The miR-17/92 Polycistron Is Up-regulated in Sonic Hedgehog–Driven Medulloblastomas and Induced by N-myc in Sonic Hedgehog–Treated Cerebellar Neural Precursors

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Abstract

Medulloblastoma is the most common malignant pediatric brain tumor, and mechanisms underlying its development are poorly understood. We identified recurrent amplification of the *miR-17/92* polycistron proto-oncogene in 6% of pediatric medulloblastomas by high-resolution single-nucleotide polymorphism genotyping arrays and subsequent interphase fluorescence *in situ* hybridization on a human medulloblastoma tissue microarray. Profiling the expression of 427 mature microRNAs (miRNA) in a series of 90 primary human medulloblastomas revealed that components of the miR-17/92 polycistron are the most highly up-regulated miRNAs in medulloblastoma. Expression of miR-17/92 was highest in the subgroup of medulloblastomas associated with activation of the sonic hedgehog (Shh) signaling pathway compared with other subgroups of medulloblastoma. Medulloblastomas in which miR-17/92 was up-regulated also had elevated levels of MYC/MYCN expression. Consistent with its regulation by Shh, we observed that Shh treatment of primary cerebellar granule neuron precursors (CGNP), proposed cells of origin for the Shh-associated medulloblastomas, resulted in increased miR-17/92 expression. In CGNPs, the Shh effector N-myc, but not Gli1, induced miR-17/92 expression. Ectopic miR-17/92 expression in CGNPs synergized with exogenous Shh to increase proliferation and also enabled them to proliferate in the absence of Shh. We conclude that miR-17/92 is a positive effector of Shh-mediated proliferation and that aberrant expression/amplification of this miR confers a growth advantage to medulloblastomas. [Cancer Res 2009;69(8):3249–55]

Introduction

Medulloblastoma, the most common malignant pediatric brain tumor, arises in the developing cerebellum (1). Lack of details regarding the molecular pathogenesis of medulloblastoma hinders the development of targeted therapies. MicroRNAs (miRNA) are small endogenous noncoding RNAs that play important roles in many biological processes, including cancer (2). miR-17/92 is a polycistronic cluster of highly conserved miRNAs that has been shown to contribute to tumor development in both human and murine cancers (3). miR-17/92 is located on chromosome 13 in humans (chromosome 14 in mice); paralogous clusters also exist including miR-106a/363 and miR-106b/25 (3). A role for miR-17/92 in medulloblastoma and cerebellar development has not been described.

Cerebellar granule neuron precursors (CGNP) are proposed cells of origin for a subset of medulloblastomas. CGNPs undergo rapid sonic hedgehog (Shh)–dependent expansion perinatally in mice and humans, and excessive Shh pathway activity promotes medulloblastoma (4). We show that miR-17/92 is amplified and overexpressed in medulloblastoma, particularly in the medulloblastoma subgroup driven by Shh signaling. In addition, we show that the miR-17/92 cluster is a target of Shh signaling through N-myc activity in CGNPs. Overexpression of miR-17/92 synergized with exogenous Shh in promoting CGNP proliferation and was able to drive proliferation in the absence of Shh signaling. These findings suggest that miR-17/92 is an essential component of the Shh mitogenic signaling apparatus in CGNPs and that its up-regulation downstream of aberrantly activated Shh contributes to medulloblastoma.

Materials and Methods

Fluorescence *in situ* hybridization. Interphase fluorescence *in situ* hybridization (FISH) for *hsa-mir-17/92* was carried out as previously published (5). Bacterial artificial chromosome (BAC) clones used included RP11-97P7 (hsa-mir-17/92; 13q31.3), as well as RP11-936K15 and RP11-539J14 (13q12.11) as adjacent controls.

Taqman miRNA assays. Expression of MYCN and MYC were quantified relative to ACTB using Platinum SYBR Green qPCR SuperMix UDG (Invitrogen). Taqman miRNA assays (Applied Biosystems) were used to quantify mature miRNA expression as previously described (6). See Supplementary Materials and Methods for additional details.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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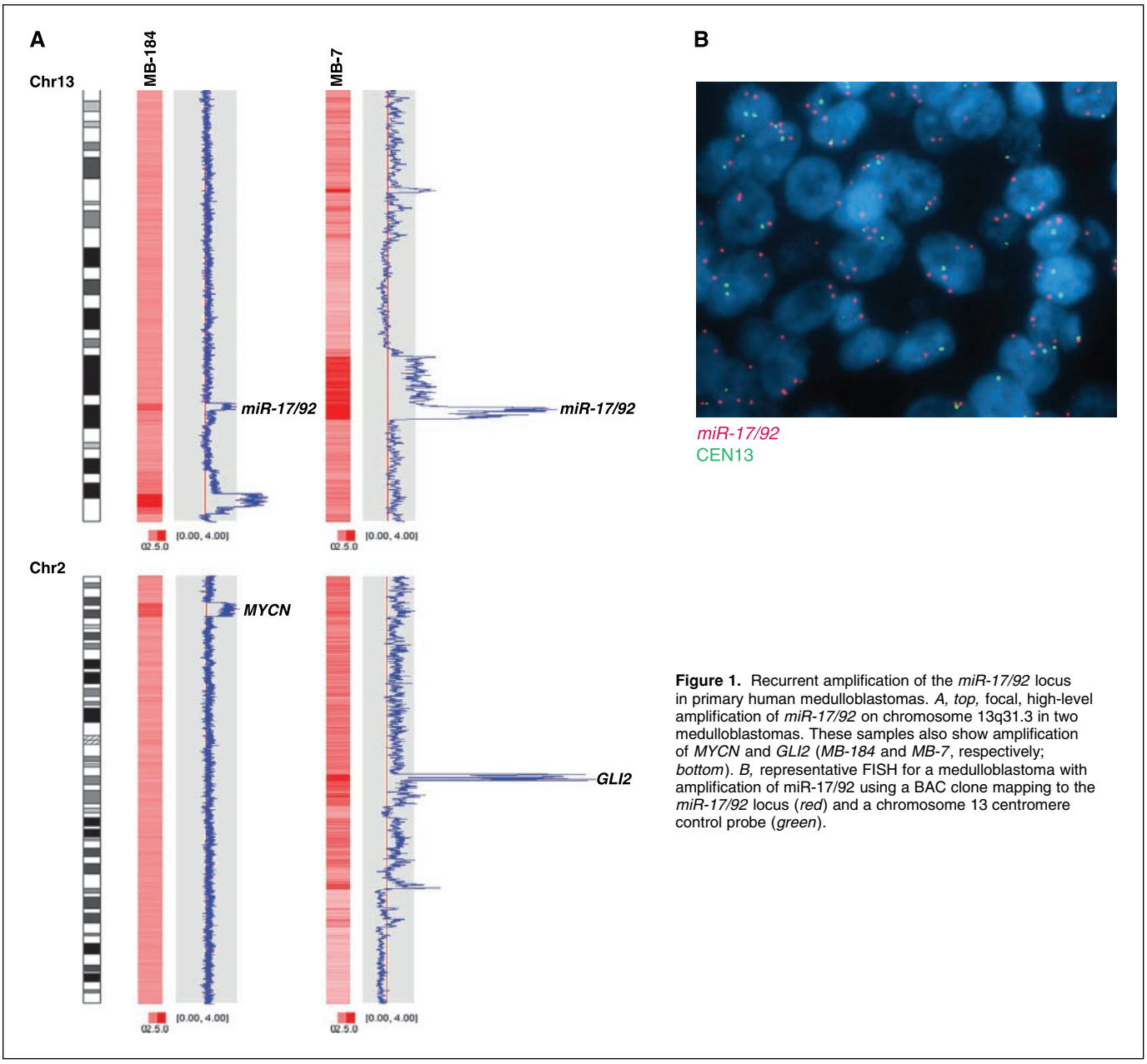


Figure 1. Recurrent amplification of the *miR-17/92* locus in primary human medulloblastomas. *A, top,* focal, high-level amplification of *miR-17/92* on chromosome 13q31.3 in two medulloblastomas. These samples also show amplification of *MYCN* and *GLI2* (*MB-184* and *MB-7*, respectively; *bottom*). *B,* representative FISH for a medulloblastoma with amplification of miR-17/92 using a BAC clone mapping to the *miR-17/92* locus (*red*) and a chromosome 13 centromere control probe (*green*).

Primary CGNP cultures. Culture and infection of CGNPs were performed as previously described (7). See Supplementary Materials and Methods for additional details.

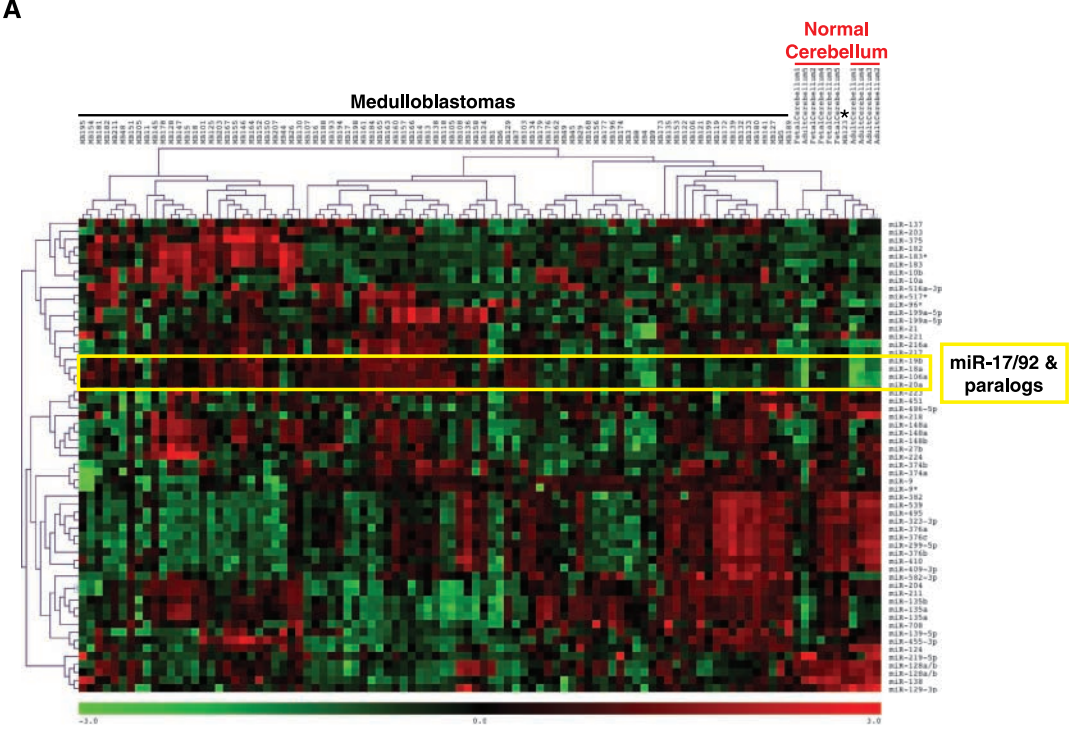
Tumor specimens, 100K and 500K single-nucleotide polymorphism arrays, miRNA arrays, and exon arrays. See Supplementary Materials and Methods.

Results and Discussion

The miR-17/92 cluster is recurrently amplified in medulloblastoma. We profiled 201 primary human medulloblastomas using Affymetrix single-nucleotide polymorphism arrays to delineate recurrent copy number aberrations that may contribute

Figure 2. Overexpression of miR-17/92 in human and murine medulloblastomas. *A,* heatmap showing that components of the miR-17/92 polycistron (*yellow box*) are expressed at low levels in the normal cerebellum, are expressed at elevated levels in medulloblastomas, and cluster together based on their common pattern of expression. Other miRs, including miR-375, miR-182, and miR-183, are also elevated in a subset of tumors. *B,* scatterplot analysis showing that components of miR-17/92 and related paralogues (miR-106a and miR-106b clusters) are the most significantly up-regulated miRNAs in human medulloblastoma compared with normal cerebellar samples (*left*). Mature miRNAs are depicted as circles with diameter determined by the ratio of expression for a given miRNA in the brain versus other tissues, and the colors reflect the parametric *P* values between the two classes. Performing the same analysis between medulloblastomas and normal cerebellum without miR-17/92, miR-106a, and miR-106b clusters shows that only a few miRNAs independent of these clusters are significantly up-regulated in medulloblastoma (*right*). *C,* summary of the top 20 most significantly overexpressed miRNAs in medulloblastoma compared with normal cerebellum samples. Eleven (of 20) of the significantly up-regulated miRNAs correspond to components of miR-17/92 or related paralogues, providing strong evidence in support of miR-17/92 overexpression in medulloblastoma. *D,* Taqman qRT-PCR analysis of murine medulloblastomas from *SmoA1* (*top*) and $Ptc^{+/-}$ (*bottom*) mice shows up-regulation of the miR-17/92 cluster in tumors from both of these mouse models. Data for components of the miR-17/92 cluster (*blue*) or controls (*red*) are represented as the fold change between medulloblastomas from either *SmoA1* or $Ptc^{+/-}$ mice and age-matched control cerebella.

A



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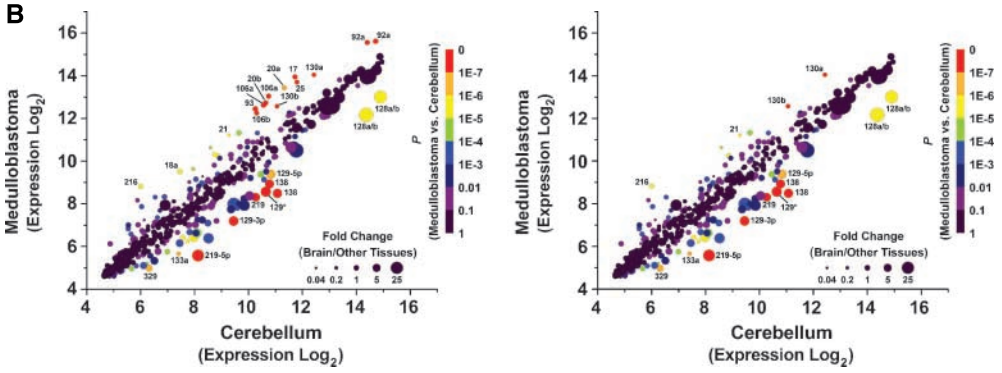

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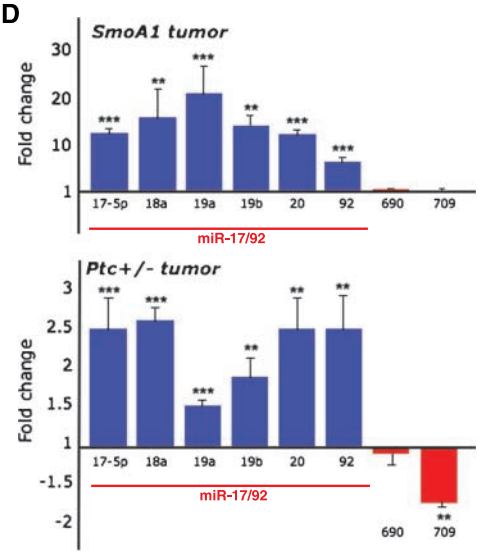
Significantly upregulated miRNAs in medulloblastoma

Mature miRNA	pValue	Fold-Change (MB vs CB)
hsa-mir-106a[#]	<1.00E-07	4.20-4.91
hsa-mir-17	<1.00E-07	4.67
hsa-mir-93	<1.00E-07	4.56
hsa-mir-20b	<1.00E-07	4.26
hsa-mir-106b	<1.00E-07	3.85
hsa-mir-25	<1.00E-07	3.75
hsa-mir-130a	<1.00E-07	3.07
hsa-mir-130b	<1.00E-07	2.85
hsa-mir-92a	<1.00E-07	2.22
hsa-mir-20a	2.00E-07	4.33
hsa-mir-21[#]	1.10E-06 to 4.81E-05	3.84-4.12
hsa-mir-18a	2.00E-06	4.15
hsa-mir-216a	2.50E-06	7.04
hsa-mir-345	4.90E-06	2.61
hsa-mir-19b[#]	6.80E-06 to 8.97E-05	2.96-3.22
hsa-mir-135a	1.33E-05	3.19
hsa-mir-33a	2.33E-04	2.46
hsa-mir-19a	2.53E-04	2.72
hsa-mir-96	4.08E-04	3.43
hsa-mir-210	6.82E-04	2.15

17/92 cluster; 106a cluster; 106b cluster.
[#]Mature miRNA supported by multiple significant probes.

D



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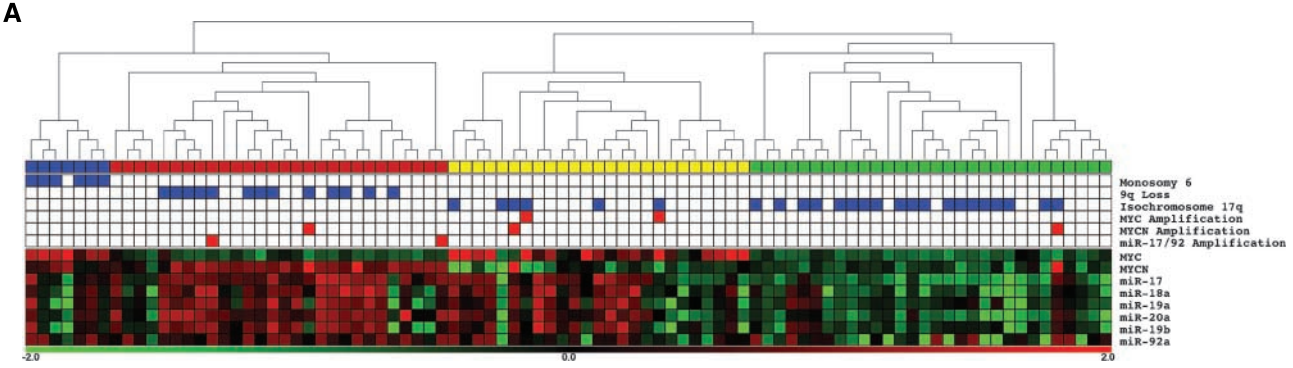
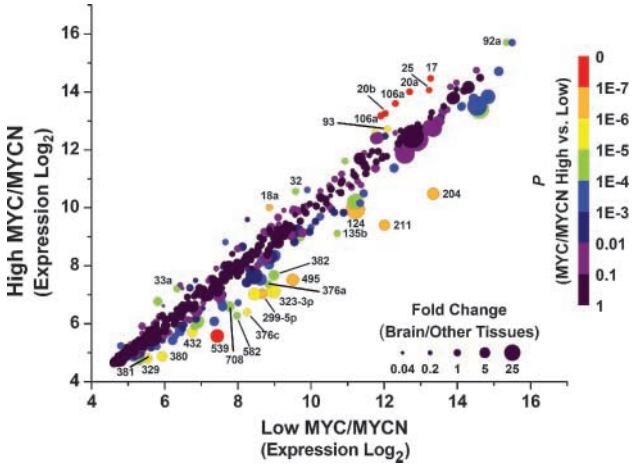


B

	WNT			SHH			Group C			Group D		
	Mature miRNA	Fold-Change	Cluster	Mature miRNA	Fold-Change	Cluster	Mature miRNA	Fold-Change	Cluster	Mature miRNA	Fold-Change	Cluster
Up	miR-224	11.23	miR-452—224	miR-20a	2.13	miR-17/92	miR-181a[#]	2.51-2.85	miR-181a-1—181b-1; miR-181a-2-181b-2	miR-323-3p	3.73	Chr14q cluster
	miR-452[#]	1.41-2.50	miR-452—224	miR-17	1.91	miR-17/92	miR-181b[#]	1.86-1.93	miR-181a-1—181b-1; miR-181a-2-181b-2	miR-376c	3.67	Chr14q cluster
	miR-24[#]	5.41-5.85	miR-23b—27b—24-1; miR-23a—27a—24-2	miR-106a[#]	2.04	miR-106/363	miR-181c	2.58	miR-181c—181d	miR-539	3.37	Chr14q cluster
	miR-27b	4.78	miR-23b—27b—24-1	miR-20b	1.92	miR-106/363	miR-181d	1.84	miR-181c—181d	miR-299-5p	3.23	Chr14q cluster
	miR-23b	2.58	miR-23b—27b—24-1	miR-199a-5p[#]	3.14-3.21	N/A	miR-375	9.23	N/A	miR-204	8.58	N/A
	miR-23a	2.60	miR-23a—27a—24-2	miR-199b-5p	2.53	N/A	miR-182	7.61	N/A	miR-211	7.38	N/A
	miR-183	8.01	miR-183—96	miR-378	2.50	N/A	miR-183[#]	3.02-3.71	miR-183—96	miR-582-3p	5.08	N/A
	miR-365[#]	4.04-5.98	N/A	miR-422a	1.90	N/A	miR-455-3p	2.98	N/A	miR-495	4.29	N/A
	miR-193b	4.77	N/A	miR-222	1.85	miR-222—221	miR-455-5p	1.81	N/A	miR-135b	4.01	N/A
	miR-193a-5p	3.74	N/A	miR-16-2	1.80	miR-15b—16-2				miR-135a[#]	2.52-3.12	N/A
Down	miR-340	0.23	N/A	miR-204	0.06	N/A	miR-376a	0.34	Chr14q cluster	miR-20a	0.41	miR-17/92
	miR-9	0.23	N/A	miR-211	0.09	N/A	miR-299-5p	0.35	Chr14q cluster	miR-17	0.43	miR-17/92
				miR-135b	0.11	N/A	miR-223	0.41	N/A	miR-18a	0.46	miR-17/92
				miR-182	0.12	N/A	miR-26a[#]	0.62-0.71	N/A	miR-106a[#]	0.40-0.42	miR-106/363
				miR-135a[#]	0.16-0.40	N/A	let-7b	0.65	let-7a-3—7b	miR-20b	0.44	miR-106/363
				miR-455-3p	0.18	N/A	miR-130a	0.66	N/A	miR-375	0.30	N/A
				miR-10b	0.19	N/A	let-7i[#]	0.68	N/A	miR-182	0.45	N/A
				miR-183[#]	0.28-0.36	miR-183—96				miR-181a[#]	0.46-0.50	miR-181a-1—181b-1; miR-181a-2-181b-2
				miR-708	0.30	N/A				miR-199a-5p	0.46	N/A
				miR-203	0.35	N/A				miR-378	0.47	N/A

17/92 cluster; 106a cluster. [#]Mature miRNA supported by multiple significant probes.

C



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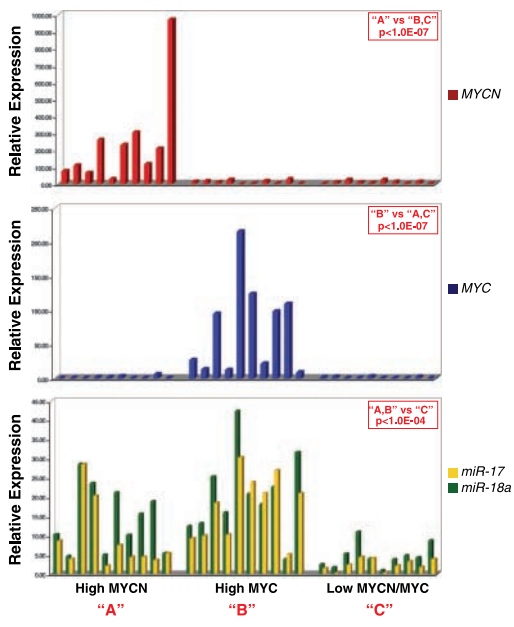


to medulloblastoma pathogenesis (8). We identified two medulloblastomas with recurrent, focal, high-level amplification on chromosome 13q31.3, sharing a minimal common region that spans ~1.82 Mb (Fig. 1*A*). The only gene mapping to this amplified locus is *miR-17/92* (NCBI Build 36.1). Amplification of this miR cluster has not previously been reported in medulloblastoma. Further examination of the tumors harboring *miR-17/92* amplification revealed amplification of *MYCN* and *GLI2* (Fig. 1*A*). We subsequently carried out interphase FISH on a medulloblastoma tissue microarray to determine the incidence of miR-17/92 amplification in a nonoverlapping series of 80 medulloblastomas. Low-level amplification of miR-17/92 was identified in ~6% (5 of 80) of cases (Fig. 1*B*). Taken together, these results suggest that miR-17/92 functions as an oncogene in a subset of medulloblastomas.

miR-17/92 is overexpressed in human and murine medulloblastomas. We performed a genome-wide survey of 427 mature miRNAs in a series of 90 primary human medulloblastomas and 10 normal human cerebella (five fetal and five adult). Unsupervised hierarchical clustering of samples and differentially expressed miRNAs in the data set could easily discriminate medulloblastomas from normal cerebella samples (Fig. 2*A*). Notably, components of miR-17/92, including miR-18a, miR-19b, and miR-20a, as well as the paralogous miR-106a (miR-106a/363 cluster) clustered together, were expressed at low levels in the normal cerebella and showed considerably higher levels of expression in most medulloblastomas (Fig. 2*A*).

Statistical comparison of miRNA profiles for medulloblastomas versus normal cerebella samples revealed consistent overexpression of miR-17/92 and its related paralogues (miR-106a/363 and miR-106b/25) in medulloblastoma (Fig. 2*B, left* and *C*; Supplementary Table S1). Reanalysis of the data after removing the 22 probe sets, which detect components of miR-17/92 or paralogous clusters, shows that there are relatively few remaining overexpressed miRs in medulloblastoma compared with normal cerebella (Fig. 2*B, right*).

As miR-17/92 was overexpressed in a large percentage of human medulloblastomas compared with normal cerebella, we examined its expression in murine medulloblastoma. Medulloblastomas from *NeuroD2-SmoA1* and $Ptc^{+/-}$ mice showed marked overexpression of the miR-17/92 cluster compared with cerebellum from age-matched tumor-free littermates (Fig. 2*D*).

miR-17/92 up-regulation is associated with activated Shh signaling in human medulloblastoma. Aberrant activation of the Shh pathway through mutation of pathway members has been documented in 25% to 30% of medulloblastomas (5, 9). To subclassify the 90 medulloblastomas used in miRNA expression profiling above, we performed mRNA expression analysis for 17,881 mRNAs on the same cohort of medulloblastomas. Unsupervised hierarchical clustering using 1,300 differentially expressed mRNAs segregated four unique molecular subgroups: WNT (*blue*), SHH (*red*), Group C (*yellow*), and Group D (*green*; Fig. 3*A* and Supplementary Fig. S1*A* and *B*). These four subgroups were supported by their expression pattern (Supplementary Table S2) and specific genomic features, including monosomy 6 (WNT), chromosome 9q loss (SHH), and isochromosome 17q (Group C and Group D; Fig. 3*A*). miR-17/92 was most highly expressed in the SHH subgroup, followed by Group C, and the WNT subgroup (Fig. 3*A* and *B* and Supplementary Fig. S2; Supplementary Table S3).

Confirming previous reports (10), we observed high MYCN expression in the SHH tumors, whereas MYC levels were most elevated in WNT and Group C tumors (Fig. 3*A* and Supplementary Fig. 3). MYC and MYCN have both been reported to transcriptionally regulate miR-17/92 (11, 12). We compared miR-17/92 expression between tumors with higher MYCN/MYC expression to tumors with lower expression to determine whether miR-17/92 regulation might also be myc-dependent in medulloblastoma. As shown in Fig. 3*C*, components of miR-17/92 (miR-17, miR-20a, miR-92a) and related paralogues (miR-106a, miR-20b, miR-25, miR-93) represented the majority of up-regulated miRNAs in medulloblastomas with higher MYCN/MYC (n = 51) expression compared with lower expressing MYCN/MYC (n = 39) tumors (Supplementary Table S4).

We carried out Taqman miRNA assays to validate the correlation between MYCN/MYC and miR-17/92 expression observed on the array platforms. Samples were divided into three groups of 10 tumors each: higher MYCN, higher MYC, and lower MYCN/MYC and then quantitative reverse transcription-PCR (qRT-PCR) was performed for MYCN, MYC, miR-17, and miR-18. As predicted from the mRNA array data, the high MYCN (P = 3.33E−08) and high MYC (P = 3.33E−08) tumors did not overlap (Fig. 3*D, top* and *middle*). Importantly, miR-17 (P = 4.92E−05) and miR-18 (P = 3.75E−05) were significantly up-regulated in both the higher MYCN and higher MYC expressing groups compared with the lower MYCN/MYC–expressing group (Fig. 3*D, bottom*). These results provide strong evidence that up-regulation of the miR-17/92 polycistron may be MYCN/MYC–dependent in medulloblastomas.

miR-17/92 is up-regulated by Shh signaling in primary CGNP cultures. To determine whether the relationship between activated Shh signaling and miR-17/92 up-regulation we observed in medulloblastomas reflected co-option of developmental programs, we cultured murine CGNPs with or without exogenous Shh (±cyclohexamide) for 24 hours and then performed array-based miRNA profiling. Of 599 mouse miRNAs assayed, 19 were significantly changed, 9 were up-regulated, and 10 were down-regulated (Fig. 4*A*; Supplementary Table S5). The miR-17/92 polycistron was up-regulated in Shh-treated CGNPs, but not in the presence of cycloheximide, indicating that a new protein intermediate needs to be synthesized to regulate *miR-17/92* expression.

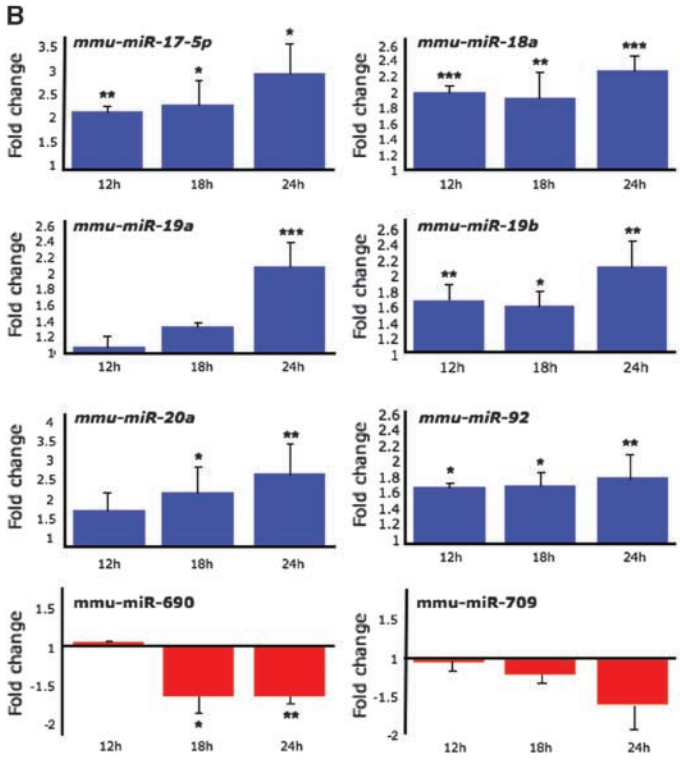
Validation by qRT-PCR in Fig. 4*B* shows that the six miRNAs within the miR-17/92 cluster were consistently up-regulated by Shh, which was abrogated by cyclohexamide (data not shown). These results indicate that the association between activated Shh signaling and miR-17/92 expression is conserved between normal

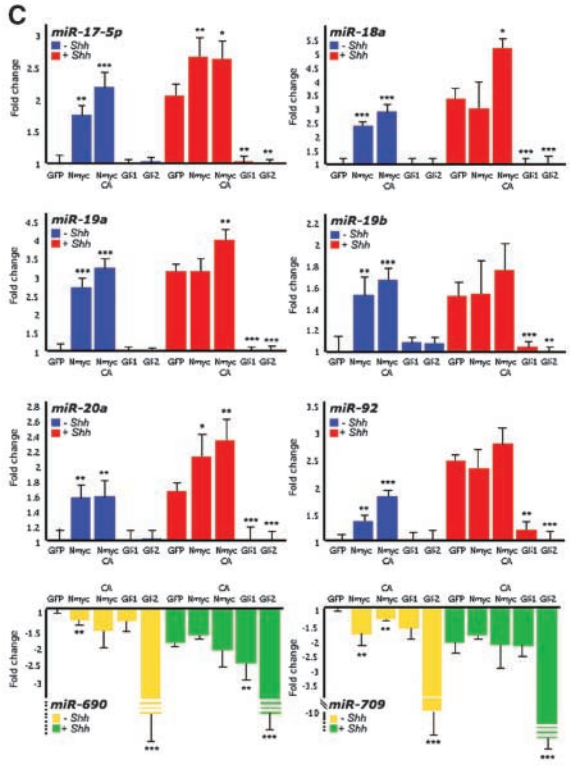
Figure 3. miR-17/92 is overexpressed in SHH-dependent medulloblastomas and tumors with elevated MYC family expression. *A,* heatmap showing expression of MYC, MYCN, and the miR-17/92 cluster suggests a strong correlation between miR-17/92 overexpression and the SHH subgroup as defined by molecular classification of the 90 primary medulloblastomas used for miRNA profiling. Expression array analysis of protein-coding genes identifies four consistent subgroups: WNT (*blue*), SHH (*red*), Group C (*yellow*), and Group D (*green*). Classification of the subgroups into WNT, SHH, Group C, and Group D was achieved by comparing each of the respective subgroups to the other three using *t* test statistics (see Supplementary Table S2). miR-17/92 expression is also elevated in the WNT and Group C subgroups, both of which exhibit higher MYC expression, compared with Group D, which is characterized by lower MYC levels. *B,* representative miRNAs of the four molecular subgroups described in *A* identified using *t* test statistics. miR-17/92 expression is highest in the SHH subgroup and lowest in Group D. *C,* division of the medulloblastoma series into higher MYC/MYCN (n = 51) and lower MYC/MYCN (n = 39) expressing groups identifies highly significant up-regulation of miR-17/92 and related paralogues in the higher MYC/MYCN expressing tumors. Scatterplot analysis shows significant, differentially expressed miRNAs between the two groups of medulloblastomas. *D,* qRT-PCR validation of the expression array data in a subset of medulloblastomas (n = 30) confirming the consistent correlation between MYC/MYCN status and miR-17/92 expression.

A

	Significant miRNAs in Shh-treated CGNPs		
	Mature miRNA	pValue	Fold-Change (SHH vs Vehicle)
Up	mmu-miR-19a	1.33E-04	3.74
	mmu-miR-216a	<1.00E-07	3.16
	mmu-miR-18a	<1.00E-07	1.86
	mmu-miR-19b	<1.00E-07	1.74
	mmu-miR-872	1.36E-03	1.77
	mmu-miR-30e	5.13E-06	1.69
	mmu-miR-106a	<1.00E-07	1.56
	mmu-miR-103	8.36E-06	1.51
	mmu-miR-106b	<1.00E-07	1.51
Down	mmu-miR-714	<1.00E-07	0.66
	mmu-miR-671-5p	<1.00E-07	0.66
	mmu-miR-374	<1.00E-07	0.63
	mmu-miR-342-3p	<1.00E-07	0.56
	mmu-miR-98	<1.00E-07	0.55
	mmu-miR-805	<1.00E-07	0.28
	mmu-miR-690	<1.00E-07	0.54
	mmu-miR-483	1.69E-04	0.48
	mmu-miR-711	<1.00E-07	0.41
	mmu-miR-709	<1.00E-07	0.47

17/92 cluster; 106a cluster; 106b cluster.





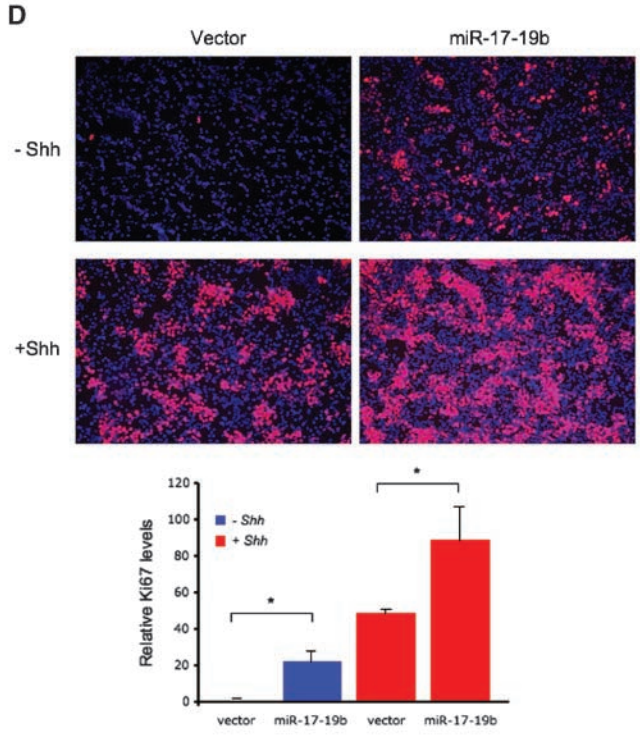


Figure 4. Shh and N-myc drive the expression of miR-17/92 in cerebellar neural precursor cells resulting in mitosis. *A,* table showing miRNAs whose expression was significantly altered in CGNPs by Shh treatment. The miR-17/92 cluster is up-regulated, as are its paralogues miR106a and miR106b. *B,* qRT-PCR quantification to validate Shh-mediated up-regulation of miR-17/92 members in CGNPs. *C,* quantification of miR-17/92 expression in CGNPs transduced with retroviruses expressing N-myc, stabilized N-myc (*CA*), Gli1, or Gli2. N-myc, but not Gli, drives miR-17/92 expression. *D,* immunofluorescence staining for Ki67 showing that viral transduction of CGNPs with miR-17/92 increases Ki67 labeling of P7 cerebellar neural precursor cells and that miR-17/92 can synergize with Shh. Graph shows results of automated quantification of Ki67 staining.

Shh mitogenic activity in CGNPs and oncogenic Shh signaling in medulloblastoma.

miR-17/92 induces proliferation of CGNPs downstream of N-myc. We have previously shown that N-myc is a downstream target of Shh whose induction is not protein synthesis dependent and which can drive CGNP proliferation in the absence of Shh signaling (7, 13). We asked whether miR-17/92 was regulated by N-myc in CGNPs. We infected CGNPs with retroviruses carrying N-myc or the stabilized mutant N-myc^{T50A} that can prolong CGNP proliferation *in vitro* (14). N-myc transduction resulted in increased expression of the miR-17/92 cluster in the presence and absence of Shh (Fig. 4*C*). In contrast, neither Gli1 nor Gli2 expression induced miR-17/92 in the absence of Shh; indeed, Gli1 and Gli2 suppressed Shh-mediated miR-17/92 expression. These results indicate that the Shh pathway effectors N-myc and Gli regulate different miRNA targets.

Because N-myc expression alone is sufficient to drive CGNP proliferation, we asked whether miR-17/92 contributes to the N-myc–regulated proliferation program. We infected CGNPs with retroviruses expressing five of the six miRNAs within the miR-17/92 cluster (pWzl-miR-17-19b; ref. 15). After 48 hours, we measured CGNP proliferation by quantifying Ki67 staining. Overexpression of the miR-17/92 cluster increased proliferation in Shh-treated cells (Fig. 4*D*). miR-17/92 alone was able to maintain cell proliferation in the absence of Shh, albeit not at the same levels as Shh alone, suggesting that its expression does not recapitulate the complete Shh/N-myc proliferative response.

In summary, we have shown that miR-17/92 amplification and overexpression are a hallmark of SHH-associated medulloblastoma in humans and in mice and that its expression correlates with high levels of MYC family proto-oncogenes. We also show that, in normally proliferating CGNPs, miR-17/92 is a Shh target whose expression is regulated by N-myc. Our finding that Shh regulates expression of an oncogenic miRNA provides additional insights as to the mechanisms through which Shh drives cell cycle progression. Our observation that miR-17/92 expression increases Shh-mediated CGNP proliferation provides insight into its role in human medulloblastoma, suggesting that high levels of miR-17/92 can provide cells with a selective growth advantage through an enhanced proliferative capacity. A role for miR-17/92 in tumor cell survival may also be at play, as its targets identified in lymphoma include PTEN and the proapoptotic p53 target TP53INP1 (16, 17).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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