

Control of oligodendroglial cell number by the miR-17-92 cluster

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SUMMARY

The generation of myelinating cells in the central nervous system requires the initiation of specific gene expression programs in oligodendrocytes. We reasoned that microRNAs (miRNAs) could play an important role in this process by regulating crucial developmental genes. Microarray profiling of cultured oligodendrocytes identified the miR-17-92 miRNA cluster as highly enriched in oligodendrocytes. We specifically deleted the *miR-17-92* cluster in oligodendrocytes using 2',3'-cyclic nucleotide 3' phosphodiesterase (*Cnp*)-Cre mice. Absence of miR-17-92 leads to a reduction in oligodendrocyte number in vivo and we find that the expression of these miRNAs in primary cultures of oligodendrocyte precursor cells promotes cell proliferation by influencing Akt signaling. Together, these results suggest that the miRNA pathway is essential in determining oligodendroglial cell number and that the miR-17-92 cluster is crucial in this process.

KEY WORDS: Oligodendrocytes, Proliferation, Dicer, miRNA, miR-17-92 (Mir17-92), Mouse

INTRODUCTION

Rapid saltatory conduction of axons in the vertebrate central nervous system (CNS) requires myelin formation by oligodendrocytes (Sherman and Brophy, 2005; Simons and Trotter, 2007). The first step in this process is the generation of oligodendrocyte precursor cells (OPCs) from pluripotent neuroepithelial precursors. OPCs then migrate into the developing white matter until they reach their target axons (Richardson et al., 2006). At their final resting sites, OPCs exit the cell cycle, become non-migratory and differentiate into myelin-forming oligodendrocytes (Rivers et al., 2008; Zhu et al., 2008).

Oligodendrocytes are greatly overproduced and only by competing for the limiting amounts of target-derived growth factors is the cell number adjusted to the number and length of the axons requiring myelination. Several intracellular and extracellular factors, including oligodendrocyte differentiation inhibitors and cell cycle inhibitors, have been shown to regulate oligodendrocyte number (Li et al., 2009).

In this study, we asked whether microRNAs (miRNAs) play a role in this process.

MATERIALS AND METHODS

Dicer and miR-17-92 knockout mice

Mice carrying floxed *Dicer* alleles (*Dicer*^{fllox/fllox}) [The Jackson Laboratory (Harfe et al., 2005)] were bred with mice heterozygous for Cre recombinase expression under the control of the *Cnp* promoter (*Cnp*^{+/Cre}) (Lappe-Siefke et al., 2003). Offspring with the genotype *Dicer*^{+/fllox} *Cnp*^{+/Cre} were mated with *Dicer*^{fllox/fllox} mice to generate *Dicer*^{fllox/fllox} *Cnp*^{+/Cre} mutant mice. Mice with loxP sites flanking the *miR-17-92* cluster (*miR-17-*

92^{fllox/fllox}) [The Jackson Laboratory (Ventura et al., 2008)] were bred with *Cnp*^{+/Cre} mice. Offspring with the genotype *miR-17-92*^{+/fllox} *Cnp*^{+/Cre} were bred with *miR-17-92*^{fllox/fllox} mice to obtain *miR-17-92* cluster knockout mice (*miR-17-92*^{fllox/fllox} *Cnp*^{+/Cre}). Littermates were used as controls.

Cell culture and transfection

Astrocytes were prepared by trypsinization of mixed glial cultures after shaking of oligodendrocytes. Mouse oligodendrocyte primary cultures and Oli-neu cells were prepared as previously described (Fitzner et al., 2006; Trajkovic et al., 2006). Cells were transfected 4–24 hours after shaking using Lipofectamine 2000 (Invitrogen), when cells were in a precursor stage. Transfection efficiency was confirmed using FITC-labeled antisense oligonucleotides (see Fig. S1 in the supplementary material). For the downregulation of specific miRNAs, miRCURY LNA knockdown probes (Exiqon) for miR-17 and miR-19b were used in combination (anti-17-19b). Pre-miR miRNA precursors (Ambion) for miR-17 and miR-19b were used in combination (pre-17-19b). The miRCURY LNA Scramble-miR (Exiqon) and the Pre-miR negative control (Ambion) were used as negative controls.

Immunofluorescence, immunoblotting, BrdU and TUNEL assay

Immunofluorescence and immunohistochemistry were performed as described previously (Simons et al., 2000). The following antibodies were used: anti-Mbp (Sternberger Monoclonals), anti-O1, anti-Olig2 (IBL), A2B5 (Millipore), anti-Pten, anti-phospho-Akt, anti-Akt (Cell Signaling) and anti-actin (Sigma).

Cell proliferation was measured by detecting the incorporation of bromodeoxyuridine (BrdU). Cells were grown in 10 ng/ml platelet-derived growth factor (Sigma); BrdU (20 µM) was added 24 hours after shaking and cells fixed 24 hours later.

For inhibition of Akt phosphorylation, 10 µM Akt1/2 kinase inhibitor (Sigma) was added to the culture medium.

Statistical significance was assessed by Student's *t*-test; *P* < 0.05 was defined as statistically significant.

miRNA microarray

miRCURY LNA microRNA arrays (Exiqon) or mouse miRNA microarrays (G4472A, Agilent Technologies) were used (three replicates for each experiment). The microarray data are deposited in NCBI Gene Expression Omnibus with accession GSE21801.

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RNA isolation and real-time quantitative PCR

Expression levels of miR-17, miR-19b, miR-125b and miR-34b were measured by quantitative real-time reverse transcriptase PCR (qRT-PCR). For normalization, internal snoRNA-234 levels were used. The quantitative PCR was performed using the iQTM5 Real-Time PCR Detection System (Bio-Rad) and TagMan Universal Master Mix (Applied Biosystems).

Luciferase assay

To monitor the downregulation of miR-19b, the firefly luciferase-based pMiR19b-Luc reporter vector (Signosis) with an miR-19b binding site was used. For normalization, the vector was cotransfected with a *Renilla* luciferase vector without an miRNA binding site. The luciferase activity of both constructs was determined 48 hours post-transfection.

RESULTS AND DISCUSSION

Reduction of oligodendrocyte cell number by Cnp-Cre-mediated deletion of *Dicer*

In order to understand the roles of miRNAs in oligodendrocyte development, we deleted *Dicer* (*Dicer1* – Mouse Genome Informatics) using the Cre-loxP recombination system. We used the 2',3'-cyclic nucleotide 3' phosphodiesterase (*Cnp*) promoter to drive Cre expression. *Cnp* is detectable in OPCs at E11.5 to E12 and is maintained in mature oligodendrocytes. When floxed *Dicer* mice (*Dicer*^{fllox/fllox}) were crossed with *Cnp*-Cre mice (Fig. 1A) we found that out of more than 250 newborns, only three *Cnp*-Cre *Dicer*^{fllox/fllox} (*Cnp*-Cre-*Dicer*) survived into adulthood, possibly owing to the expression of *Cnp*-Cre in the peripheral nervous system (Dugas et al., 2010). Since *Cnp*-Cre-*Dicer* mice were detected at a normal ratio at late embryonic stages, we used mice at E18.5 or directly after birth for our analysis.

When primary cultures of oligodendrocytes were prepared from E18.5 *Cnp*-Cre-*Dicer* embryos and compared with control littermates, we did not detect any major changes in the number of myelin basic protein (Mbp)-positive cells (Fig. 1G). Next, we analyzed whether the number of oligodendrocytes was changed in *Dicer* knockout brains by staining coronal P0 sections with antibodies against Olig2. Olig2 is expressed in both OPCs and mature oligodendrocytes and also in some astrocytes (Ono et al., 2008). There was a ~40% reduction of Olig2-positive cells in the brains of *Cnp*-Cre-*Dicer* as compared with control animals (Fig. 1E). Similar results were obtained when *Dicer* was depleted by RNA interference (RNAi) in primary cultures of oligodendrocytes (see Fig. S2 in the supplementary material). Surprisingly, the numbers of Olig2-positive cells were not altered in the spinal cord of *Cnp*-Cre-*Dicer* animals (Fig. 1F), pointing to distinct mechanisms of miRNA action in oligodendrocyte development within the spinal cord and the brain. By TUNEL staining, we found a small increase in apoptotic cells in *Dicer* knockout brains, but the numbers did not attain statistical significance (Fig. 1H). The number of apoptotic Olig2-positive cells was similar in knockout brains and controls (Fig. 1I).

Identification of the miR-17-92 cluster as highly enriched in oligodendrocytes by microarray profiling

In order to identify the responsible miRNAs, we performed miRNA expression profiling of oligodendrocytes using miRNA microarrays. Oligodendroglia-enriched miRNAs were identified by comparing the expression of miRNAs in primary cultures of oligodendrocytes and

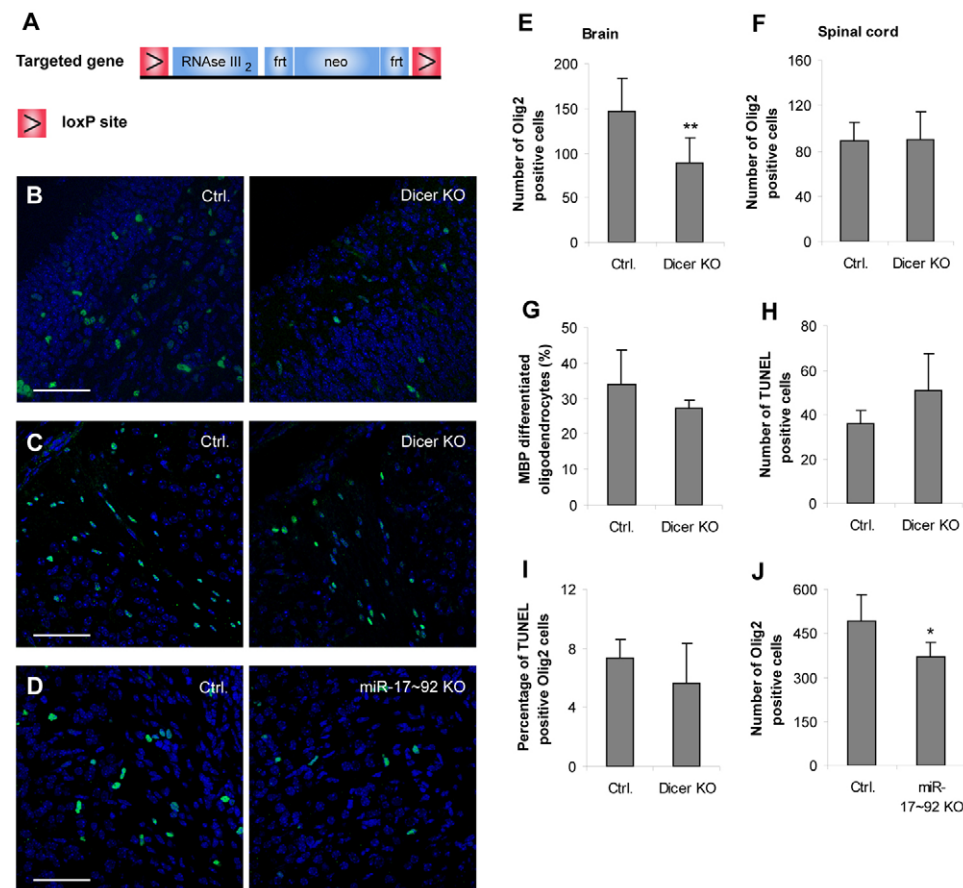


Fig. 1. Conditional deletion of *Dicer* or *miR-17-92* using *Cnp*-Cre reduces oligodendroglial cell number.

(A) The targeting strategy for *Dicer* conditional deletion in mouse oligodendrocytes. (B–D) Coronal sections of P0 brain (B), cross-sections of spinal cord of *Cnp*-Cre-*Dicer* embryos (C) and coronal sections of *miR-17-92*^{fllox/fllox} *Cnp*^{+/Cre} embryos (D) and control littermate P0 brain were analyzed by immunohistochemistry with Olig2 antibodies. (E,F,J) Changes in Olig2-positive cell number in brain and spinal cord in *Dicer* and *miR-17-92* knockouts (KO) are shown as mean \pm s.d. *, $P < 0.05$; **, $P < 0.01$; $n = 4$ –5 animals. (G) Primary cultures of oligodendrocytes were prepared from E18.5 *Cnp*-Cre-*Dicer* embryos and compared with control littermate cultures by labeling for O1 and Mbp. The percentage of Mbp-positive O1-positive cells is shown as mean \pm s.d. ($n = 3$ independent cultures). (H) Number of TUNEL-positive cells in coronal sections of the periventricular region of *Cnp*-Cre-*Dicer* and control littermates ($n = 5$ animals). (I) Percentage of TUNEL-positive Olig2-labeled cells in brain slices ($n = 3$). Scale bars: 60 μ m.

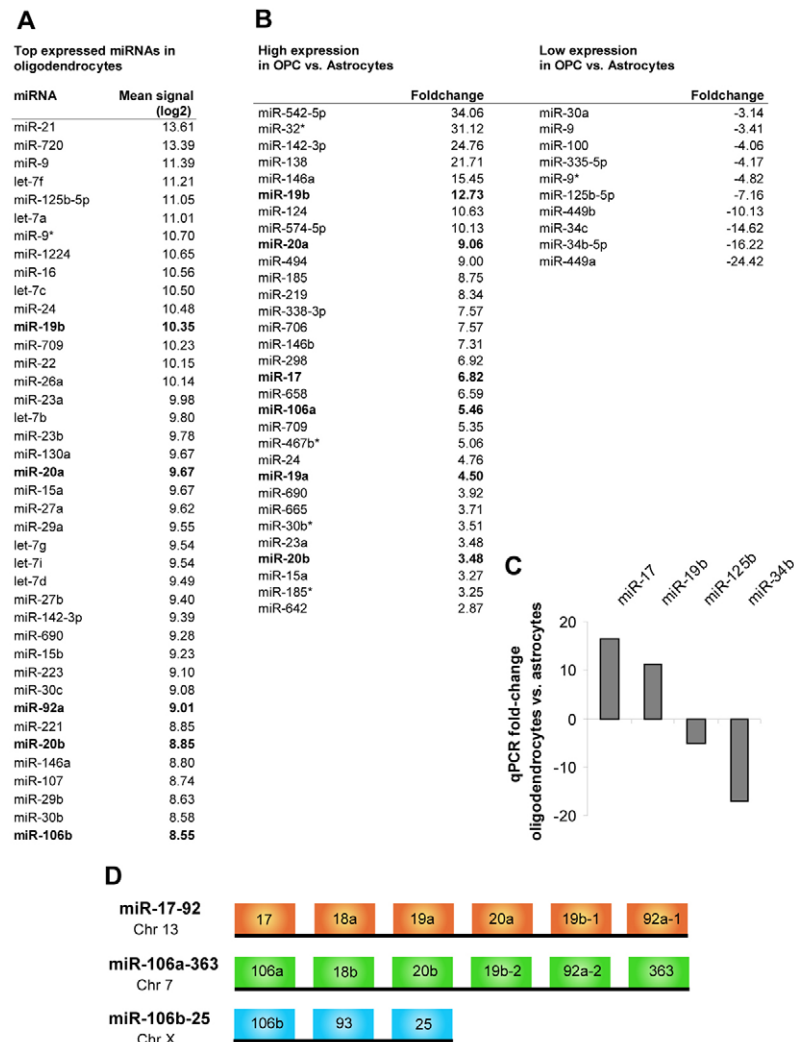


Fig. 2. Identification of the miR-17-92 cluster as highly enriched in oligodendrocytes by miRNA microarray profiling. (A) The top 40 miRNAs continuously expressed in cultured mouse primary oligodendrocytes (cultured for 0, 1 or 6 days). miRNAs of the miR-17-92 cluster family are in bold. (B) miRNA profiling of primary cultures of oligodendrocytes and astrocytes to identify highly (left) and poorly (right) expressed miRNAs in oligodendrocytes. The fold enrichments of miRNAs are shown. The miRNAs of the miR-17-92 cluster family are in bold. (C) Validation of differentially expressed miRNAs by qRT-PCR. (D) Schematic representation of the miR-17-92 cluster and two known paralogs.

astrocytes. First, we compared the mRNA transcriptome of cultured oligodendrocytes and astrocytes to verify that this approach identifies oligodendrocyte-enriched transcripts. Indeed, among the top 20 miRNAs expressed by oligodendrocytes were many of the previously described oligodendroglia-enriched genes (see Table S1 in the supplementary material) (Cahoy et al., 2008).

When the miRNA expression was profiled, we detected 31 miRNAs that were expressed at least 2-fold higher in cultured oligodendrocytes than astrocytes. The 31 miRNAs with the largest differences are shown in Fig. 2B.

Interestingly, the miR-17-92 cluster appeared to be enriched in oligodendrocytes. This cluster consists of six miRNAs that are processed from a common precursor transcript (Mendell, 2008) (Fig. 2D). Furthermore, there are two known paralogs: miR-106a-363 and miR-106b-25 (Tanzer and Stadler, 2004). The miRNAs from the three miRNA clusters can be grouped into four different functional groups based on their seed sequence. Our microarray analysis revealed that four miRNAs of the miR-17-92 cluster and two miRNAs of the miR-106a-363 cluster were enriched in oligodendrocytes (Fig. 2B).

To confirm the miRNA microarray profiling data, we performed real-time PCR analysis of some of the differentially expressed miRNAs: miR-17, miR-19b, miR-125b and miR-34b. The

differential expression of all of these miRNAs was confirmed by real-time PCR (Fig. 2C). Most importantly, real-time PCR analysis revealed that the two miRNAs of the miR-17-92 cluster (miR-19b and miR-17) were ~11- to 16-fold enriched in oligodendrocytes as compared with astrocytes (Fig. 2C).

To analyze how the expression of these miRNAs changes during oligodendrocyte differentiation, microarray profiling was performed on cultured oligodendrocytes. The expression of the miR-17-92 cluster was already high in OPCs (see Table S2 in the supplementary material). Only a relatively small number of miRNAs changed in expression during oligodendrocyte differentiation in culture (see Table S3 in the supplementary material).

When the miRNAs were sorted according to their mean signal intensities to identify the most highly expressed miRNAs in oligodendrocytes, we observed that four miRNAs of the miR-17-92 cluster were among the 35 most abundant miRNAs (Fig. 2A).

These data are consistent with a previous microarray analysis that identified miR-17 and miR-20a as highly enriched miRNAs both in A2B5 (ganglioside GQ1c)-positive and GalC-positive cells of the oligodendroglial lineage (Lau et al., 2008).

Together, these data show that the miR-17-92 cluster is one of the most abundant family of miRNAs in oligodendrocytes.

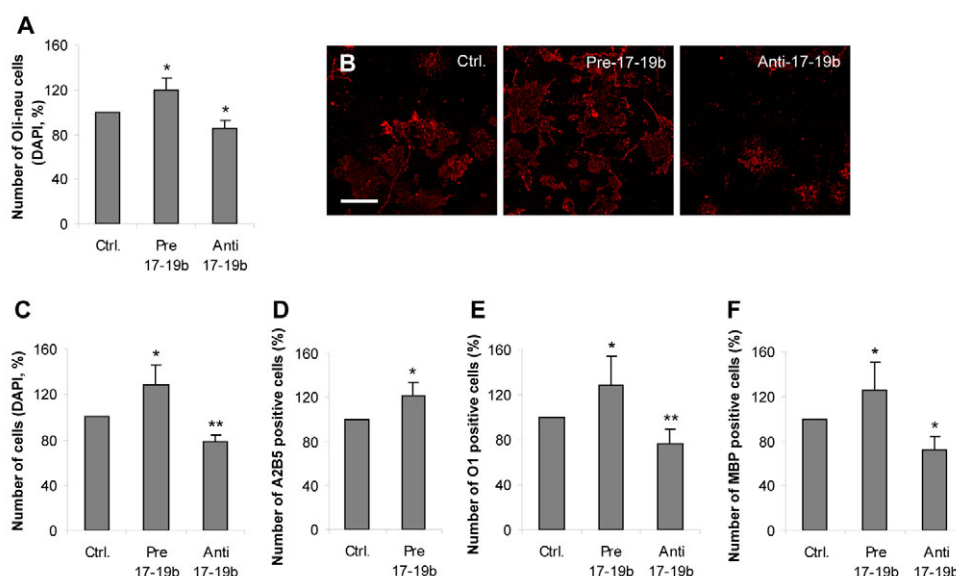


Fig. 3. The miR-17-92 cluster regulates oligodendrocyte cell number. (A) Pre-17-19b or oligonucleotides directed against miR-17 and miR-19b (anti-17-19b) were expressed in the mouse oligodendroglial cell line Oli-neu. $n=3$; *, $P<0.05$. (B-F) Pre-17-19b or anti-17-19b were expressed in primary oligodendrocytes. (B) Cells were stained for O1 (red). (C-F) Changes in total cell number (DAPI stained) and in those positive for A2B5, O1 or Mbp. Mean \pm s.d.; $n=3-4$; *, $P<0.05$; **, $P<0.01$. Scale bar: 100 μ M.

The miR-17-92 cluster regulates oligodendrocyte cell number

Previous studies have shown that the miR-17-92 cluster is essential for the development of the heart, lung and immune system (Mendell, 2008; Ventura et al., 2008; Koralov et al., 2008; Xiao et al., 2008). To address the role of miR-17-92 in oligodendrocyte development, mice with loxP sites flanking the *miR-17-92* cluster (*miR-17-92^{fllox/fllox}*) were bred with *Cnp^{+/-Cre}* mice to delete the *miR-17-92* cluster (*miR-17-92^{fllox/fllox} Cnp^{+/-Cre}*). We stained coronal P0 sections with antibodies against Olig2 to analyze whether the number of oligodendrocytes was changed in *miR-17-92^{fllox/fllox} Cnp^{+/-Cre}* knockout brains. There was a ~25% reduction of Olig2-positive cells in the brains after *miR-17-92* deletion, as compared with control animals (Fig. 1D,J).

To address the mechanism of miR-17-92 action in oligodendrocytes, we expressed miR-17 and miR-19b in the oligodendroglial cell line, Oli-neu. Overexpression of miR-17-19b resulted in a greater number of Oli-neu cells (Fig. 3A). Conversely, in a loss-of-function approach using antisense oligonucleotides, the cell number decreased. To efficiently interfere with the function of the miR-17-92 cluster, oligonucleotides directed against miR-17 and miR-19b were combined in order to target different functional groups of the miR-17-92 cluster. Similar results were obtained when the experiments were performed with primary cultures of oligodendrocytes (Fig. 3B,C). The delivery of the oligonucleotides into the cells was confirmed by a luciferase-based assay and real-time PCR (see Figs S3 and S4 in the supplementary material). Overexpression of miR-17-19b increased, whereas the antisense treatment reduced, the number of oligodendrocytes. The number of A2B5-, oligodendrocyte marker O1- and Mbp-positive oligodendrocytes changed to a similar degree, suggesting that the differentiation of oligodendrocytes was not impaired (Fig. 3D-F).

The changes in oligodendrocyte cell number upon miR-17-19b overexpression could indicate a role in the regulation of cell proliferation and/or cell survival. To distinguish between these

two possibilities, we established an oligodendrocyte survival assay based on the withdrawal of growth factors. When oligodendrocytes are switched to serum-free medium without supplemental growth factors (–B27), ~80% of the cells die within 48 hours by apoptosis (Fig. 4A). As previously shown, the addition of insulin or neuron-conditioned medium almost completely prevents cell death (Fig. 4A) (Barres et al., 1992). In order to determine whether miR-17-19b is able to rescue oligodendrocytes from apoptosis, miR-17-19b precursors were expressed in OPCs before growth factor deprivation. However, a rescue by miR-17-19b from cell death was not observed (Fig. 4B). Next, we tested whether insulin or neuron-conditioned medium, which efficiently protects oligodendrocytes from cell death, induced miR-17-19b expression. By real-time PCR we did not detect any differences in the level of miR-17 or miR-19b upon addition of insulin or conditioned neuronal medium (data not shown). Furthermore, the number of apoptotic cells did not change after transfection of miR-17-19b precursors, as evaluated by TUNEL staining (Fig. 4C; see Fig. S5 in the supplementary material). Together, these data argue against a role of miR-17-19b in regulating oligodendrocyte survival.

In order to resolve whether miR-17-19b functions in the regulation of oligodendroglial cell proliferation, we performed BrdU assays. BrdU was incorporated for 24 hours into OPCs that had been transfected with the miR-19b or control miRNA 4 hours after plating. Cells were fixed and the incorporation of BrdU into DNA was detected using a monoclonal antibody against BrdU. We found that the expression of miR-19b significantly increased the OPC proliferation rate, as compared with the control miRNA (Fig. 4D).

We searched for negative regulators of cell proliferation using Gene Ontology (www.geneontology.org) (242 gene products) that were also computationally predicted targets of miR-19 [TargetScanS (www.targetscan.org) and PicTar (pictar.mdc-berlin.de)]. Among the three targets (Ereg, Pten and Smarca2), the tumor suppressor Pten

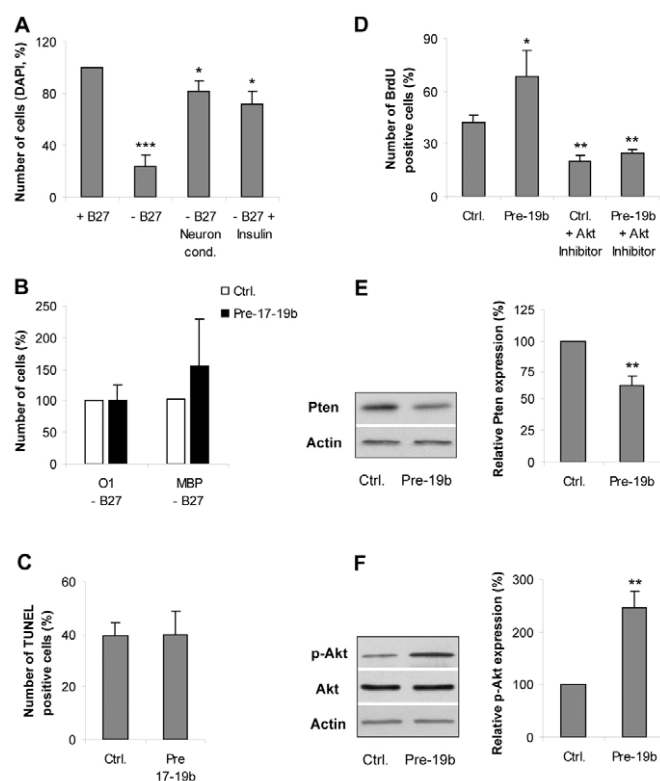


Fig. 4. miR-19b controls oligodendrocyte precursor cell proliferation by regulating Akt signaling. (A) Primary cultures of mouse oligodendrocytes were grown in serum-free medium containing B27 supplement (+B27), without B27 (–B27), with neuron-conditioned medium (neuron cond.) or supplemented with 10 µg/ml insulin. Changes in cell number upon B27 deprivation or rescue by insulin or neuronal conditioning are shown as mean \pm s.d. $n=3-4$; *, $P<0.05$; ***, $P<0.001$. (B,C) Pre-17-19b expression does not rescue oligodendrocyte death induced by B27 supplement deprivation ($n=4$) (B) and does not change the number of TUNEL-positive cells ($n=3$) (C). (D) Pre-19b expression in oligodendrocytes increases the number of BrdU-positive cells. Akt1/2 kinase inhibitor reduces cell proliferation. Changes in cell number are shown as the mean for three to four experiments; *, $P<0.05$; **, $P<0.01$. (E,F) Delivery of pre-19b into primary oligodendrocytes results in a reduction of Pten protein levels (E) and increased phosphorylation of Akt without affecting overall levels of Akt ($n=3-4$; **, $P<0.01$) (F).

seemed to be of particular importance, owing to its known function in regulating oligodendrocyte development (Barros et al., 2009; Narayanan et al., 2009). Overexpression of miR-19b resulted in a reduction of Pten protein levels in OPCs (Fig. 4E) (Olive et al., 2009). Furthermore, transfection of miR-19b into OPCs led to activation of Akt signaling, an essential downstream target of Pten. We observed increased phosphorylation of Akt, without affecting overall levels of Akt (Fig. 4F). In addition, incubating OPCs with the Akt1/2 kinase inhibitor reduced cell proliferation (Fig. 4D). Importantly, the increase in cell proliferation induced by miR-19b overexpression was blocked by the Akt kinase inhibitor. The effects of miR-19b are thus at least in part explained by the inactivation of Pten, which in turn stimulates cell proliferation and growth by Akt signaling.

In summary, we provide evidence that the miR-17-92 cluster miRNA family is enriched in cells of the oligodendroglial lineage and is involved in the control of oligodendrocyte number by

regulating their proliferation. The effects of the miR-17-92 knockdown in oligodendrocytes are strikingly similar to the phenotype observed after the conditional loss of *Dicer* using a *Cnp-Cre* line (Fig. 1). *Dicer* deletion reduces oligodendroglial cell number in the brain of P0 mice. It is interesting to compare our *Cnp-Cre-Dicer* mice with recently developed *Nestin-Cre-Dicer* mice, in which *Dicer* is depleted throughout the entire CNS (Kawase-Koga et al., 2009). Most of these mice die at an early embryonic stage and the few that survive until E18.5 have a lower number of cortical neurons and oligodendrocytes. Importantly, recent reports show that miRNAs also play a role in oligodendrocyte differentiation (Dugas et al., 2010; Zhao et al., 2010). Deletion of *Dicer* in oligodendrocytes using *Olig1-Cre* or *Olig2-Cre* results in an inhibition of oligodendrocyte differentiation and myelin formation (Dugas et al., 2010; Zhao et al., 2010). miR-219 and miR-338 were identified as oligodendrocyte-specific miRNAs that repress negative regulators of oligodendrocyte differentiation, including transcription factors such as Sox6. Interestingly, an expansion of the progenitor pool was observed at P7 and P14 when *Dicer* was deleted using an *Olig2-Cre* line. These results suggest that miRNAs control the expression of transcription factors that are crucial for oligodendrocyte differentiation, and they seem to be involved in the regulation of cell proliferation at earlier stages of development. Furthermore, post-natal *Dicer* ablation in mature oligodendrocytes using a *Plp-Cre* line results in another interesting phenotype, consisting of an inflammatory response and neuronal damage (Shin et al., 2009).

Together, these studies provide evidence for miRNAs as important regulators of oligodendrocyte development. Our work establishes the role of the miR-17-92 cluster in regulating oligodendrocyte proliferation and provides a resource for future studies on miRNA function in oligodendrocytes.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.050633/-DC1>

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