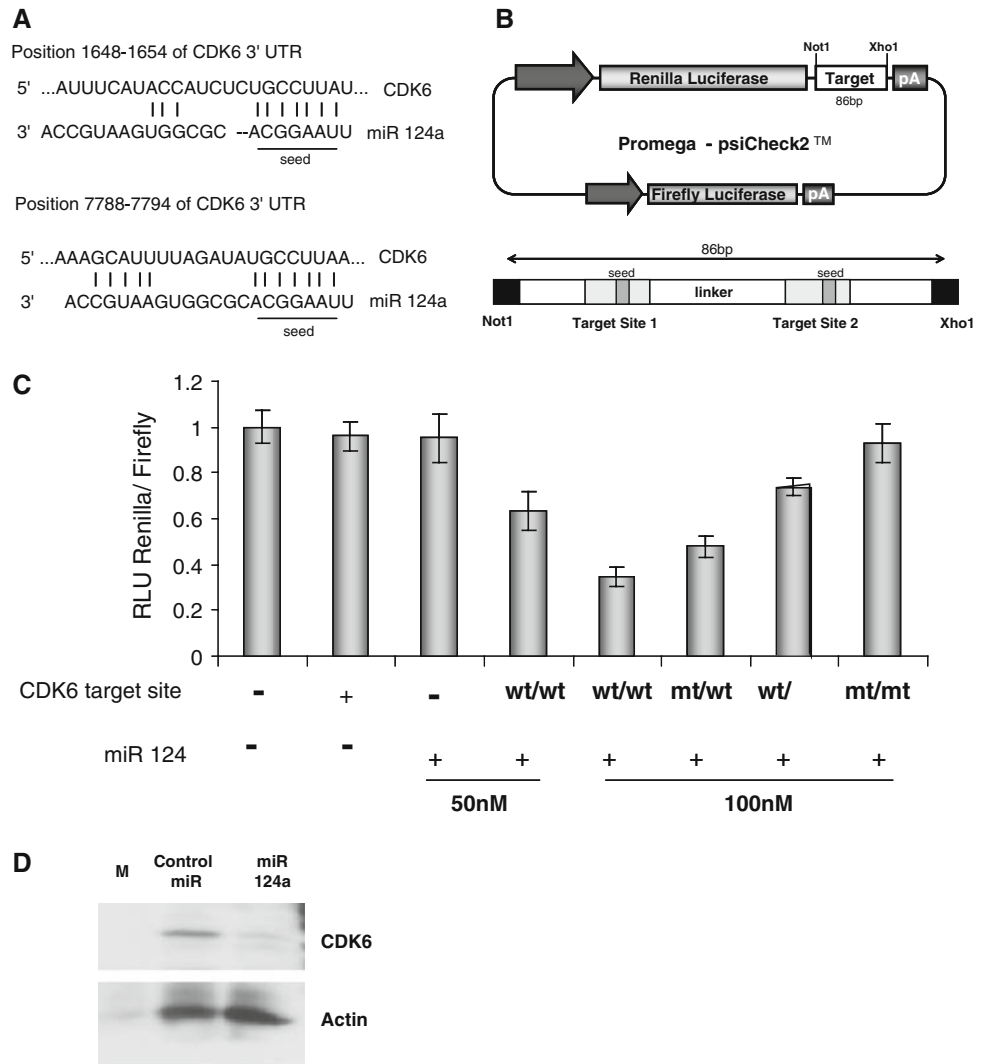
**Fig. 1** Expression of microRNA 124 in medulloblastoma as measured by stem-loop qPCR. **(a)** MiR 124 expression is decreased in common medulloblastoma cell lines Daoy, D283 and D341 compared to normal cerebellum (CB). MiR 32, a non brain enriched microRNA is unchanged. **(b)** MiR 124 expression is significantly decreased in four freshly isolated medulloblastoma cell explants (MB) compared to cerebellum (CB) and embryonic brain (EB). Error bars indicate standard error

medulloblastoma we performed analysis of the putative miR 124 binding sites from the CDK6 3'UTR using luciferase assays as previously described [16]. Target sites were amplified and cloned into the Renilla luciferase 3'UTR of the siCHECK vector (Fig. 2b). The siCHECK vector was then transfected alone or with miR 124 precursor oligonucleotides into Daoy cells. These microRNA precursor molecules are designed to mimic endogenous microRNAs (Ambion, TX). Luciferase activity measured after 48 h. Renilla luciferase activity was normalized to firefly luciferase activity. If miR 124 targets CDK6 then we would expect to see a decrease in the Renilla/firefly luciferase ratio. Indeed, co-transfection of siCHECK-CDK6 with miR 124 oligonucleotides (100 nM) decreased firefly luciferase activity to 34% of the vector alone control (Fig. 2c).

A key component of microRNA binding to its targets is the "seed" region [17]. To confirm the specificity of miR 124 binding to CDK6 target sites and to evaluate the relative contribution of each target site to microRNA mediated silencing, we introduced mutations in each of the target seeds alone or in combination (Fig. 2b). Mutations consisted of inversion of the 5 central nucleotides of the seed match. Mutation of site 1 alone (mutS1, position 1648–1654) resulted in luciferase activity that was 50% of control compared to 34% for the wild type sites [22] (Fig. 2c). This

**Fig. 2** MiR 124 targets CDK6 In Medulloblastoma cell lines.

(a) MiR 124 target sites in the CDK6 3'UTR as predicted by TargetScan. (b) Schematic of Luciferase vector used to assay miR 124 targeting. An 86 bp DNA segment containing both target sites with wild type [22] or mutated (mt) seed matches was cloned into the Not1-Xho1 sites in the Renilla luciferase UTR. (c) Luciferase reporter experiments with the above siCHECK vector containing the Renilla luciferase-CDK6-3'UTR and the control firefly luciferase and the corresponding mir 124 oligonucleotides. Data is a summary of 3 independent experiments, each performed in triplicate. (d) Western blot analysis of CDK6 protein in Daoy cells transfected with control or miR 124 oligonucleotides



difference was statistically significant ( $P < 0.01$ , t-test). Mutation of site 2 (mutS2, position 7788–7794) alone increased activity to 73% of control, and mutation of both sites (mutS1, S2) restored luciferase activity to 93% of control. Both were statistically significant increases compared to the wild type sites (ANOVA,  $P < 0.001$ ).

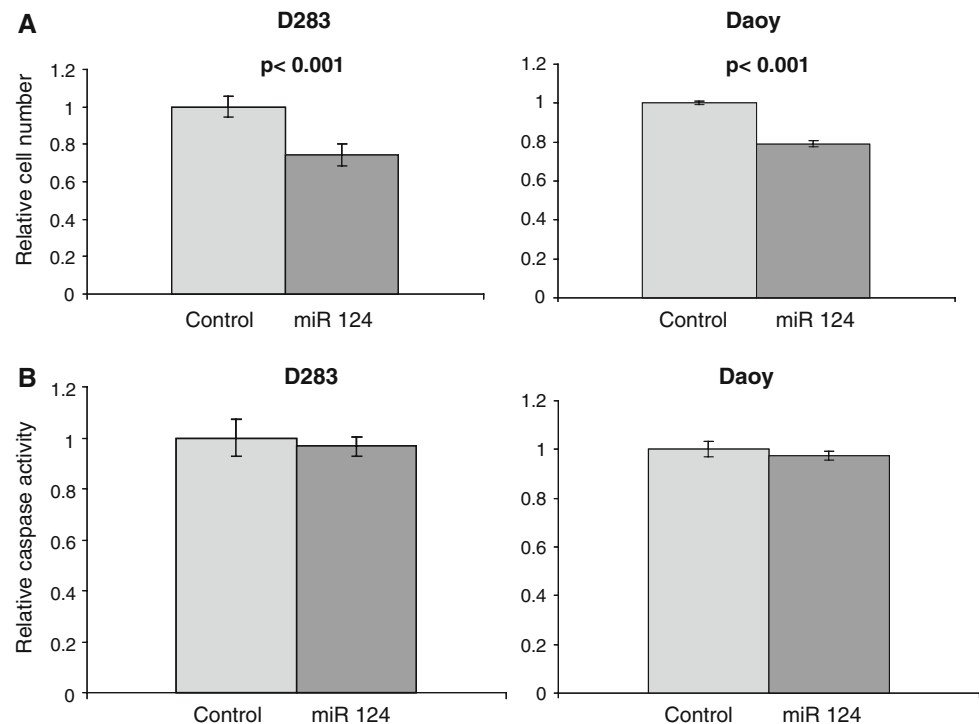
To further substantiate CDK6 as a target of miR 124, we transfected Daoy cells with miR 124 precursors followed by western blotting for CDK6. Re-expression of miR 124 in Daoy medulloblastoma cells decreased expression of CDK6 protein (Fig. 2d). Together these data indicate that CDK6 is a target for miR 124 in medulloblastoma.

#### MicroRNA 124 alters medulloblastoma cell proliferation but not apoptosis

We next examined the impact of miR 124 re-expression on medulloblastoma cell growth. We transfected 50 nM of miR

124 RNA oligonucleotides (Ambion) into D283 and Daoy medulloblastoma cells followed by analysis using the Cell Titer AQueous MTS assay 48 h later. Scrambled oligonucleotides purchased from Ambion were used as a negative control. All transfections and MTS assays were performed in triplicate in 96 well plates. As shown in Fig. 3a, transient transfection of miR 124 RNA oligonucleotide significantly decreases medulloblastoma cell growth by 24% in D283 ( $P < 0.001$ ) and 20% ( $P < 0.001$ ) in Daoy cells. This decrease is consistent with other studies of microRNA effects on cell growth [23]. We sought to evaluate whether the effect of cell growth was related to apoptosis. D283 and Daoy cells were transfected as above and harvested after 48 h. Cells were assayed for caspase 3/7 activity using the Caspase-Glo kit (Promega). There was no change in caspase activity suggesting that caspase mediated apoptosis was not a key factor in the miR 124 mediated decrease in medulloblastoma cell growth (Fig. 3b).

**Fig. 3** Suppression of Growth by miR 124. **(a)** Analysis of D283 and Daoy medulloblastoma cell growth using the MTS assay. Y axis is arbitrary units corresponding to metabolically active cells 48 h after transfection with microRNA. Data is summary of 2 independent experiments each done in triplicate. **(b)** Activity of Caspase 3/7 in control or miR 124 transfected cells. Y axis is arbitrary units corresponding to luminescence released by caspase activity. Data is summary of 2 independent experiments each done in triplicate. Error bars indicate standard error



#### MicroRNA 124 is significantly decreased in medulloblastoma patient samples

To extend these findings to medulloblastoma tumors, we compared miR 124 expression in 10 patient tissue samples relative to normal cerebellum by quantitative RT-PCR. When compared to normal cerebellum, all 10 samples expressed less miR 124 (Fig. 4a). Analysis of variance confirmed that this difference was statistically significant ( $P < 0.001$ ). Interestingly, seven samples had greater than 85% reduction while three samples had only 50–60% reduction. This could be due to the heterogeneity of the biopsy material. We also measured CDK6 expression by qRT-PCR in the same sample set. Compared to normal cerebellum, CDK6 expression was elevated in 6/10 samples with 2 samples demonstrating greater than 6 fold increase (Fig. 4b). Interestingly six samples with very low miR 124 expression all had significantly increased CDK6 mRNA expression (Fig. 4c). On the other hand one of the intermediate level miR 124 expression samples also had very elevated levels of CDK6 mRNA possibly due to genomic amplification. One drawback to our studies was that we were unable to measure CDK6 protein levels from our clinical samples which would have been a better reflection of miR 124 activity. This will be an important point to address as we further investigate the connection between miR 124 and CDK6.

#### Discussion

Our data strongly indicate that CDK6 is regulated by microRNA 124 in medulloblastoma. MiR 124 expression is

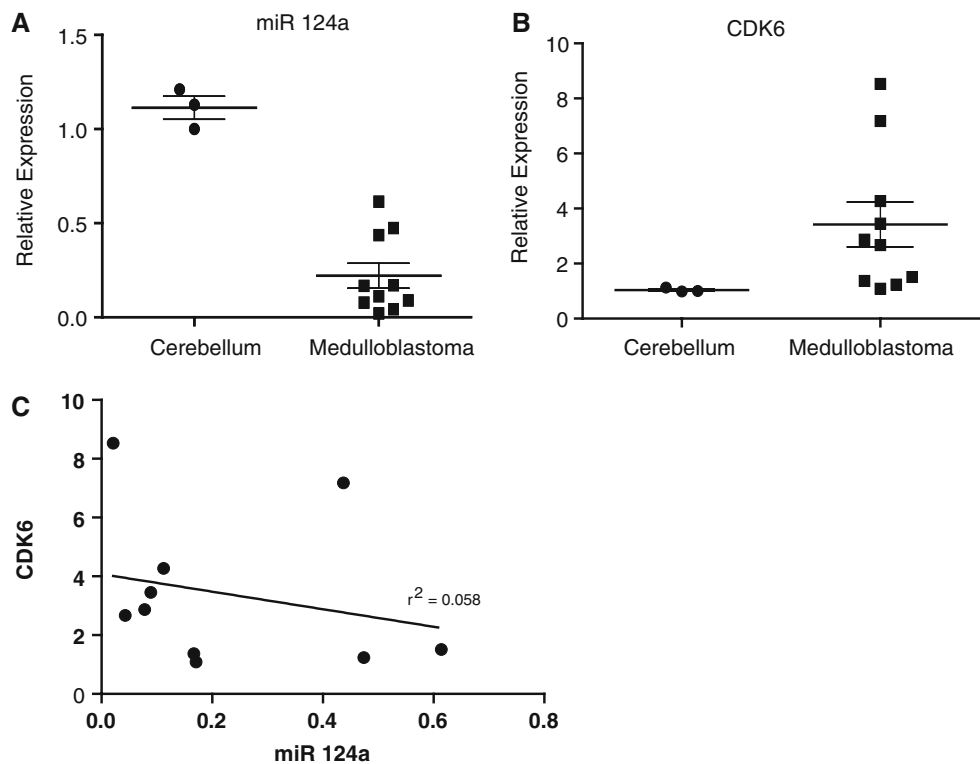
significantly decreased in medulloblastoma and re-expression of miR 124 in medulloblastoma cells significantly decreases proliferation.

MicroRNAs are key regulators of many neuronal functions [24]. MicroRNA 124 constitutes 26% of all microRNAs in the mature cerebellum [25]. Neural progenitors express very low levels of miR-124. However it is highly expressed in differentiating and mature neurons [26, 27]. The exact function of miR 124 in the CNS is unclear. In HeLa cells miR 124 decreases the levels of many non-neuronal transcripts and promotes a neuronal-like gene expression profile [20]. In addition miR 124 expression is regulated by RE1 silencing transcription factor (REST) [21]. Interestingly, REST is over expressed in many medulloblastoma tumors and can induce medulloblastoma in conjunction with myc in mouse models [28]. It will be interesting to examine if the REST induced tumor formation is mediated in part by suppression of miR 124. In addition miR-124 targets small C-terminal domain phosphatase 1(SCP1), suppresses SCP1 expression and induces neurogenesis in P19 cells [29]. These and other findings indicate that miR 124 is involved in the transition from proliferation to differentiation in neuronal tumors.

We show that miR 124 is expressed at very low levels in medulloblastoma cell lines and tumor tissues. Given that miR 124 is associated with differentiated neuronal phenotypes, one explanation is that the medulloblastoma cells and tissues have a less differentiated phenotype than the adult cerebellum controls. Furthermore we demonstrate that miR 124 targets CDK6, a critical pro-proliferative protein that is an established adverse prognostic indicator in medulloblastoma. Our data is



**Fig. 4** Dysregulated miR 124 expression in medulloblastoma patient samples. **(a)** Expression of miR 124 in 10 patient samples compared to normal cerebellum as determined by stem-loop qRT-PCR. **(b)** Expression of CDK6 mRNA in the same set of 10 medulloblastoma samples compared to normal cerebellum. **(c)** Comparison of miR 124 expression (x axis) to CDK6 mRNA expression (y axis) in the medulloblastoma samples



consistent with a recent report that miR 124 targets CDK6 in colon cancer cells [30]. Interestingly they reported only one miR 124 target site (position 1648–1654) in CDK6. This site is the most highly conserved site across multiple species. We experimentally established that there are two sites and that the distal site (position 7788–7794) contributes more to CDK6 silencing in human medulloblastoma cells. Our data are consistent with the bio-informatic predictions (TargetScan) [17].

In addition to its role in cell proliferation, CDK6 is also postulated to be involved in cell differentiation [31]. This is different from other cyclin dependent kinases such as CDK2 and CDK4. For example, over-expression of inhibitor resistant CDK6 prevents murine erythroid leukemia cells from undergoing differentiation upon stimulation [32].

It is possible to envision a model where miR 124 down regulates CDK6 in the cerebellum to promote orderly cellular differentiation. Deregulation of such control could lead to unchecked cellular proliferation thus predisposing cells to increased tumor forming potential.

Our data suggest that miR 124a dose not affect apoptosis in medulloblastoma. MicroRNAs have recently been shown to have many other functions in cancer phenotypes. For example the miR 17p cluster controls angiogenesis in colon adenocarcinoma, while let-7 seems to control tumor stem cell self renewal in breast cancer [33, 34]. Identification of the cellular phenotypes controlled by miR 124a will be critical in further understanding its role in medulloblastoma.

In summary, our data suggest that miR 124 may play an important role in medulloblastoma pathogenesis by acting as a tumor suppressor and regulating among other genes, CDK6. We are now investigating the biological impact of miR 124 expression on medulloblastoma formation in vivo to further elucidate its role in tumor formation in the cerebellum.

**Acknowledgements** This work was supported by the Department of Pediatrics, University of Iowa and the Bear Necessities Cancer Research Fund. We thank Dr. B.L. Davidson for her mentorship and guidance, and Zita Sibenaller PhD, Department of Neurosurgery, University of Iowa for tumor banking and isolation of primary explants.

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