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Research Paper

The miR-183~96~182 cluster promotes tumorigenesis in a mouse model of medulloblastoma

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Abstract

Medulloblastoma is the most common malignant pediatric brain tumor. Some are thought to originate from cerebellar granule neuron progenitors (CGNPs) that fail to undergo normal cell cycle exit and differentiation. The contribution of microRNAs to the initiation and progression of medulloblastoma remains poorly understood. Increased expression of the miR-183~96~182 cluster of microRNAs has been noted in several aggressive sub−groups. We identified that expression of miR-183~96~182 was higher in medulloblastomas with *Pten* gene loss in the background of the activated sonic hedgehog (Shh) signaling pathway. Ectopic miR-183~96~182 expression in CGNPs synergized with exogenous Shh to increase proliferation and its role depended on hedgehog signaling ac−tivation. Our findings suggest a new microRNA cluster, the miR-183~96~182, functionally collaborates with the Shh signaling pathway in the development of medulloblastomas in mice.

Keywords: medulloblastoma, microRNAs, Shh pathway, granule neuron progenitors, proliferation

INTRODUCTION

Medulloblastoma is a tumor of the cerebellum and the most common malignant pediatric brain tumor. Medulloblastoma occurs most frequently in children between the ages of 5 and 10 years but may occur in adults as well[1-3]. Although 5-year in overall survival rates have reached 60%-80%, the prognosis for many medulloblastoma patients remains bleak. Many pa−tients who develop the disease die from it and survi−vors, after conventional treatment regimens (surgery, radiotherapy, and chemotherapy), often face a variety of long-term neurological, neuroendocrine, and social sequelaes[2-4]. It is therefore imperative for us to gain a deeper understanding of the molecular pathogenesis driving medulloblastomas, in order to improve ap−proaches for treating medulloblastomas.

MicroRNAs (miR) are small endogenous noncod−ing RNAs that play important roles in many bio−logical processes, including tumorigenesis[5]. These 21-23 nucleotide (nt) RNAs are matured through a 2-step biogenesis mechanism from long, RNA polII transcripts[6]. Mature microRNAs are incorporated into the RNA-induced silencing complex, where they anneal to their recognition sequences in the 3'-UTR of mRNA genes to attenuate gene expression through translational repression or mRNA degradation[7]. Sev−eral genomic studies have revealed the differential ex−pression of microRNAs across various molecular sub−groups of medulloblastoma. As previously reported, the miR-17/92 polycistron was identified as a putative medulloblastoma oncomir and associated with the

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sonic hedgehog-driven medulloblastoma compared with other subgroups[8,9].

In different comprehensive analyses of the human and mouse medulloblastoma oncomir, the miR-183~96~182 cluster is upregulated in several subgroups, including particularly aggressive clinical course involving high rates of metastasis and overall poor survival[10]. Based on the studies of cell lines, Weeraratne et al. found that the knockdown of the full miR-183~96~182 cluster results in the enrichment of genes associated with apoptosis and dysregulation of the PI3K/AKT/mTOR signaling axis. However, the specific role of miR-183~96~182 cluster in medulloblastoma and cerebellar development has not been described[11,12].

Cerebellar granule neuron progenitors (CGNPs) are proposed cells of origin for a subset of medulloblastomas. CGNPs undergo rapid Shh-dependent expansion perinatally in mice and humans, and excessive Shh pathway activity promotes medulloblastoma[13,14]. We used the $Ptch1^{+/-}$; $Pten^{Floxp/+}$; $GFAP$-Cre mouse model of medulloblastoma to study the effect of the PI3K/Akt signaling pathway activated on medulloblastoma tumorigenesis. We found that the miR-183~96~182 cluster was highly expressed in this $Pten$ and $Ptch1$ loss associated medulloblastoma and promote tumorigenesis by controlling CGNP proliferation.

MATERIALS AND METHODS

Animal

$Ptch1^{+/-}$ mice were purchased from Jackson Laboratory[15]. C57BL/6 and $Pten^{Floxp/Floxp}$ mice were purchased from the Model Animal Research Center of Nanjing University[16]. $GFAP$-Cre mice were gifts from Minsheng Zhu′s laboratory (Nanjing University, Nanjing, Jiangsu, China). $PtenFloxp/Floxp$ mice were crossed with $Ptch1^{+/-}$ mice. The,n $Ptch1^{+/-}$; $Pten^{Floxp/Floxp}$ offspring mice were crossed with $GFAP$-Cre mice to obtain $Ptch1^{+/-}$; $Pten^{Floxp/+}$; $GFAP$-Cre mice. Mice were observed for symptoms of medulloblastoma at least twice-weekly for 12 months. Transgenic expression patterns of $GFAP$-Cre were examined with a R26R reporter line (129S4/SvJaeSorGt(ROSA)26Sortm1(FLP1)Dym, (Jackson Laboratory, Bar Harbor, Maine, USA) carrying a $lacZ$ gene whose expression requires excision of loxP-flanked stop sequences. All mice were housed in a specific pathogen free animal room. The study protocol was approved by the local institutional review board at the authors′ affiliated institutions. Animal welfare and the experimental procedures were carried out strictly in accordance with the Guide for Care and Use of Laboratory Animals (National Research Council of USA, 1996).

Histological study

For histological analysis, mice were perfused with PBS followed by 4% paraformaldehyde. The cerebella were removed, fixed in 4% paraformaldehyde overnight, and transferred to gradient ethanol for dehydration and embedded in paraffin. Sections (5 m) were stained with hematoxylin and eosin (Sigma, St Louis, MO, USA). Frozen sections (20 mm) were stained for β-galactosidase according to a previously reported method[17].

RT-PCR

Total RNA from mouse tissues was extracted using the RNAiso reagent (TaKaRa, Osaka, Japan). Stem-loop RT-PCR primers were designed as previously reported[18]. The U6 RNA was used for normalization. Stem-loop RT primers and PCR primers were synthesized by Invitrogen (Carlsbad, CA, USA). Reverse tran scription was performed using the PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa). The cycling condition of PCR was 95°C for 5 minutes, followed by 30 amplification cycles of 95°C for 15 seconds and 60°C for 1 minute. Stem-loop RT primers sequences for these microRNA were listed as follows: mmu-mir-183-RT: 5′- GTCG−TATCCAGTGCAGGGTCCGAGGTATTCGCACT−GGATACGACAGTGAA-3′; mmu-mir-96-RT: 5′-GTCGTATCCAGTGCAGGGTCCGAGGTAT−TCGCACTGGATACGACAGCAAA-3′; and mmu-mir-182-RT: 5′-GTCGTATCCAGTGCAGGGTC−CGAGGTATTCGCACTGGATACGACCGGTGT-3′. Forward PCR primers sequences for these microRNAs are: mmu-mir-183-FR: 5′-AGCCGTATGGCACT−GGTAGAA-3′; mmu-mir-96-FR: 5′-AGCCGTTT−GGCACTAGCACATT-3′; mmu-mir-182-FR: 5′-AGCCGTTTGGCAATGGTAGAACTC-3′.

Culture of CGNPs

Granule neuron progenitors were purified from the cerebellum of 7-day-old C57BL/6 pups by using a modified protocol as previously reported[19,20]. Briefly, postnatal cerebella were triturated into single-cell suspensions that were loaded onto a step gradient of 35% and 65% Percoll (Amersham-Bioscience, Uppsala, Sweden) and separated by high-speed centrifugation for 10 minutes at 4°C. Granule cells and precursors were harvested from the 35/65% interface, washed in PBS/DNase, and further purified by depleting adherent cells with 2-hour incubation on normal tissue culture dishes. Their purity (> 95%) was assessed by immunostaining with markers of neurons and glia. Cells (5×10^5 per well) were plated in four-well Lab-Tek

chamber slides precoated with 100 μg/mL poly-D-lysine (Millipore, Billerica, MA, USA) and Matrigel (Beckton Dickinson, Bedford, MA, USA) in neural basal medium supplemented with 0.45% glucose, SPITE (Sigma), oleic acid albumin/linoleic acid (Sigma), B27 (Invitrogen), and N-acetyl cysteine (Sigma).

Retrovirus production and infection

To generate a mmu-miR-183~96~182 cluster expression clone, the micoRNA cluster was PCR-amplified from genomic DNA of C57BL/6 mouse and cloned into pENTR/D-TOPO plasmid (Invitrogen), then transferred into MSCV-P1G-gateway vector (Clontech, Mountain View, CA, USA) by Gateway LR Clonase II enzyme mix (Invitrogen). When CGNPs became attached, 250 μL medium was removed and 250 μL medium containing virus was added. Infection was repeated 3 times with 2 hours each time. The next day, the medium was changed for fresh complete medium with or without 10% ShhN conditional medium (ShhN CM), which was generated by transfecting 293T cells with a ShhN expression construct[21].

EdU staining

Identification of purified granule cells in the S phase was performed by incubation with Click-iT EdU Alexa Fluor 555 Imaging Kit (Invitrogen). EdU was added for 12 hours before fixation. EdU detection was performed according to the manufacturer´s instructions (Invitrogen). Stained chambers covered with mounting medium containing DAPI (Sigma) were enumerated under a microscope. TIFF images of 4 random fields were taken for each experimental group by using the 20 × objective lens. The counting of EdU-positive and DAPI-positive CGNPs number was quantified by using ImageJ software. The kinetics of the proliferative response to mmu-miR-183~96~182 was analyzed by using GraphPad Prism 5.

Statistical analysis

CGNPs proliferation at each time point was measured as the mean ratio (EdU-labeling to DAPI-labeling cell number) from 4 random fields. Statistical significance of proliferation was determined by using Student´s *t*-test.

RESULTS

Heterozygosity for *Pten* promotes medulloblastoma in cooperation with Hedgehog signaling pathway

The founders of GFAP-Cre and their offspring were identified by PCR genotyping. Transgenic expression patterns of Cre were examined with a Rosa26 reporter line, 129S4/SvJaeSor-Gt(ROSA)26Sortm1(FLP1)Dym, carrying a *lacZ* gene whose expression requires excision of *Floxp*-flanked stop sequences. β-galactosidase staining revealed GFAP-Cre mediated recombination both in astrocytes and immature precursor cells of the external granular layer (EGL) in the developing cerebellum of 7-day old mice (**Fig. 1A**). To explore the role of *Pten* loss in the background of *Ptch1*^{+/-} mice, we administered the GFAP-Cre transgenic mice to delete Pten in CNS lineages by using conditional knockout mice *Pten*[Floxp/Floxp]. Broad CNS deletion of *Pten* results in lethal hydrocephalus in early postnatal life; modeling efforts henceforth emphasized the *Pten*[Floxp/+] GFAP-Cre genotype.

In *Pten*[Floxp/+] mice, part of *Ptch1* exon 1 (including putative start codon) and all of exon 2 were replaced with *lacZ* and a neomycin resistance gene in one allele[15]. Approximately 17.6% (3 out of 17 *Pten*[Floxp/+]) of mice expressing one allele of *Ptch1* developed symptoms of medulloblastoma by 1 year of age. Most of these mice died 5 months after birth[22,23]. However, *Pten*[Floxp/+] *Pten*[Floxp/+] *GFAP-Cre* mice died of medulloblastoma from 2 months after birth. We found that, between 2 to 8 months of ages, 18/23 (78.2%) of the *Pten*[Floxp/+] *Pten*[Floxp/+] *GFAP-Cre* mice presented with acute-onset neurological symptoms. Similarly to *Ptch1*^{+/-} mice, these mice began to display signs of illness, including domed head, hunched back, abnormal gait and/or decreased movement (data not shown). Histopathologically, these mice harbored malignant medulloblastoma. Tumor cells, regardless of whether the mouse was *Ptch1*^{+/-} or *Ptch1*^{+/-} *Pten*[Floxp/+] or *GFAP-Cre*, arose from the surface of the cerebellum and invaded the cortex and deep white matter of the cerebellum (**Fig. 1B**, panel a and b). The tumors in the *Ptch1*^{+/-} mice showed small round or ellipsoid cells with a high nuclear-to-cytoplasmic ratio and round-to-oval or triangular hyperchromatic nuclei, corresponding to patterns of classic medulloblastomas in humans (**Fig. 1B**, panel c). In contrast, the tumors from *Ptch1*^{+/-}*Pten*[Floxp/+] *GFAP-Cre* mice had a biphasic architecture that nodular areas consisted of differentiated cells with round nuclei and less condensed chromatin, and were intermingled with the undifferentiated hyperchromatic cells (**Fig. 1B**, panel d). The nodular areas were surrounded with desmoplasia, a pericellular deposition of collagen in this context, similarly to the appearance of desmoplastic medulloblastomas in humans. However, none of the *Pten*[Floxp/+] *GFAP-Cre* mice developed medulloblastoma. Therefore, in cooperation with the hedgehog signaling pathway, heterozygosity for *Pten* promoted medulloblastoma. Medul-
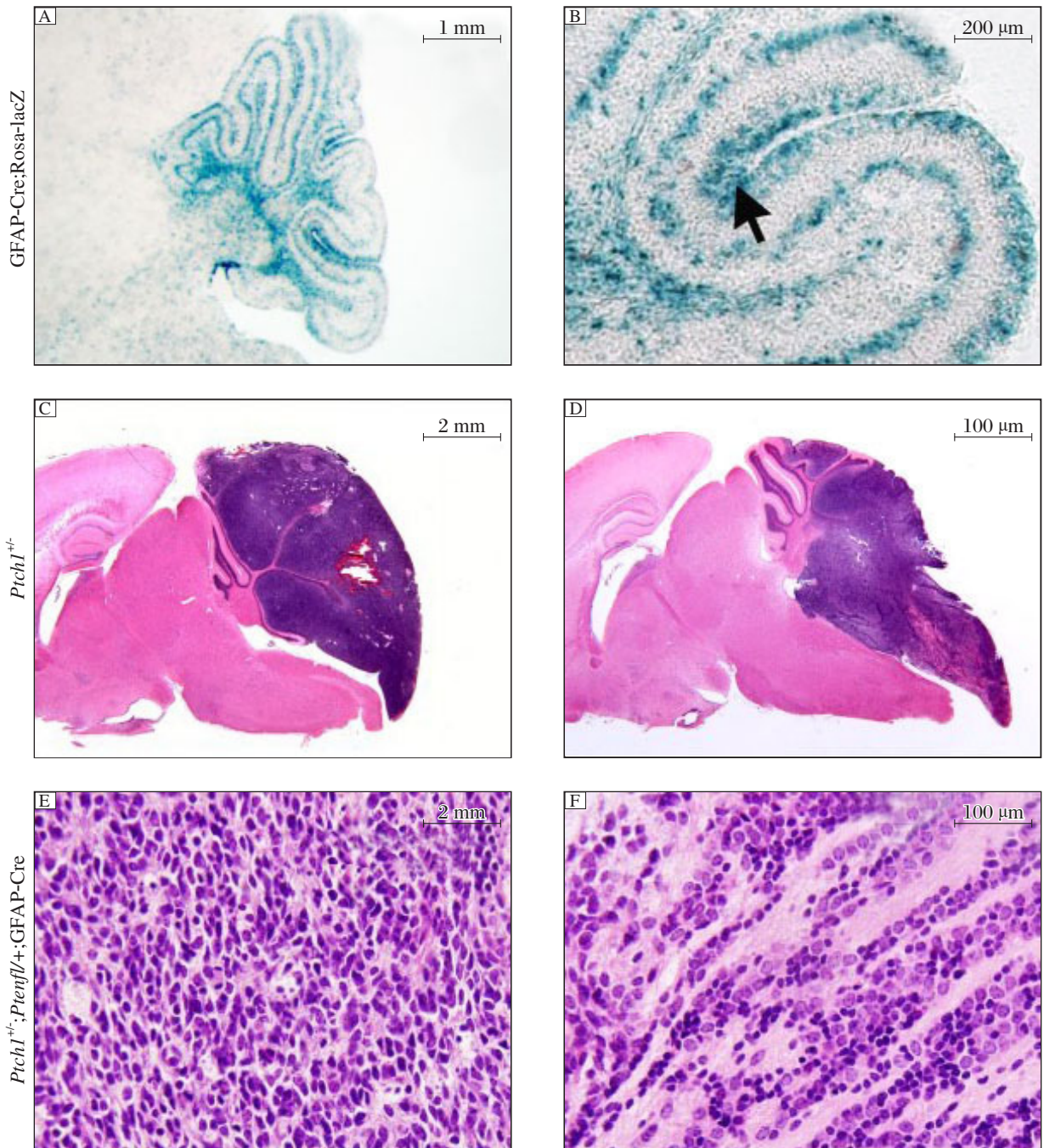
Fig. 1 Heterozygosity for Pten promotes medulloblastoma in the background of *Ptch1*. A: β-galactosidase staining in GFAP-Cre; Rosa26-lacZ mice of P7. B: Higher magnification of (A). Positive for β-galactosidase staining (blue reaction product) was found both in astrocytes and in immature precursor cells of the external granular layer (arrow) in the developing cerebellum. C: H&E staining of histological sections of rep–resentative malignancies in *Ptch1*^{+/-} medulloblastomas. D: H&E staining of histological sections of representative malignancies in *Ptch1*^{+/-}; *Pten*[Floxp/+]; GFAP-Cre medulloblastomas. E: Higher magnification of (C). F: Higher magnification of (D).

loblastomas induced by mutations in the Shh pathway has been reported to originate from long-lasting pro–liferative CGNPs and are located within the cerebellar hemispheres[13,24,25]. Therefore, we speculated that the *Ptch1*^{+/−} *Pten*[Floxp/+] *GFAP-Cre* medulloblastomas arise from CGNPs.

Mouse medulloblastoma with heterozygosity for Pten and Ptch1 overexpresses microRNAs from the miR-183~96~182 cluster

The miR-183~96~182 cluster is a highly conserved cluster of microRNAs residing on an intergenic region

of human chromosome 7q, which is commonly gained in medulloblastoma. The miR-183~96~182 cluster has been largely reported as pro-tumorigenic/prometastatic effects in cancers. Increased expression of the miR-183~96~182 cluster of microRNAs has been reported in several subgroups of medulloblastoma, including the most clinically aggressive subgroup associated with genetic amplification of *MYC* and activation of pho–toreceptor transcriptional programs[11,12]. Therefore, we investigated the expression of mmu-miR-183, mmu-miR-96 and mmu-miR-182 in these medulloblastomas. The genes for 3 microRNAs of the cluster, including miR-183, miR-96 and miR-182, were clustered within 4 kb on mouse chr6qA3, and transcribed in the same direction (telomere→centromere) (***Fig. 2A***). All the 3 microRNAs had a high degree of sequence homol–ogy and potentially overlapping mRNA targets (***Fig. 2B***). For the stem-loop RT-PCR assay, we found that the mmu-miR-183, mmu-miR-96 and mmu-miR-182

expressions were increased in $Ptch1^{+/-};Pten^{Floxp/+}$ *GFAP-Cre* medulloblastoma compared with $Ptch1^{+/-}$ medulloblastoma, especially the mmu-miR-183 and mmu-miR-96. However, as the same PCR cycles, the expression of these microRNAs had very low level in adult normal cerebellum of WT, *GFAP-Cre*, *PtenFloxp/+;GFAP-Cre*, $Ptch1^{+/-}$ and $Ptch1^{+/-}Pten^{-Floxp/+}$ *GFAP-Cre* mice (***Fig. 2C***). Therefore, we specu–lated that high expression of the miR-183~96~182 cluster was an important factor in promoting tumori–genesis in $Ptch1^{+/-}Pten^{Floxp/+}$ *GFAP-Cre* mice.

Enforced expression of miR-183~96~182 promotes CGNP proliferation in cooperation with the hedgehog signaling pathway

As CGNPs are the source of Shh-subtype medul–loblstomas, we asked that whether the overexpression of miR-183~96~182 promoted CGNP proliferation, and dysregulation of miR-183~96~182 was one of the
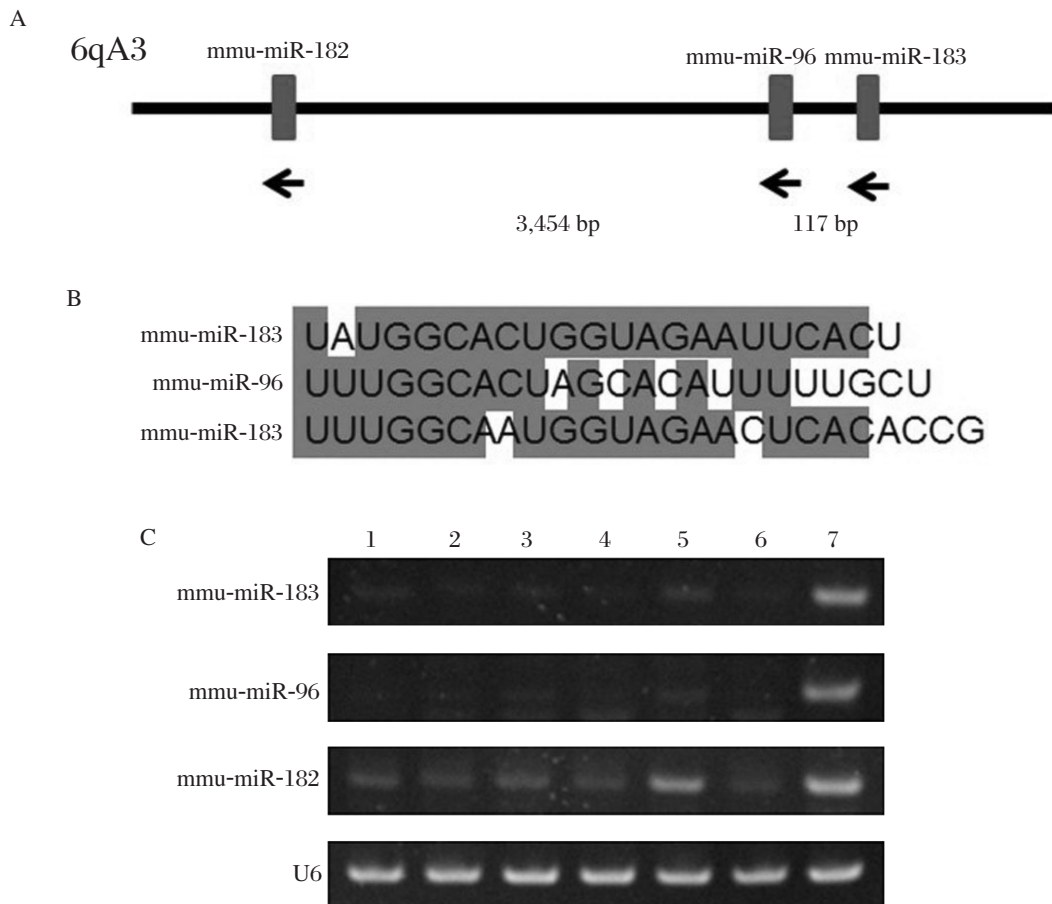

Fig. 2 **Overexpresses miRNAs from the miR-183~96~182 Cluster in $Ptch1^{+/-}$; $Pten^{Floxp/+}$; GFAP-Cre medulloblastomas.** A: Ge–nomic organization of the miR-183~96~182 cluster on mouse chr6qA3. The numbers show the spacing in each of the three species in genes. B: Sequence alignment for mouse miR-183, miR-96, and miR-182. C: RT-PCR detection of mature miR-183, miR-96, and miR-182 in adult normal cerebellum of WT, GFAP-Cre, $Pten^{Floxp/+}$;GFAP-Cre, $Ptch1^{+/-}$ and $Ptch1^{+/-}$;$Pten^{Floxp/+}$; GFAP-Cre mouse and medulloblastoma of $Ptch1^{+/-}$ and $Ptch1^{+/-}$;$Pten^{Floxp/+}$;GFAP-Cre mouse. The U6 RNA was used for normalization. Lane 1: WT cerebellum; Lane 2: *GFAP-Cre* cerebellum; Lane 3: $Pten^{fl/+}$; GFAP-Cre cerebellum; Lane 4: $Ptch1^{+/-}$ cerebellum; Lane 5: $Ptch1^{+/-}$ medullobastoma; Lane 6: $Ptch1^{+/-}Pten^{fl/+}$; GFAP-Cre cerebellum; Lane 7: $Ptch1^{+/-}Pten^{fl/+}$; GFAP-Cre medulloblastoma.

most important factors in *Ptch1*^{+/−} *Pten*[Floxp/+] *GFAP-Cre* medulloblastomas. We isolated CGNPs from the cerebellum of 7-day-old C57BL/6 pups and in−fected them with retroviruses expressing the mmu-miR-183~96~182 cluster or control with Retro-GFP for 12 hours and then cultured CGNPs with or without ShhN CM. After infecting retrovirus for 36, 48, 60 and 72 hours, we measured each CGNP proliferation by quantifying EdU staining, respectively (**Fig. 3A**). Consistent with previous studies, we found that cells maintained proliferation in the presence of shhN. Af−ter infection for 36 hours, the overexpression of miR-183~96~182 had little effect on high concentration of ShhN. During 48 to 60 hours, the overexpression of miR-183~96~182 cluster increased proliferation in ShhN CM-treated cells (**Fig. 3B**). With the prolonga−tion of the culture time, CGNPs grew with contact inhibition. The proliferation was reduced in the group of overexpressing miR-183~96~182 with ShhN CM at 72 hours. However, when cells were cultured in the absence of ShhN, they showed a decline in pro−liferation. In the beginning, the proliferation of miR-183~96~182-infected CGNPs was decreased slower than control at 36 hours, which then showed a con−tinuous decline. There were no significant differences for proliferation between miR-183~96~182-infected CGNPs and control-infected CGNPs from 48 hours (**Fig. 3C**). Therefore, miR-183~96~182 was unable to maintain cell proliferation in the absence of Shh, sug−gesting that its role in promoting CGNPs proliferation is dependent on hedgehog signaling activation.

DISCUSSION

Chromosome 10q deletion is frequently identified in human medulloblastoma, and is shown to be highly associated with survival probability. Chromosome 10q loss was primarily limited to Shh and group C tumors[26]. Adult Shh-medulloblastomas harboring 10q loss show particularly poor progression-free survival and overall survival probabilities[27]. *PTEN*, the tumor suppressor gene of phosphatase and tensin homolog, is located on the chromosomal region 10q23.31. We established a mouse model to present the impor−tant role for cross talk between *Pten* loss and sonic hedgehog signaling pathways in the pathogenesis of medulloblastoma.

We inactivated Pten during brain development in neuronal and glial cell populations in a precisely de−fined area of the cerebellum. It was achieved by us−ing GFAP-Cre transgenic mice, in which Cre is active in EGL, the Bergmann glia and white matter of the cerebellum. *Pten*[Floxp/Floxp] GFAP-Cre mice developed seizures and ataxia early in life and died prematurely,

which is similar to other previous studies[28,29]. Al−though PTEN is frequently inactivated in malignant human brain tumors, inactivation of Pten in the mouse brain does not lead to tumor development, which may due to the fact that additional genetic alterations are necessary. We used the *Ptch1-lacZ* mouse model of medulloblastoma to study the effect of *Pten* loss on medulloblastoma tumorigenesis. We found that the heterozygosity for *Pten*, in the context of hetero−zygosity for *Ptch1*, altered tumor histology and ac−celerated medulloblastoma tumorigenesis in *Ptch1*^{+/-} *Pten*[Floxp/+] GFAP-Cre mice. Although spontaneous medulloblastoma harbor only monoallelic mutations of *Pten* possess at least one functional wildtype Pten allele, they further or completely lose *Pten* protein immunoreactivity[30]. These results showed that epi−genetic silencing by aberrant promoter methylation, deregulated transcription and/or increased degradation may disrupt function and promote tumorigenesis. As a result, loss of *Pten* expression leads to activation of PI3K signaling: increased expression of activated Akt, phosphorylated on serine 473, in *Ptch1*^{+/-} *Pten*[Floxp/+] GFAP-Cre medulloblastoma (data not shown).

Here we examined the role of miR-183~96~182 in the context of heterozygosity for Pten and Ptch1 associated medulloblastoma. The miR-183~96~182 cluster that upregulated across *Ptch1*^{+/-} *Pten*[Floxp/+] *GFAP-Cre* medulloblastoma samples is relative to *Ptch1*^{+/-} medulloblastoma. MiR-183~96~182 has been implicated in retinal development and stem-cell maintenance[31-33]. With the expression of GFAP-Cre and recombination occur in scattered EGL cells be−fore neoplastic transformation, we concluded that in *Ptch1*^{+/-} *Pten*[Floxp/+] *GFAP-Cre* mouse model, medullob−lastomas arise from cerebellar granule neuron precur−sors retained on the cerebellar surface. CGNPs taken from the P6~7 cerebellums have greater proliferative capacity than those harvested later, when neuronal progenitors have already begun to exit the cell divi−sion cycle and migrate into the internal granular layer. When explanted into culture in the absence of ShhN, CGNP rapidly exit the cell cycle and differentiate, but ShhN additionally extends their proliferative po−tential[19]. CGNPs harvested from C57BL/6 mice and engineered to ectopically express miR-183~96~182 by retroviral transfer demonstrated a greater prolifera−tive advantage than their Retro-GFP counterparts in the presence of ShhN. However, its role in promoting CGNPs proliferation is dependent on hedgehog sign−aling activation. When cultured in vitro for 48 hours, CGNPs without ShhN-CM lost the effort of endog−enous ShhN and declined sharply in the proliferation, despite enforced expression of miR-183~96~182. Our
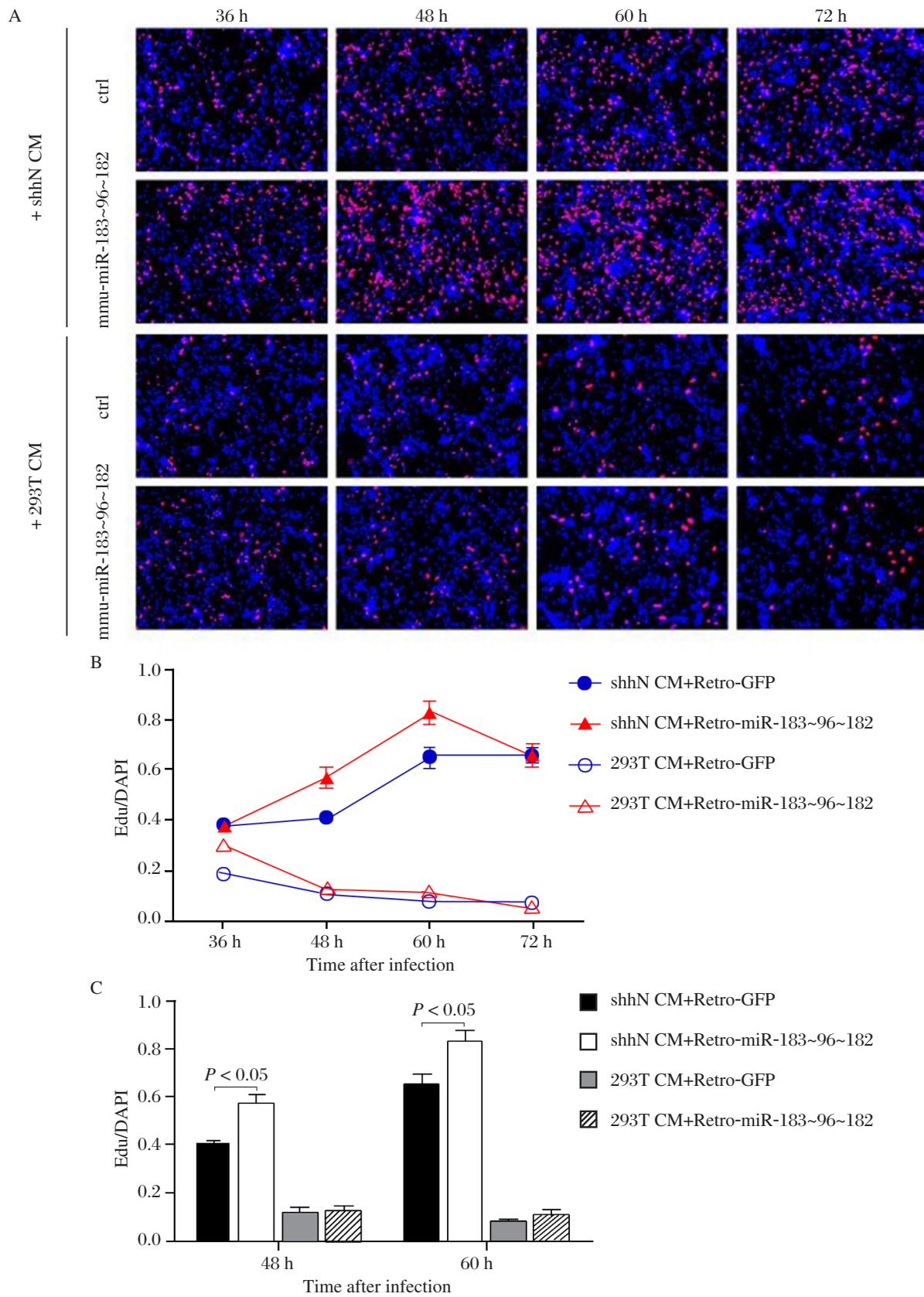
Fig. 3 Enforced expression of the miR-183~96~182 cluster promotes CGNPs proliferation. A: Immunofluorescence staining for EdU incorporation. Freshly isolated CGNPs were grown in poly-D-lysine and matrigel coated chamber slides in the presence of ShhN CM or 293T CM. The cells were infected with retroviral miR-183~96~182 or empty-control. B: Kinetics of proliferative response to miR-183~96~182 or empty-control in the presence ShhN CM or 293T CM. The numbers at each data point were counted from 4 random fields. C: Column diagram shows quantification of EdU-positive CGNPs after retroviral infection for 48 hours and 60 hours. The bars denote standard deviation.

data concluded that miR-183~96~182 was unable to maintain cell proliferation in the absence of ShhN. miR-183~96~182 could amplify the effects of Shh signaling, perhaps by inhibiting a transcriptional re−pressor of a subset of Shh signaling target genes.

In conclusion, our study provides evidence for an important role of miR-183~96~182 cluster in hetero−zygosity for *Pten* and *Ptch1* associated medullobla−tomas. Antagomirs to the miR-183~96~182 cluster might provide a potential new therapeutic strategy for patients with medulloblastomas harboring a constitu−tively activated PI3K signaling pathway.

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