MicroRNA 22 Regulates Cell Cycle Length in Cerebellar Granular Neuron Precursors

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During cerebellum development, Sonic hedgehog (Shh)-induced proliferation of cerebellar granular neuronal precursors (CGNPs) is potently inhibited by bone morphogenetic proteins (BMPs). We have previously reported the upregulation of TIEG-1 and Mash1, two antimitotic factors that modulate MYCN transcription and N-Myc activity, in response to BMP2. To gain further insight into the BMP antimitotic mechanism, we used microRNA (miRNA) arrays to compare the miRNAs of CGNPs proliferating in response to Shh with those of CGNPs treated with Shh plus BMP2. The array analysis revealed that miRNA 11 (miR-22) levels significantly increased in cells treated with BMP2. Additionally, in P7 mouse cerebellum, miR-22 distribution mostly recapitulated the combination of BMP2 and BMP4 expression patterns. Accordingly, in CGNP cultures, miR-22 overexpression significantly reduced cell proliferation, whereas miR-22 suppression diminished BMP2 antiproliferative activity. In contrast to BMP2, miR-22 did not induce neural differentiation but instead significantly increased cell cycle length. Consistent with the central role played by N-myc on CGNP proliferation, Max was revealed as a direct target of miR-22, and miR-22 expression caused a significant reduction of Max protein levels and N-myc/Max-dependent promoter activity. Therefore, we conclude that, in addition to the previously described mechanisms, miR-22 plays a specific role on downstream BMPs through cerebellum growth.

Cerebellar granular neuronal precursors (CGNPs) are generated within the external germinal layer (EGL) during development of the cerebellar cortex. Clonal expansion of CGNPs is achieved by the mitogenic activity of Sonic hedgehog (Shh) signaling emanating from the Purkinje cells (PC) to the EGL (1, 2). During cerebellum development, CGNPs exit the cell cycle and migrate through the Purkinje cells to establish the three layers of the cerebellar cortex (3). MYCN is a direct Shh target (4) and one of the main downstream effectors of the Shh pathway during the expansion of CGNPs (4–6). The MYC transcription factors have well-established roles in regulating cell cycle progression and cell survival (7). MYC proteins belong to the basic helix-loop-helix (bHLH) family of transcription factors. The mammalian MYC family includes three different genes: Myc (C-MYC), encoding Myc protein, which displays a universal distribution, MYCN, encoding N-myc, and MYCL, encoding L-myc proteins, the last two being expressed mainly in the nervous system and the lungs, respectively. Despite their different expression patterns, Myc and N-myc proteins are mostly functionally interchangeable (8), although Myc overexpression not only induced significantly more aggressive tumors than N-myc in a murine model of medulloblastoma but also induced a completely different medulloblastoma subgroup, group 3 (9).

N-myc forms heterodimers with Max to activate transcription by binding to E-box motifs (CANNTG) (10, 11) of different genes involved in cell cycle regulation such as cyclin D2 (12). Shh-dependent proliferation of CGNPs depends entirely on N-myc activity (4, 6). The existence of local signals that counteract the mitogenic effects of Shh was predicted by the fact that exit from the cell cycle and migration of CGNPs both occur in a Shh-rich environment. Bone morphogenetic proteins (BMPs) belong to the transforming growth factor β (TGF-β) family of growth factors and play important roles during the development of the central nervous system. BMP2 to -4 are expressed at the EGL during the early genesis of postnatal cerebellum, where they function as powerful inhibitors of Shh-mediated proliferation of CGNPs (13). In CGNPs, BMP2 to -4 control MYCN activity through a multifaceted mechanism. On the one hand, BMPs induce the transcriptional repressor TIEG-1, which inhibits the activity of the MYCN promoter (14). On the other hand, BMPs potently enhance the levels of the bHLH proneural protein Mash1; Mash1-E12 dimers compete with N-myc/Max for the occupancy of the E boxes on N-myc target genes (15). In addition, using a posttranscriptional mechanism, BMPs raise the protein levels of Math1 (16), a proneural transcription factor required for Shh-induced proliferation of CGNPs and medulloblastoma formation (17, 18). microRNAs (miRNAs) comprise a large family of small (21 nucleotide [nt]) noncoding RNAs that have emerged as key regulators of posttranscriptional gene expression in virtually all cellular events (19, 20). miRNAs regulate protein synthesis by base pairing to target mRNAs. In animals, the majority of known miRNAs form imperfect hybrids between the mRNA 3′ untranslated region (3′UTR) and the mRNA 5′-proximal “seed region” (positions 2 to 8) (21). Ordinarily, miRNAs inhibit protein synthesis by repressing transla-
lutation and/or inducing deadenylation and subsequent degradation of their mRNA targets (21).

In the present work, we addressed whether the signals that antagonize Shh-dependent proliferation are, at least in part, mediated by miRNA molecules. Using mouse miRNA arrays, we compared the miRNA population from CGNPs proliferating under the influence of Shh with the miRNAs of CGNPs treated with Shh plus BMP2 or dibutyryl-cyclic AMP (DBA), a PKA activator that inhibits proliferation (14, 15). The array analysis revealed that miRNA 11 (miR-22) levels increased significantly after treatment with either DBA or BMP2. Likewise, the ectopic expression of miR-22 had a potent antiproliferative effect, significantly increasing the cell cycle duration in CGNPs. In addition, we observed that in P7 mouse cerebellum, the expression pattern of miR-22 recapitulated mostly BMP2 plus BMP4 expression patterns and that the suppression of miR-22 activity significantly reduced the antiproliferative effect of BMP2 on CGNPs. Interestingly, Max, which forms heterodimers with N-Myc, was scored as one of the best targets of miR-22 expression on the basis of the four different target scan programs. In agreement, the expression of miR-22 not only decreased Max protein levels but also significantly reduced N-Myc/Max-dependent promoter activity. Consequently, miR-22 expression selectively reduced the proliferation of Shh/N-myc-dependent neural tumor cell lines. Therefore, we conclude that miR-22 acts downstream from BMPs to modulate the activity of N-myc in CGNPs during the development of the cerebellum.

MATERIALS AND METHODS

Antibodies and chemicals. (i) Mouse monoclonal antibodies. The following mouse monoclonal antibodies were procured: anti-PCNA (SC-56; Santa Cruz), anticalbindin (CB-955; Sigma), anti-HuC/D (A21271; Molecular Probes), anti-β-tubulin III/Tuj1 (MMS435P; Covance), anti-Ki67 (16667; Abcam), and anti-β-actin (AC15; Sigma).

(ii) Rat monoclonal antibodies. Anti-8-bromodeoxyuridine (anti-BrdU) (BU1/75) was obtained from AbD-Serotec.

(iii) Rabbit polyclonal antibodies. Anti-green fluorescent protein (anti-GFP) was produced in our laboratory, and the following were obtained commercially: antiretinoblastoma phospho-Ser807 and Cdc2 phospho-Tyr15 (9308 and 9111; Cell Signaling), anti-Max (SC197; Santa Cruz), anti-cleaved-caspase 3 (55965; BD), and anti-histone 3-phospho-Ser10 (06-570; Millipore).

(iv) Immunocytochemical analysis. For immunocytochemical analysis, fluorescence-conjugated secondary antibodies anti-rabbit antibody–Alexa Fluor 488 and anti-mouse antibody–Alexa Fluor 594 (Molecular Probes) were used. Protein A/G-coupled peroxidase was obtained from Pierce. Human BMP2 produced in CHO cells was from R&D. Shh N-terminal peptide was produced in our laboratory as previously reported (14). 8-Bromodeoxyuridine was obtained from Sigma. DBA was obtained from Calbiochem.

Cell cultures and transfection. The preparation of cerebellar cultures was performed using a modification of the Papain method as described previously (13). For transient-transfection experiments, freshly isolated cells were electroporated in suspension and plated in Neurobasal plus B-27 medium (Invitrogen) supplemented with KCl (25 mM), glucose (1 mM), and Shh (3 µg/ml) on poly-l-lysine–plus laminin–coated dishes. Twenty-four hours later, the medium was replaced by fresh medium containing the corresponding treatments, considering this moment as time zero. Electroporation was performed using the Microprotor MP-100 (Digital Bio, Seoul, South Korea) according to the manufacturer’s instructions, with a single pulse of 1,700 V for 20 milliseconds. Neuroblastoma and medulloblastoma cell lines were grown on poly-l-lysine coated dishes in Dulbecco’s modified Eagle medium (DMEM)–10% fetal bovine serum (FBS) and DMEM–F-12–10% FBS, respectively.

DNA constructs. The miRNA expression vector pMICRO was created from pCIG, a GFP polycistronic expression vector, by the insertion of a new multiple-cloning site between the GFP-coding region and the rabbit β-globin polyadenylation site. To generate miRNA expression constructs, pre-miRNAs region, flanked by 200 additional nucleotides, were PCR-amplified from murine genomic DNA and cloned into pMICRO expression vector. The expression efficiency of pMICRO was confirmed through a real-time PCR that specifically detected the mature form of miR-22. A miR-22 decoy construct (dec22) was created by adapting the directions published in reference 22. Briefly, four repeats of a “bulged” (imperfectly complementary) miR-22 sequence (ACAGTTCTTCTACGCGAGCTT) separated by spacer sequences (CGAT) were cloned into pMICRO polylinker, and the capacity of dec22 to neutralize miR-22 activity was checked in HEK cells using the pLUCMAX construct. pLUCMAX vector was created from pGL3 basic vector (Promega) to study the effect of miR-22 expression on MAX 3'UTR. Briefly, using the pGL3 backbone (no promoter), a simian virus 40 (SV40) promoter and the entire MAX 3'UTR were cloned upstream and downstream, respectively, of the LUC gene. pLUCMAX-Scr is a version of pLUCMAX in which the miR-22 binding site on MAX 3'UTR (GCCAGCU, nucleotides 333 to 339 of mouse MAX 3'UTR) was scrambled by site-directed mutagenesis (UACCGG). Similarly, the miR-22 seed sequence (CCCGUCGA) was scrambled (CGUC GAC) and cloned into pMICRO to create Scr22 (scrambled miR-22). Empty pMICRO and pMICRO expressing Scr22 were both used as transfection controls.

The cyclin D2 promoter reporter plasmid containing the regions −1624 to +1 was obtained from Rene H. Medema (University Medical Center Utrecht, Utrecht, Netherlands). The 5X E-box reporter promoter was constructed within the pGL3 (Promega) plasmid and includes five repetitions of the CACGTG motif and a minimal TATA box. The cytomegalovirus (CMV)-Renilla luciferase was purchased from Promega. To generate a Max expression vector (pCIG-Max), the entire MAX coding sequence was PCR amplified from human brain cortex cDNA and cloned into pCIG.

BrdU incorporation assay and immunocytochemistry. For the BrdU incorporation assay, cells were pulsed with 50 ng/ml of BrdU 6 h prior to fixation with 4% paraformaldehyde. Cells were then permeabilized with methanol for 5 min, washed twice with phosphate-buffered saline (PBS), and incubated for 10 min with DNase I in DNase buffer (10 mM Tris-HCl [pH 7.4], 2.5 mM MgCl2, 0.1 mM CaCl2). Finally, cells were washed once with PBS, incubated overnight at 4°C with a mouse monoclonal anti-BrdU antibody, and developed with anti-mouse antibody–Alexa Fluor 555.

Immunohistochemistry and in situ hybridization. Mouse cerebella were fixed overnight with 4% paraformaldehyde, rinsed three times, and embedded in agarose blocks (5% agarose, 10% sucrose). Sections (50 µm) were obtained by vibratome. Exon miRCURY LNA double digoxigenin-labeled (DIG) (5'-3')-labeled probes of 22 nucleotides were used to perform in situ hybridizations against miR-22. According to the manufacturer’s directions, scrambled microRNA and miR-22 probes were used at 40 nM, the U6 snRNA probe was used at 1 nM, and hybridization was done overnight at 52°C. In situ hybridizations for MYCN were performed with DIG-labeled riboprobes (~500 nt) using standard protocols previously published (13). Immunohistochemistry was realized using vibrationate sections in “free-floating” conditions. The images were taken with a Leica optical microscope for in situ hybridization and with a Leica TCS SP5 confocal microscope for immunohistochemistry.

Immunoprecipitation and immunoblotting. Cultures grown in 6-well dishes were lysed with radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate [DOC], 0.1% SDS, 50 mM Tris [pH 7.5], and 1 mM phenylmethylsulfonyl fluoride [PMSF]) 72 h after electroporation. Following sonication, insoluble material was removed by centrifugation, 1/10 of the resulting supernatant was reserved as “input,” and the remaining part was immunoprecipitated overnight at 4°C with anti-Max rabbit polyclonal antibody. Antibody-antigen com-
plexes were collected with protein A-Sepharose beads (Amersham Biosciences). Then, beads were washed three times with Tris-buffered saline (25 mM Tris, pH 7.5, 140 mM NaCl), boiled in 1× SDS Laemmli sample buffer, resolved in 12% SDS-PAGE, transferred to nitrocellulose membranes, blocked with 8% nonfat dry milk in TTBS (150 mM NaCl, 0.05% Tween 20, and 20 mM Tris-HCl, pH 7.4), and then incubated with the same anti-Max antibody. The “inputs” were separated in a parallel gel and blotted with anti-β-actin monoclonal antibody. The blots were developed using protein A/G-coupled peroxidase plus the enhanced chemiluminescence (ECL) system and captured with the Versadoc imaging system from Bio–Rad. Expression values were quantified with Quantity One software (Bio–Rad); values were actin normalized and referenced toScr22 (scrambled miR-22)-transfected controls.

Luciferase assays. Luciferase reporter constructs (pGL3MAX, cy-cin-D2 promoter, or 5′X E-box) were coelectroporated with a CMV- Renilla vector and a 3-fold excess of the indicated expression constructs. Cells were cultured for 48 h and then lysed and analyzed with the dual-luciferase reporter assay system (Promega) according to the manufacturer’s instructions. Luciferase activity was detected with an Orion II microplate luminometer (Berthold). Luciferase data were normalized to the Renilla values, and results were plotted and expressed in arbitrary units as the means and standard deviations (SD) of three different experiments.

Cell counting and statistical analysis. Cell counting was calculated as the percentage of positive cells (BrDU, HuC/D, etc.) among the transplanted population (GFP-expressing cells). Duplicate wells from at least three different cultures were counted (minimum, 6 wells). The number of cells counted for each data point is indicated in the bars in the figures. Quantitative data were expressed as the means ± SD. Significant differences between groups were tested by one-way analysis of variance (ANOVA) followed by Tukey’s multiple-comparison test (except in the experiment shown in Fig. 2D, where a two-way ANOVA followed by a Bonferroni posttest was applied).

RNA isolation, RT-PCR, and real-time PCR. Total RNA was isolated from 10-cm culture plates using the mirVANA miRNA isolation kit according to the manufacturer’s indications. To study MAX mRNA levels by semiquantitative reverse transcription (RT)–PCR, total mRNA was extracted using TRIzol reagent (Invitrogen). Real-time PCR analysis of miRNAs was performed using the miCURY microRNA PCR system (Exiqon). Real-time PCR was performed in the MyiQ Single Color Real-Time PCR Detection system (Bio–Rad).

Microarray preparation and statistical analysis. Agilent mouse miRNA microarrays V2 (G4470B) were used to analyze independent samples (control, DBA-treated, and BMP-treated cells). Briefly, 500 ng of total RNA from each sample was chemically labeled with cyanine 3-pCp using the Agilent miRNA Complete labeling and hybridization kit (p/n5190-0456). Labeled samples were dried and resuspended in nuclease-free water and cohybridized with in situ hybridization buffer (Agilent) for 20 h at 55°C and washed at room temperature for 5 min in Gene Expression Wash Buffer 1 (Agilent) and for 5 min at 37°C in Gene Expression Wash Buffer 2 (Agilent). The images were generated on a confocal microarray scanner (G2505B; Agilent) at 5-μm resolution and quantified using Feature Extraction (Agilent). Extracted log2-transformed intensities were quantile normalized to make all data comparable. To assess differential expression, significance analysis of microarrays (SAM) was used. Results of SAM analysis were corrected for multiple testing using the false-discovery rate (FDR) method. Relevant probe significance cutoff was set as a combination of a q-value below 5% and an absolute fold change above 1.2. Final relative expression values were computed by taking the median log2 ratio of the respective probes for each miRNA.

RESULTS

miR-22 expression is induced by two independent antiproliferative pathways in CGNPs. Shh-induced proliferation of CGNPs can be reversed by growth factors such as basic fibroblast growth factor (b-FGF) (2) and BMPs (13) or by direct activation of cyclic AMP (cAMP)-dependent protein kinase A (PKA) with either forskolin, which directly stimulates adenylyl cyclase (AC) (2), or the cAMP analogue DBA (13). Activation of the Shh pathway promotes the translocation of active forms of Gli2-3 transcription factors to the nucleus, which in turn stimulate the production of other transcription factors, including Gli1 and N-myc (4, 6). PKA inhibits the Shh pathway through phosphorylation-dependent generation of the repressor forms of Gli2-3 transcription factors (23), whereas the BMPs act through a PKA-independent mechanism that requires downregulation of MYCN function (14). Therefore, to identify miRNAs participating in these pathways that modulate CGNP proliferation, we compared miRNA levels in proliferating CGNPs (Shh, 3 μg/ml) with those of CGNPs treated with 1 mM DBA or 100 ng/ml of BMP2 for 2 or 24 h (Fig. 1A). The heat map in Fig. 1B shows all the miRNA species that varied significantly between control cultures and those treated for 24 h with DBA or BMP2 (fold change values are indicated on the left of each heat map cell). Thus, BMP2 reduced the expression of miRNAs 17*, 19b, and 18a and increased miR-22. Interestingly, although the number of miRNAs regulated by DBA treatment was greater than with BMP-2, all miRNAs regulated by BMP-2 were also regulated similarly by DBA (Fig. 1B). Notably, miR-22 was the miRNA most changed under these two treatment conditions and was the only miRNA consistently upregulated by the four experimental conditions tested (DBA or BMP2, 2 or 24 h of treatment, heat map of 2-h treatment) (data not shown).

miR-22 is expressed in the cerebellar EGL. Although most of the cell types found in the adult cerebellum proliferate only during embryonic stages, more than 90% of the final cellular content of a mouse cerebellum consists of granular cells generated during the first 2 weeks of postnatal development. During this period, the CGNPs proliferate extensively at the EGL and migrate to the internal granular layer (IGL). Despite the intense proliferation, the thickness of the EGL remains nearly constant for more than 1 week, due to the balance between proliferation and migration processes, causing though an enormous increase in IGL size. Given our miRNA array results, we next confirmed the effect of DBA and BMP2 treatments on miR-22 expression by quantitative real-time PCR (Fig. 1C). In agreement with the array predictions, both treatments significantly increased miR-22 levels in CGNP cultures after 24 h, with BMP2 exerting a stronger effect than DBA. Previously, we reported that in P7 rat cerebellum, BMP2 and BMP4 are expressed predominantly at the IGL and the inner EGL, respectively (13). Thus, we next questioned whether the pattern of miR-22 expression during normal cerebellum development was related to BMP expression. So, we performed “in situ” hybridization (IH) on sagittal sections of mouse cerebellum using a commercial probe specifically designed against miR-22 (miRCURY LNA). We observed that in P7 mouse cerebellum, miR-22 was expressed at the inner EGL, at the Purkinje cells (PC), and especially at the IGL compared to the negative control (scrambled miRNA probe). We used a commercial probe directed against U6 SnRNA (which labels all cells in cerebellum) as a positive control and a riboprobe directed against MYCN (which labels the external EGL (4)) as a landmark (Fig. 1D). In addition, we observed that miR-22 expression was restricted to the IGL in P21 mice and barely detectable in the adult animals (P60) (Fig. 1E). Interestingly, miR-22 distribution mostly recapitulated the combination
of BMP2 and BMP4 expression patterns previously reported by our group (13).

Expression of miR-22 decreases the cell proliferation rate. BMPs have been shown to simultaneously repress proliferation and stimulate terminal neuronal differentiation of CGNPs. During this process, the levels of TIEG-1 and Mash1 increased considerably. Notably, although both transcription factors exhibited a potent antiproliferative activity, only Mash1 triggered neuronal differentiation of CGNPs (14,15). These results indicated that the effect of BMPs on CGNPs is accomplished through signals that differentially modulate proliferation and differentiation processes. Therefore, we wondered whether miR-22 expression would alter the proliferation and/or differentiation of CGNPs. So, we next planned to test the levels of different cell cycle and neuronal differentiation markers in CGNP cultures overexpressing miR-22. Pilot experiments indicated that the performance of the commercially available miRNA expression vectors was very poor in CGNPs. To circumvent this problem, we created a new miRNA expression vector (pMICRO) based on pCIG (an enhanced GFP [EGFP]-expressing bicistronic vector) (see Materials and Methods for vector creation details). We also used pMICRO to express dec22, a 4× decay sequence designed to neutralize miR-22 activity (see Materials and Methods for sequence and cloning details). To study cell proliferation, freshly isolated CGNPs were electroporated with scrambled miR-22 (Scr22) (used as a control) (see Materials and Methods for the sequence), miR-22, miR-361 (an miRNA upregulated by DBA but not by BMP2, used as an additional control), or dec22. Transfected cells were cultured for 48 h in a medium containing a saturating concentration of Shh (3 μg/ml), and a portion of these cells were treated with BrdU for the last 4 h. Finally, the cultures were fixed and stained with antibodies against BrdU (Fig. 2A and B). A similar culture was stained with anti-PCNA, another proliferation marker (Fig. 2C). Interestingly, the percentage of cells labeled with BrdU or PCNA was significantly lower in cultures transfected with miR-22 than in cultures transfected with empty vector, Scr22, or miR-361. Notably, dec22 consistently reversed the antiproliferative effect induced by miR-22, and in most cases it significantly elevated the basal proliferation rate, most likely due to the inhibition of the endogenous miR-22 (Fig. 2B).

miR-22 knockdown diminishes BMP antiproliferative potency. The good performance shown by the dec22 construct allowed us to explore the contribution of miR-22 to the antiproliferative effect developed by BMPs. Thus, control (Scr22) or miR-22-depleted
CGNPs were cultured for 48 h in a medium containing 3 μg/ml of Shh plus different concentrations of BMP2 (0, 10, 50, or 100 ng/ml). The cultures were then pulsed with BrdU for the last 4 h. Remarkably, BrdU counting showed a significant increase in cell proliferation in dec22-transfected cells at all BMP2 concentrations tested, including the control conditions (no BMP2 added) (Fig. 2D). This result demonstrates that endogenous miR-22 regulates cell proliferation in CGNPs and that miR-22 activity is necessary for the proliferation arrest induced by BMP2.

miR-22 prolongs cell cycle duration but does not induce neuronal differentiation of CGNPs. To better understand the effect of miR-22 on cell cycle progression, we next probed CGNP cultures with antibodies against retinoblastoma PS807 (phosphorylation indicates progression through G1 restriction point) (Fig. 3A), Cdc2 PY15 (dephosphorylation is required for G1-to-S and G2-to-M progression) (Fig. 3B), and histone 3 phosphorylation (M phase marker) (Fig. 3C). Notably, miR-22 transfection significantly decreased the number of Rb PS807- and PH3-labeled cells and increased the number of cells stained with Cdc2 PY15, indicating a reduction in cell cycle progression. In agreement, EGFP-sorted fluorescence-activated cell sorter (FACS) analysis of similar CGNP cultures demonstrated an accumulation of cells in G1/G0 and a reduction in G2 and M phases in miR-22-transfected cells compared to pMICRO (Fig. 3D). Because antiproliferative pathways are often associated with apoptosis, we next stained Scr22- or miR-22-transfected CGNPs with anticaspase 3 to identify apoptotic cells (Fig. 3E). In both cases, the percentage of apoptotic cells was very low (0.72% ± 0.23% and 0.63% ± 0.41%, respectively) compared to the positive control, where Scr22-transfected cells were cultured for the last 12 h with Neurobasal minus B27 supplement (12.03% ± 0.6%). Since the FACS results could equally indicate an accumulation of cells in G1 phase or an increase in cell differentiation (G0), we next wondered whether the cell proliferation arrest induced by miR-22 was accompanied by an increase in neuronal differentiation; therefore, we labeled miR-22-transfected CGNP cultures with Tuj1 (Fig. 3F and H) and HuC/D (Fig. 3G), two neuronal differentiation markers. Cell counting demonstrated that, in spite of its antiproliferative effect, miR-22 did not promote neuronal differentiation of CGNPs (Fig. 3F, G, and H). On the contrary, the percentage of differentiated cells was significantly increased by BMP2 treatment (100 ng/ml) (Fig. 3F and H). Altogether, these results indicated that the main miR-22 function in BMP signaling was related to cell cycle speed control rather than to cell survival or differentiation. Thus, using a procedure based on cumulative BrdU labeling, first described by Nowakowski et al. (24) and recently used to calculate cell cycle duration during cerebral cortex development (25), we calculated the growth fraction (i.e., the proportion of cells that comprise the proliferating population), the length of the cell cycle (Tc), and the length of the DNA-synthetic phase (Ts). Freshly isolated CGNPs were electroporated and cultivated for 24 h in medium containing 3 μg/ml of Shh. At this point, a dose of 50 ng/ml of BrdU was added to the cultures, and an additional dose of 50 ng/ml was added every 12 h. The cultures were fixed at the indicated time points and stained with anti-BrdU. For this experiment, scrambled miR-22 (miR-22-Scr) rather than empty vector was used as miR-22 control to maximize the similarities between the two constructs. The percentage of cells that incorporated BrdU among the GFP-positive population was calculated for each time point and plotted against time as indicated in reference 24 (Fig. 3I). The tendency lines were generated for each group with the program GraphPad Prism, and the equation formula and the \( R^2 \) coefficient of determination are
shown in the colored boxes. Notably, the growth fraction, indicated in the plot by the point where the BrdU/GFP index reaches the “plateau” (dotted line and arrowheads), was very similar in cells transfected with miR-22-Scr (42.9 ± 0.4) and miR-22 (42.6 ± 0.1), confirming the absence of prodifferentiation effects of miR-22. Plotting the data in this way, the slope of the tendency lines is proportional to the cell cycle speed, and the intersection between the tendency and plateau lines shows the total cycle duration minus the DNA synthesis phase (Tc - Ts) (18.5 h for miR-22-Scr and 31.9 h for miR-22, indicated with arrows on the x axis). The duration of the S phase (Ts) is obtained from the intersection between the tendency lines and the y axis. Therefore, total cycle duration (Tc) can be calculated by adding Ts to Tc - Ts. In conclusion, miR-22 expression increased the total cycle duration from 24.9 h to 36.1 h and decreased S-phase extent from 6.4 h to 4.2 h. This result is totally coherent with all the previous data obtained by FACS analysis or with cell cycle and cell fate markers. Altogether, these two groups of experiments demonstrate that the main effect of miR-22 on cell cycle progression consists of a slowdown of the G1 phase advance.

Max, the obligate partner of N-myc, is a target of miR-22 in CGNPs. Various lines of evidence support the essential role played by the transcription factor N-myc in mediating the Shh-dependent proliferation of CGNPs (4–6). In addition, it is also well established that MYCN is one of the main targets of BMPs in these cells (14,15). Interestingly, Max, the obligate partner of N-myc, was scored as one of the best targets of miR-22 by three different target prediction programs: miRANDA (www.microrna.org/microrna/home.do), Targetscan (www.targetscan.org), and PicTar (http://pictar.mdc-berlin.de). Whereas MYCN mRNA has
previously been detected at the outer EGL in P7 mouse cerebellum (4), little is known about the developmental expression pattern of Max. Thus, before performing studies to define the functional relationship between miR-22 and Max, we wanted to first assess whether the expression pattern of Max is compatible with that of miR-22 during cerebellum development in vivo (Fig. 4A). We first confirmed the specificity of the anti-Max antibody by immunoprecipitation (IP) followed by Western blotting (WB). Anti-Max antibody immunoprecipitated and blotted a single band in CGNP lysates, which was coincident in molecular weight with recombinant human Max. (D) Max protein expression levels were measured by IP/WB in cultures transfected with miR-22 or dec22 (miR-22 decoy), and values were actin normalized and referenced to Scr22 (scrambled miR-22)-transfected controls. Fold change ± SD from three different experiments is indicated above each lane. (E) The effects of miR-22 expression on MAX mRNA levels were studied by semiquantitative RT-PCR in CGNP cultures transfected for 48 h. PCR cycles were optimized for each set of primers to ensure linearity. (F) pLUCMAX (see Materials and Methods for construct details) luciferase activity was studied in CGNPs transfected for 48 h with either empty vector (pMICRO) or miR-22 or its scrambled control (Scr22). (G) In a similar experiment, the effect of miR-22 expression on pLUCMAX activity was compared to that on pLUMAX-Scr, a variant of pLUCMAX in which the miR-22 binding site on MAX3’UTR was scrambled. Graph bars represent the means ± SD.

FIG 4 MAX is a target of miR-22. (A) Max protein expression was studied in sagittal sections of mouse cerebellum at P4 and P8 developmental stages. To better define Max protein distribution, slices were costained with anticalbindin, a Purkinje cell marker, and the nuclear stain DAPI. (A’) Similar P8 mouse cerebellum sections were stained with TO-PRO, a DNA dye, and with the proliferation marker Ki67 to expose proliferating cells. (B) Enlargements of the areas boxed in white in panels A and A’. The locations of the different cerebellar layers are indicated by gray bars above the pictures: PC, Purkinje cell layer; ML, molecular layer; EGL, external germinal layer. (C) The specificity of the anti-Max antibody was assessed by immunoprecipitation (IP) followed by Western blotting (WB). Anti-Max antibody immunoprecipitated and blotted a single band in CGNP lysates, which was coincident in molecular weight with recombinant human Max. (D) Max protein expression levels were measured by IP/WB in cultures transfected with miR-22 or dec22 (miR-22 decoy), and values were actin normalized and referenced to Scr22 (scrambled miR-22)-transfected controls. Fold change ± SD from three different experiments is indicated above each lane. (E) The effects of miR-22 expression on MAX mRNA levels were studied by semiquantitative RT-PCR in CGNP cultures transfected for 48 h. PCR cycles were optimized for each set of primers to ensure linearity. (F) pLUCMAX (see Materials and Methods for construct details) luciferase activity was studied in CGNPs transfected for 48 h with either empty vector (pMICRO) or miR-22 or its scrambled control (Scr22). (G) In a similar experiment, the effect of miR-22 expression on pLUCMAX activity was compared to that on pLUMAX-Scr, a variant of pLUCMAX in which the miR-22 binding site on MAX3’UTR was scrambled. Graph bars represent the means ± SD.
independent functions in differentiated neurons. In fact, it has been previously reported in both developing brain and the P19 proneural cell line that upon cell cycle exit, Myc is rapidly downregulated while Mad is upregulated, forming complexes with Max to induce differentiation (26, 27). Next, we studied the capacity of miR-22 to target Max in CGNPs, observing that the Max protein level was significantly reduced in miR-22-transfected cells compared to cultures transfected with Scr22 or dec22 (Fig. 4D). In addition, by semiquantitative RT-PCR we also observed that the Max mRNA level was consistently reduced by miR-22 expression (Fig. 3E). Thus, we next performed two sets of experiments to demonstrate the relevance of the predicted miR-22 binding motif located on the 3'UTR of MAX mRNA. First, we created an SV40-driven modification of pGL3 to enable the cloning of the entire MAX mRNA 3'UTR downstream of the LUC sequence (pLUCMAX) (see Materials and Methods for vector creation details and mutant sequences). Using this tool, we observed that luciferase activity was significantly reduced by miR-22 expression but not by scrambled control sequence (Scr22) (Fig. 4D). In the second set of experiments, we observed that the inhibitory effect of miR-22 on pLUCMAX luciferase activity was lost in cells transfected with pLUCMAX-Scr, in which the miR-22 binding site on Max 3'UTR had been scrambled (Fig. 4G). Collectively, these results demonstrate that Max is a direct target of miR-22 in CGNPs.

miR-22 inhibits N-myc-dependent transcription and proliferation. Although it is well established that N-myc transcriptional activity requires Max, it remains controversial whether the availability of Max itself constitutes a mechanism for regulating N-myc activity. Depending on the cell system used, it has been observed that an excess of ectopic Max could either enhance or inhibit N-myc activity. Whereas N-myc appears to interact exclusively with Max, Max is less selective, as it binds to members of the Mad family, which function as transcriptional repressors (26, 28). Therefore, we studied whether miR-22 modulates N-myc-dependent transcriptional activity. Using CGNP cultures growing in a saturating concentration of Shh (3 μg/ml), we cotransfected miR-22 and N-myc, along with a reporter construct where luciferase was driven either by an artificial 5'E-box or by the natural human cyclin D2 promoter (Fig. 5A and B). In both cases, the expression of miR-22 significantly reduced N-myc-dependent transcription. As other miR-22 targets involving or not MYC activity have been reported to modulate cell proliferation (29–32), we explored to what extent the inhibitory effect of miR-22 on Shh-induced cell proliferation was due to reduction of Max expression. Therefore, we analyzed proliferation in CGNPs treated with Shh and transfected either with miR-22 alone or with miR-22

FIG 5 miR-22 reduces MYC-dependent transcription and proliferation. The bar graphs show the means ± SD of at least 3 independent experiments. The total number of cells counted for each data point is indicated in each bar in panels C through G. (A) CGNPs electroporated with a 5'E-box-driven luciferase reporter vector plus miR-22, MYCN, or their combination were cultured for 24 h with Shh (3 μg/ml) and for an additional 24 h period without Shh; cells were then lysed, and luciferase activity was measured. Empty pMICRO vector was used as a control and was also employed to equalize the amount of transfected DNA among wells. (B) Transfections were performed as described for panel A, but in these experiments the luciferase activity was controlled by the cyclin D2 promoter (which contains 2 natural E-boxes). (C) CGNPs were transfected with miR-22 or miR-22 plus a nontargetable form of Max and cultured with Shh (3 μg/ml). Proliferation was studied at 48 h by BrdU incorporation. (D, E) The effect of miR-22 expression on cell proliferation was assessed in two different human neuroblastoma cell lines, SK-BE (bearing a MYCN amplification) and SH-SY-5Y (no MYCN amplification). Proliferation of cells was estimated by BrdU incorporation 48 h after transfection. (F, G) In a similar experiment, the effect of miR-22 expression was evaluated in two cell lines for which proliferation has been reported to depend on MYC activity, C17-2, a murine neuroectodermal cell line created through v-myc transformation, and D283, a human medulloblastoma cell line with high levels of Myc but without MYC or MYCN amplifications.
DISCUSSION

BMP2 and PKA activation downregulate the miR-17-92 cluster expression. The miR-17-92 cluster, also called Oncomir-1, was among the first miRNAs to be validated as showing oncogenic potential and was shown to collaborate with Myc in B cell lymphoma formation (36). Since then, several lines of evidence have suggested a positive functional relationship between Shh pathway and miR-17-92 expression during physiologic and pathologic cerebellum development. Thus, the miR-17-92 cluster was found to be expressed in the developing mouse cerebellum and in proliferating CGNPs but not in postmitotic differentiated neurons (37). In addition, the miR-17-92 cluster level was observed to be very high in mouse and human medulloblastomas with an aberrantly activated Shh pathway. Moreover, medulloblastoma pentrace in immunocompromised mice orthotopically transplanted with CGNPs purified from P6 Patch^+/−; Ink4c^−/− mice was about 30% but increased to 100% when the CGNPs were transduced with the miR-17-92 cluster before transplantation (37). On the contrary, no medulloblastomas were formed when the CGNPs were obtained from Ink4c^−/−; p53^−/− mice. Therefore, considering that the miR-17-92 cluster has been shown to be a direct target of Myc (38) and that the Shh pathway increased MYCN expression in CGNPs (4) and in medulloblastomas (39), it is logical to think that Shh-dependent regulation of miR-17-92 expression in CGNPs and medulloblastoma is mediated by N-myc. Previous reports demonstrated that BMP2 treatment (14, 16) and PKA activation (40) decreased N-myc levels in P6 CGNPs. In addition, in the present work we show a downregulation of the miR-17-92 cluster expression induced by BMP2 and DBA (PKA activation). It therefore suggests that Shh and BMP pathways converge over NMYC to regulate miR-17-92 cluster expression. In agreement, we previously reported a significant decrease of MYCN mRNA (at 12 h) and N-myc protein (at 24 h) induced by BMP2 treatment (14). On the contrary, however, Zhao et al. (16) did not find differences in N-myc expression until 72 h of BMP treatment (but no time points between 24 and 72 h were studied), suggesting that BMP2 regulation of miR-17-92 could occur prior to N-myc variation. In any case, a probable explanation for this apparent discrepancy may be found in the way the P6 CGNPs cultures are prepared in the different laboratories. In our group, we always leave the culture to recover for the first 24 h (in Shh at 3 μg/ml) before starting any experiment, for we observed that either due to the aggressiveness of the disaggregation treatment itself or due to inherited inhibitory signals, the responsiveness of CGNPs during this period is diminished.

miR-22 operates downstream from BMPs controlling cell cycle length but does not induce differentiation. miRNAs are emerging as master regulators of development that control cellular proliferation and differentiation and have therefore also been linked to cancer (41). Previously, we reported that BMPs induce a rapid exit from the cell cycle and thereby induce the differentiation of CGNPs in vitro and in culture (13). Here, using the same cellular model, we have observed a marked upregulation of miR-22 expression in response to BMP2 (Fig. 1B and C), coincident with cell cycle arrest and induction of the differentiation process. Whether the BMPs are the only extracellular signals that regulate miR-22 expression in cerebellum remains to be determined, since we cannot yet exclude the possibility that other factors that promote the differentiation of CGNPs also upregulate miR-22 expression. The fact that miR-22 expression in CGNP cultures was also increased by PKA activation suggests that miR-22 may be a common element of different antiproliferative/prodifferentiation pathways. In 2009, Roussel’s group performed a very extensive study in which the miRNomes from wild-type mouse cerebella (P6, P30, and purified CGNPs) were compared to cerebella obtained from different mouse models of medulloblastoma and to medulloblastomas from these models. Although some of the miRNAs that were increased in P30 cerebellum compared to P6 were also increased in our DBA treatment (miR300 and miR128), no significant differences in miR-22 levels were reported (37), suggesting that compared to other miRNAs, miR-22 is not specifically linked to the terminal differentiation status. In agreement with Uziel et al., our IHC study shows that miR-22 is expressed at the inner EGL and IGL of P7 mouse cerebellum, being still abundant at the IGL of P21 but almost undetectable in adult mice (P60) (Fig. 1E), altogether suggesting a role during the differentiation process but not in maintaining the differentated condition. Similar to neuronal progenitors, cell cycle arrest is normally accompanied by terminal differentiation in many other cell types as hematopoietic cells or myocytes. Interestingly, miR-22 was previously reported to be induced by tetradecanoyl phorbol acetate (TPA) treatment of the hematopoietic HL-60 cell line, which triggers their differentiation (42), and was in the differentiation-associated miRNA group in myoblast-myotube differentiation; as observed in CGNPs, miR-22 reduced myoblast proliferation (29). Additionally, miR-22 was identified as a signature miRNA for erythrocyte maturation (43). However, our results clearly demonstrate that at least in CGNPs, miR-22 slows down the cell cycle progression but does not induce neuronal differentiation, indicating that the reported presence of miR-22 in various differentiating cell types does not necessarily imply a direct role of miR-22 in the differentiation process. Moreover, miR-22 has also been demonstrated to have a potent antiproliferative effect in differ-
ent cancer cell lines (30) and to induce senescence in fibroblasts (32). Thus, given that compounds that promote differentiation or senescence exert their effects primarily on the cell cycle, miR-22 may reflect a common antiproliferative element working in different cellular processes whereby a slowdown in the cell cycle is required. Similarly, TIEG-1 and Mash1 are both antiproliferative transcription factors reported to increase during BMP-induced neuronal differentiation of CGNs, and although in overexpression experiments only Mash1 induced neuronal differentiation, the dose of BMP2 required to induce neural differentiation was 100 times lower in TIEG-1-overexpressing cells (14, 15), suggesting that proliferation-arrested neuronal progenitors were more prone to differentiate. Now that we have observed that the miR-22 effect is very similar to that carried out by TIEG-1, it will be interesting to assess whether these two molecules work in parallel or alternatively in a consecutive manner. Coherent with the effect of miR-22 on proliferation, we observed that expression of miR-22 decreases Max levels and MYCN-dependent signaling in primary cultures of CGNs, and these data are in agreement with a recent report (30) identifying Max as a target of miR-22 in the A549 lung carcinoma cell line. However, the fact that the antiproliferative effect of miR-22 was only partially rescued by restoring Max expression strongly suggests that miR-22, similar to other known miRNAs, develops its activity through a multitarget mechanism. Indeed, MYCBP, another regulator of MYC signaling, is also targeted by miR-22 (31). Thus, more studies will be required to reveal the individual contribution of each miR-22 target on the cell cycle.

Possible role of miR-22 in regulating neoplastic growth. The increasing information regarding miRNA target networks has begun to reveal a new level of complexity in cell physiology. These networks function together with transcription factors to more precisely regulate different cellular process. Thus, oncogenic factors like MYC proteins initiate both transcription (44) and miRNA cascades (45) to control a range of biological activities, including proliferation, differentiation, and cellular energy production. Moreover, a bidirectional cross talk between these two types of regulators most likely exists, whereby oncogenes regulate the expression of specific miRNAs, and in turn, certain groups of miRNAs regulate parameters of oncogene signaling. Although it was shown initially that Myc oncogenic activity was highly dependent on the upregulation of a protumorigenic group of miRNAs known as the miR-17-92 cluster (46), a more recent study has demonstrated that Myc also represses the expression of an important group of antiproliferative/differentiation-related miRNAs; ectopic expression of these miRNAs diminishes the tumorigenic potential of Myc-induced lymphoma cells (47). Notably, miR-22 was one of the miRNAs whose expression decreased most significantly in response to MYC activation (47). On the other hand, it has been shown that components of the MYC signaling complex, including MYCBP, a positive regulator of Myc (31), or the Myc partner Max (30) are direct targets of miR-22. Recombinant expression of miR-22 significantly reduces Myc-dependent signaling and tumorigenic effects in different cell types (30, 31). Thus, a reciprocal negative autoregulatory loop between MYC signaling and miRNAs such as miR-22 seems to exist. Compared to proto-oncogenes such as Ras, where oncogenicity is usually caused by mutations in the protein, MYC proteins very rarely bear mutations, and thus, their oncogenic potential depends rather on expression levels. Deregression of MYC expression due to mechanisms including retroviral insertion, chromosomal translocation, and gene amplification is known to be the cause of various neoplastic processes (7). Therefore, it is plausible that loss of even a single element controlling MYC levels would be sufficient to initiate or maintain neoplastic growth. The miR-22 locus in humans is situated at position 1,220,571,1,220,655 (NCBI Genome Browser or Sanger miRNA database numbering) in the 17p13.3 band. Interestingly, loss of the 17p region is a rather common chromosomal aberration in different tumor types. In medulloblastomas, a malignancy arising from cerebellar cell precursors, loss of 17p is found in up to 50% of the tumors and is considered an indicator of a poor prognosis (48–50), and although 17p loss is more frequent in medulloblastoma groups 3 (~45%) and 4 (~60%), up to 20% of Shh group medulloblastomas present deletions in 17p (51), converting it into a common feature among different medulloblastoma types. However, the fact that tp53 gene is located in the 17p13.1 band led to the hypothesis that tumorigenicity associated with 17p loss was due mostly to a lack of p53 expression. Nevertheless, it was observed that in a proportion of medulloblastomas the deletion was limited to the 17p13.3 band and tp53 was not mutated (52–54). Thus, it seems very probable that other tumor suppressors are lost in 17p deletions. Consistent with this, several other genes were proposed to be responsible for the tumorigenic effect of 17p deletions (55). In agreement, a recent work has demonstrated that miR33b, a statin-regulated microRNA located at 17p11.2, targets MYC and regulating c-Myc-dependent medulloblastoma proliferation (35). In any case, additional studies will be required to define the precise contributions of miR-22 loss to neoplastic growth.

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