Title: Mir-22 regulates cell-cycle length in Cerebellar Granular Neuron Precursors.

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ABSTRACT

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 up-regulation of TIEG-1 and Mash1, two anti-mitotic factors that modulate MYCN transcription and N-Myc activity, in response to BMP2. To gain further insight into BMP anti-mitotic mechanism, we used 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 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 CGNPs cultures Mir-22 over-expression significantly reduced cell proliferation, whereas Mir-22 suppression diminished BMP2 anti-proliferative activity. Opposite to BMP2, Mir-22 did not induce neural differentiation, instead it significantly increased cell-cycle length. Consistent with the central role played by N-myc on CGNPs 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 downstream BMPs through cerebellum growth.
INTRODUCTION

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) signalling emanating from the Purkinje cells to the EGL (13, 52). During cerebellum development, CGNPs exit the cell-cycle and migrate through the Purkinje cells to establish the three layers of the cerebellar cortex (1). MYCN is a direct Shh target (27) and one of the main downstream effectors of the Shh pathway during the expansion of CGNPs (23, 27, 39). The MYC transcription factors have well-established roles in regulating cell-cycle progression and cell survival (34). 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 which are mainly expressed in the nervous system and the lungs, respectively. Despite their different expression patterns, Myc and N-myc proteins are mostly functionally interchangeable (31), although Myc over-expression not only induced significantly more aggressive tumours than N-myc in a murine model of Medulloblastoma, but also induced a completely different medulloblastoma subgroup, Group 3 (26).

N-myc forms heterodimers with Max to activate transcription by binding to E-box motifs (CANNTG) (6, 7) of different genes involved in cell-cycle regulation such as cyclin D2 (8). Shh-dependent proliferation of CGNPs depends entirely on N-myc activity (27, 39). The existence of local signals which 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 TGF-β family of growth
factors and play important roles during development of the central nervous system. BMP2-4 are expressed at the EGL during the early genesis of postnatal cerebellum where they function as powerful inhibitors of Shh-mediated proliferation of CGNPs (42). In CGNPs, BMP2-4 control MYCN activity through a multi-faceted mechanism. On the one hand, BMPs induce the transcriptional repressor TIEG-1 which inhibits the activity of the MYCN promoter (2). On the other hand, BMPs potently enhance the levels of the β-HLH proneural protein Mash1; Mash1-E12 dimers compete with N-myc/Max for the occupancy of the E-boxes on N-myc target genes (3). In addition, using a post-transcriptional mechanism, BMPs rise the protein levels of Math1(55), a pro-neural transcription factor required for Shh induced proliferation of CGNPs and medulloblastoma formation (18, 22). MicroRNAs (miRNAs) comprise a large family of small (~21 nucleotides) non-coding RNAs that have emerged as key regulators of posttranscriptional gene expression in virtually all cellular events (5, 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’ untraslated region (3’UTR) and the miRNA 5’-proximal “seed region” (positions 2-8) (16). Ordinarily, miRNAs inhibit protein synthesis either by repressing translation and/or inducing deadenylation and subsequent degradation of their mRNA targets (16).

In the present work, we addressed whether the signals which 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 BMP-2 or DBA (Dibutyryl-cAMP), a PKA activator that inhibits proliferation (2, 3). The array analysis revealed that Mir-22 levels increased significantly after treatment with either DBA or BMP-2. Likewise, the ectopic expression of Mir-22 had a potent anti-proliferative effect,
significantly increasing the cell-cycle duration in CGNPs. In addition, we observed that in
P7 mouse cerebellum, the expression pattern of Mir-22 mostly recapitulated BMP2 plus
BMP4 expression patterns, and that the suppression of Mir-22 activity significantly reduced
the anti-proliferative effect of BMP2 on CGNPs. Interestingly, Max, which forms
heterodimers with N-Myc, was scored as one of the best targets of Mir-22 using three
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 tumour cell lines. Therefore, we conclude that Mir-22 acts downstream
BMPs to modulate the activity of N-myc in CGNPs during development of cerebellum.

MATERIALS AND METHODS

Antibodies and Chemicals

Mouse monoclonal antibodies: anti-PCNA (Santa Cruz, SC-56), anti-Calbindin (Sigma,
CB-955), anti-HuC/D (Molecular Probes, A21271), anti-βTubulin III/Tuj1 (Covance,
MMS435P), anti-Ki67 (Abcam, 16667) anti-β-Actin (Sigma, AC15).
Rat monoclonal antibodies: anti-bromodeoxyuridine (BrdU) (AbD-Serotec, BU1/75).
Rabbit polyclonal antibodies: anti-GFP was produced in our laboratory, anti-
Retinoblastoma phospho-Ser807 and Cdc2 phospho-Tyr15 (Cell Signalling 9308 and
9111), anti-Max (Santa Cruz, SC197), anti-cleaved-Caspase 3 (BD 559565), anti-Histone3-
phospho-Ser10 (Millipore, 06-570).
For immunocytochemical analysis, fluorochrome-conjugated secondary antibodies
anti-rabbit Alexa 488 and anti-mouse Alexa 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 (2). 8-Bromodeoxyuridine was purchased from Sigma. DBA was purchased from Calbiochem.

**Cell cultures and transfection**

The preparation of cerebellar cultures was performed using a modification of the Papain method as described previously (42). For transient-transfection experiments, freshly isolated cells were electroporated in suspension and plated in Neurobasal plus B-27 media (Invitrogen) supplemented with KCl 25 mM, glutamine 1 mM and Shh 3 µg/ml on poly-L-Lys plus laminin coated dishes. Twenty four hours later, the media was replaced by fresh media containing the corresponding treatments, considering this moment as time 0. Electroporation was performed using the Microporator MP-100 (Digital Bio, Seoul, Korea) according to the manufacturer’s instructions, with a single pulse of 1700 V for 20 milliseconds. Neuroblastoma and medulloblastoma cell lines were grown on poly-L-Lys coated dishes in DMEM-10% FBS and DMEM/F12 -10% FBS, respectively.

**DNA constructs**

The miRNA expression vector pMICRO, was created from pCIG, a GFP poly-cistronic 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 reaction which specifically detected the mature form of Mir-22. A Mir-22 decoy construct (dec22), was created by adapting the directions published in (19), briefly, four repeats of a "bulged" (imperfectly complementary) Mir-22 sequence (ACAGTTCTTCATGCGAGCTT) separated by spacer sequences (CGAT) were cloned into pMICRO poly-linker, the capacity of dec22 to neutralize Mir-22 activity was checked in HEK cells using pLUCMAX construct. pLUCMAX vector was created from pGL3 basic vector (Promega) to study the effect of Mir-22 expression on MAX3'UTR. Briefly, using the pGL3 backbone (no promoter), an SV40 promoter and the entire MAX 3'UTR were cloned upstream and downstream of LUC gene, respectively. pLUCMAX-Src, is a version of pLUCMAX where the Mir-22 binding site on MAX3'UTR (GGCAGCU, nucleotides 333 to 339 of mice MAX3' UTR) was scrambled by site-directed mutagenesis (UACCGCG). Equally, Mir-22 seed sequence (CCGUCGA) was scrambled (CGUCGAC) and cloned into pMICRO to create Src22 (scrambled Mir-22). Empty pMICRO or 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, The 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 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 50ng/ml of 8-bromodeoxyuridine (BrdU) 6 hours prior to fixation with 4% paraformaldehyde. Cells were then permeabilized with methanol for 5 minutes, washed twice with PBS and incubated for 10 minutes with DNAse I in DNAse buffer (10 mM Tris-HCl, pH 7.4, 2.5 mM MgCl$_2$, 0.1 mM CaCl$_2$). Finally, cells were washed once with PBS, incubated overnight at 4°C with a mouse monoclonal anti-BrdU antibody and developed with anti-mouse Alexa-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). 50 μM sections were obtained by vibratome. Exiqon miRCURY LNA™ double DIG (5'-3') labelled probes of 22 nucleotides, were used to perform in situ hybridizations against Mir-22. Following manufacturer directions, scrambled microRNA and Mir-22 probes were used at 40 nM and U6 snRNA probe at 1nM, and hybridization was done overnight at 52°C. In situ hybridizations for **MYCN** was performed with DIG labelled riboprobes (~500 nt) using standard protocols previously published (42). Immunohistochemistry was realized using vibratome sections in “free flouting” 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 RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 50 mM Tris, pH 7.5, and 1 mM PMSF) 72 hours after
electroporation. Following sonication, insoluble material was removed by centrifugation, 1/10th of the resulting supernatant was reserved as “input” and the remaining part was immunoprecipitated overnight at 4 °C with anti-Max rabbit polyclonal antibody. Antigen-antibody complexes 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 X SDS Laemmli sample buffer, resolved in a 12% SDS-PAGE, transferred to nitrocellulose membranes, blocked with 8% non-fat 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 ECL system and captured with Versadoc Imaging System from Bio-Rad. Expression values were quantified with Quantity OneTM software (Bio-Rad) values were Actin –normalized and refereed to Scr22 (scrambled Mir-22) transfected controls.

Luciferase assays

Luciferase reporter constructs (pLUCMAX, cyclin-D2 promoter or 5x E-box) were co-electroporated with a CMV-Renilla vector and a three-fold excess of the indicated expression constructs. Cells were cultured for 48h hours and then lysed and analysed with the Dual-Luciferase Assay System (Promega) following 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 mean and standard deviation 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 transfected 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. Quantitative data was expressed as the mean ± standard deviation (SD). Significant differences between groups were tested by one-way ANOVA followed by the Tukey’s multiple comparison test except in the experiment shown in Fig 2D where a two-way ANOVA followed by a Bonferroni post-test was applied.

RNA isolation, RT PCR and Real-time PCR

Total RNA was isolated from 10 cm culture plates using miRVANA miRNA isolation kit according to the manufacturer’s indications. To study MAX mRNA levels by semi-quantitative RT-PCR, total mRNA was extracted using Trizol reagent (Invitrogen). Real-time PCR analysis of miRNAs was performed using miRCURY™ microRNA PCR system (Exiqon). Real-Time PCR was performed in a MyiQ Single Colour Real-Time PCR Detection System (Bio-Rad).

Microarray preparation and statistical analysis

Agilent mouse miRNA microarrays V2 (G4470B) were used to analyse independent samples (control, DBA and BMP treated cells). Briefly, 500 ng of total RNA from each sample were chemically labelled with Cyanine3-pCp using Agilent miRNA Complete Labelling and Hybridization Kit (p/n5190-0456). Labelled samples were dried and resuspended in nuclease-free water and co-hybridized 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 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 cut off was set as a combination of 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 per each miRNA.

RESULTS

Mir-22 expression is induced by two independent anti-proliferative pathways in CGNPs.

Shh-induced proliferation of CGNPs can be reversed by growth factors such as b-FGF (52) and BMPs (42) or by direct activation of cAMP-dependent protein kinase A (PKA) with either forskolin, which directly stimulates adenylate cyclase (AC) (52), or the cAMP analogue DBA (42). 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 (27, 39). PKA inhibits the Shh pathway through phosphorylation-dependent generation of the repressor forms of Gli2-3 transcription factors (51), whereas the BMPs act through a PKA-independent mechanism that requires down-regulation of MYCN function (2). Therefore, to identify miRNAs which
participate in these pathways that modulate CGNP proliferation, we compared miRNA levels in proliferating CGNPs (Shh 3μg/ml) with those of CGNPs treated with 1mM of DBA or 100 ng/ml of BMP2 for 2 or 24h (Fig 1A). The heat map of Figure 1B shows all miRNA species that varied significantly between control cultures and those treated for 24 h with DBA or BMP-2 (fold change values are indicated at the right of each heat map cell). Thus, BMP-2 reduced the expression of miRNAs 17*, 19b, and 18a and only 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 by both treatment conditions and was the only miRNA consistently up-regulated by the four experimental conditions tested (DBA or BMP2, 2 or 24h of treatment, heat-map of 2h treatment, 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 are granular cells generated during the first two 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 one 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 BPM2 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 24h, 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 (42) 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” hybridizations (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 specially at the IGL, as compared to the negative control (Scrambled miRNA probe). We used a commercial probe directed against U6 SnRNA as positive control (labels all cells in cerebellum) and a riboprobe directed against MYCN as a landmark (labels the external EGL (27)) (Fig 1 D). 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 (42).

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 anti-proliferative activity, only Mash1 triggered neuronal differentiation of CGNPs (2, 3). 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 CGNPs cultures over-expressing 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 EGFP expressing bicistronic vector, see methods for vector creation details). We also used pMICRO to express dec22, a 4X decoy sequence designed to neutralize Mir-22 activity (see methods for sequence and cloning details). To study cell proliferation, freshly isolated CGNPs were electroporated with scrambled Mir-22 (Scr22) (used as control, see methods for sequence), Mir-22, miR-361 (a miRNA up-regulated by DBA but not by BMP2 used as an additional control) or dec22. Transfected cells were cultured for 48h in a medium containing a saturating concentration of Shh (3 μg/ml), and a part of them were treated with BrdU for the last 4h. Finally, the cultures were fixed and stained with antibodies against BrdU (Fig 2 A-B). A similar culture was stained with anti-PCNA, another proliferation marker (Fig 2C). Interestingly, the percentage of cells labelled with BrdU or PCNA was significantly lower in cultures transfected with Mir-22 compared to empty vector, Scr22 or miR-361. Notably, dec22 consistently reverted the anti-proliferative 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 2 B).

**Mir-22 knock-down diminishes BMP anti-proliferative potency.**

The good performance shown by dec22 construct, allowed us to explore the contribution of Mir-22 on the anti-proliferative effect developed by BMPs. Thus, control (Scr22) or Mir-22 depleted (dec22) CGNPs, were cultured for 48h in a medium containing 3μg/ml of Shh plus...
different concentrations of BMP2 (0, 10, 50, 100 ng/ml). The cultures were then pulsed with BrdU for the last four hours. 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 CGNPs cultures with antibodies against Retinoblastoma PS807 (phosphorylation indicates progression through G1 restriction point) (Fig 3A), Cdc2 PY15 (de-phosphorylation is required for G1-S and G2-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 labelled cells and increased the number of cells stained with Cdc2 PY15, indicating a reduction in cell cycle progression. In agreement, EGFP-sorted FACS analysis of similar CGNPs cultures, demonstrated an accumulation of cells in G1/G0 and a reduction in G2 and M phases in Mir-22 transfected cells as compared to pMICRO (Fig 3D). Because anti-proliferative pathways are often associated with apoptosis we next stained Scr22 or Mir-22 transfected CGNPs with anti-Caspase 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 12h 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 labelled Mir-22 transfected CGNPs cultures with Tuj1(Fig 3 F,H) and HuC/D (Fig 3G), two neuronal differentiation markers. Cell counting demonstrated that, in spite of its anti-proliferative effect, Mir-22 did not promote neuronal differentiation of CGNPs (Fig 3 F,G,H). On the contrary, the percentage of differentiated cells was significantly increased by BMP2 treatment (100 ng/ml) (Fig 3 F,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, firstly described by Nowakowski, et al (37) and recently used to calculate cell cycle duration during cerebral cortex development (4), 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 24h 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 12h. The cultures were fixed at the indicated time points and stained with anti-BrdU. For this experiment, scrambled Mir-22 (Mir-22-Src) 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 (37) (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 is shown in the coloured boxes. Notably, the Growth Fraction, indicated in the plot by the point where the BrdU/GFP index reaches the “plateau” (grey dotted line and arrow heads), was very similar in cells
transfected with Mir-22-Scr (42.9 ±0.4) or Mir-22 (42.6 ± 0.1), confirming the absence of pro-differentiation 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) (18.5h for Mir-22-Src and 31.9 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 24.9h to 36.1h and decreased S-phase extent from 6.4h to 4.2h. 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 in 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 (23, 27, 39). In addition, it is also well-established that MYCN is one of the main targets of BMPs in these cells (2, 3). 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 (27), 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 \textit{in vivo} (Fig 4A). We first confirmed the specificity of the anti-Max antibody by immunoprecipitation of CGNPs lysates and subsequent Western blotting. This antibody recognized a single band coincident in size with over-expressed recombinant Max (Fig 4C). Therefore, we proceeded to study the distribution of Max protein by immunohistochemistry using sections from 2 different stages of mouse cerebellum development (P4, P8). We used anti Calbindin, a Purkinje cell maker, and DAPI to better delineate the different layers of the cerebellum. Max expression was observed in the entire EGL at P4 and concentrated to the outer EGL at P8 (Fig 4A and enlarged in Fig 4B). In P8 cerebella, this expression pattern was coincident with the reported \textit{MYCN} mRNA expression (27) and with the layer of highly proliferating cells of the EGL (labelled with the proliferation marker Ki67). (Fig 4A’ and enlarged in Fig 4B). Compared to the expression pattern of \textit{MYCN}, Max expression was more widely distributed and was also very abundant in Purkinje cells at all stages and in the differentiated granular cells at the IGL (Fig 4A,B). Consistent with this, the Brain Gene Map Database from the Allen Institute (http://mouse.brain-map.org) reports very high levels of \textit{MAX} mRNA in the cerebellar IGL of adult mice. Thus, in addition to its role as N-myc partner during proliferation, Max may also exert N-myc-independent functions in differentiated neurons. In fact, it has been previously reported in both developing brain and P19 pro-neural cell line, that upon cell cycle exit, Myc is rapidly down-regulated while Mad is up-regulated forming complexes with Max to induce differentiation (21, 41). Next, we studied the capacity of Mir-22 to target Max in CGNPs, observing that Max protein level was significantly reduced in Mir-22 transfected cells compared to cultures transfected with Scr22 or dec22 (Fig 4D). In addition, by semi quantitative RT-PCR we also observed that \textit{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 a SV40-driven modification of pGL3 to enable the cloning
of the entire MAX mRNA 3’UTR downstream of LUC sequence (pLUCMAX) (see
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 4F). 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-Src, where the Mir-22 binding site on Max 3’UTR had
been scrambled (Fig 3G). 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 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 (21, 43). Therefore, we studied whether
Mir-22 modulates N-myc-dependent transcriptional activity. Using CGNPs cultures
growing in a saturating concentration of Shh (3 µg/ml), we co-transfected Mir-22 and N-
myc, along with a reporter construct where luciferase was driven either by an artificial 5x
E-box or by the natural human cyclin D2 promoter (Fig 5 A,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 (32, 47, 53, 54) 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 analysed proliferation in CGNPs treated with Shh and transfected with either Mir-22 alone or with Mir-22 plus a vector containing a non-targetable form of Max (Fig 5C). Although proliferation was significantly rescued by co-transfection of Max, it did not reach the levels of control cells, suggesting that our transfection conditions failed to achieve the optimal N-myc /Max ratio needed for proliferation or alternatively, that other targets of Mir-22 contribute additionally to the inhibition of Shh-mediated proliferation. As mentioned above, N-myc dependent transcription plays a key role in the physiologic expansion of different neuronal populations including CGNPs. In addition, genomic amplifications of MYCN are found in a sub-set of neuroblastomas with a particularly poor prognosis. Thus, we postulated that the expression of Mir-22 in neuroblastoma derived cell lines (50) would inhibit proliferation, similar to our observations in CGNPs. Interestingly, ectopic expression of Mir-22 significantly reduced proliferation in SK-BE(2C) cell line where MYCN is highly amplified, but not in SH-SY-5Y cells where MYCN gene expression is normal (Fig 5D,E). To further substantiate the inhibitory effects of Mir-22 on MYCN-dependent proliferation, we tested Mir-22 expression in two unrelated cells lines where MYC proteins have a key role in proliferation. Thus, cell proliferation was reduced by ectopic expression of Mir-22 in both C17-2, a murine neuroectodermal cell line generated through v-myc transformation, and D283 a human medulloblastoma cell line with high levels of Myc but without MYC or MYCN amplifications (44, 45) (Fig 5F,G).


DISCUSSION

BMP2 and PKA activation down-regulate 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 (24). 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 (48). In addition, miR-17–92 cluster level was observed to be very high in mouse and human medulloblastomas with aberrantly activated Shh pathway. Moreover, medulloblastoma penetrance 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 (48). On the contrary, no medulloblastomas were formed when the CGNPs were obtained from Ink4c−/−; p53−/− mice. Therefore, considering that miR-17–92 cluster has been shown to be a direct target of Myc (38) and that Shh pathway increased MYCN expression in CGNPs (27) and in medulloblastomas (35), 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 (2, 55) and PKA activation (28) decreased N-myc levels in P6 CGNPs. In addition, in the present work we show a down-regulation of 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 12h) and N-myc protein (at 24h) induced by BMP2 treatment (2). On the contrary, however, Zhao et al. (55) did not find differences in N-myc expression until 72h of BMP treatment (but no time points between 24 and 72h 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 to inherited inhibitory signals, the responsiveness of CGNPs during this period is diminished.

Mir-22 operates downstream BMPs controlling cell cycle length but does not induce differentiation. miRNAs are emerging as master regulators of development which control cellular proliferation and differentiation and therefore, have also been linked to cancer (15). Previously, we reported that BMPs induce a rapid exit from the cell cycle and thereby, induce the differentiation of CGNPs in vivo and in culture (42). Here, using the same cellular model, we have observed a marked up-regulation of Mir-22 expression in response to BMP2 (Fig 1B,C), coincident with cell-cycle arrest and induction of the differentiation process. Whether the BMPs are the only extracellular signals that regulate mirR-22 expression in cerebellum remains to be determined, since we cannot yet exclude the possibility that other factors which promote the differentiation of CGNPs may also up-regulate Mir-22 expression. The fact that Mir-22 expression in CGNPs cultures was also increased by PKA activation suggests that Mir-22 may be a common element of different
anti-proliferative/pro-differentiation pathways. In 2009, Dr Roussel’s group performed a very extensive study where 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 as compared to P6, were also increased in our DBA treatment (miR300, miR128), no significant differences in Mir-22 levels were reported (48), suggesting that compared to other miRNAs, Mir-22 is not specifically linked to the terminal differentiation status. In agreement with Uziel et al, our IH 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 differentiated 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 TPA-treatment of the hematopoietic HL-60 cell line which triggers their differentiation (30) and was among the differentiation-associated miRNA group in myoblast-myotube differentiation, interestingly, as observed in CGNPs, Mir-22 reduced myoblasts proliferation (32). Additionally, Mir-22 was identified as a signature miRNA for erythrocyte maturation (12). 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 anti-proliferative effect in different cancer cell lines (47) and to induce senescence in fibroblasts (54). Thus, given that compounds which promote differentiation or senescence
exert their effects primarily on the cell cycle, Mir-22 may reflect a common anti-
proliferative element working in different cellular processes where a slowdown in the cell
cycle is required. Similarly, TIEG-1 and Mash1 are both anti-proliferative transcription
factors reported to increase during BMP induced neuronal differentiation of CGNPs, and
although in over-expression experiments only Mash1 induced neuronal differentiation, the
dose of BMP2 required to induce neural differentiation was 100 times lower in TIEG-1
over-expressing cells (2, 3) suggesting that proliferation arrested neural progenitors were
more prone to differentiate. Now we have observed that 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 signalling in primary cultures of CGNPs, this data is in agreement with a recent
report (47) identifying Max as a target of Mir-22 in the A549 lung carcinoma cell line.
However, the fact that the anti-proliferative 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 multi-target mechanism. Indeed, MYCBP, another regulator
of MYC signalling, is also targeted by Mir-22 (53). 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 (49) and miRNA cascades (9) to control a range of
biological activities including proliferation, differentiation, and cellular energy production. Moreover, a bi-directional cross-talk between these two types of regulators most likely exists, where oncogenes regulate the expression of specific miRNAs, and in turn, certain groups of miRNAs regulate parameters of oncogene signalling. Although it was shown initially that Myc oncogenic activity was highly dependent on the up-regulation of a pro-tumorigenic group of miRNAs known as the miR-17~92 cluster (14), a more recent study has demonstrated that Myc also represses the expression of an important group of anti-proliferative/differentiation related miRNAs; ectopic expression of these miRNAs diminishes the tumorigenic potential of Myc-induced lymphoma cells (11). Notably, Mir-22 was one of miRNAs whose expression decreased most significantly in response to MYC activation (11). On the other hand, it has been shown that components of MYC signalling complex including MYCBP, a positive regulator of Myc (53), or the Myc partner Max (47) are direct targets of Mir-22. Recombinant expression of Mir-22 significantly reduces Myc dependent signalling and tumorigenic effects in different cell types (47, 53). Thus, a reciprocal negative auto-regulatory loop between MYC signalling 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. Deregulation of MYC expression due to mechanisms including retroviral insertion, chromosomal translocation and gene amplification is known to be the cause of various neoplastic processes (34). 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 tumour types. In medulloblastomas, a malignancy arising from cerebellar cell precursors, loss of 17p is found in up to 50% of the tumours and is considered an indicator of a poor prognosis (36, 40, 46) 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 (29) 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 mostly due 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 (10, 25, 33). Thus, it seems very probable that other tumour suppressors are lost in 17p deletions. Consistent with this, several other genes were proposed to be responsible for the tumorigenic effect of 17p deletions (17). 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 (45). In any case, additional studies will be required to define the precise contributions of Mir-22 loss to neoplastic growth.

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FIGURE LEGENDS

Figure 1. Experimental design, array results and expression of Mir-22 in CGNPs. (A) Flow chart showing the miRNA array experimental design. (Shh 3µg/ml, BMP2 100 ng/ml and DBA 1 mM) (B) The panel shows the fold change in gene expression relative to the untreated control and the corresponding heat map of all miRNA species which were changed significantly upon 24h treatment with DBA or BMP2. The Venn diagram summarizes the common and differentially regulated miRNAs. (C) Mir-22 levels were analysed by Real time PCR in proliferating CGNP cultures treated for 24h with 1 mM of DBA or 100 ng/ml of BMP2. Graph bars represent the mean ± standard deviation (SD). (D) Low and high magnification images showing in situ hybridizations on 50 µm sagittal sections of P7 mouse cerebella, using either 22 nucleotides Exiqon miRCURY LNA™ double DIG (5’-3’) labelled probes (scrambled-miR 40 nM (negative control), Mir-22 40 nM and U6 snRNA 1nM (positive control)) or a ~500 nucleotides DIG labelled riboprobe directed against MYCN (used as a landmark labelling the outer EGL). The relative location of the different cerebellar layers is indicated at the right of the panel, external germinal layer (EGL), Purkinje cell layer (PC), internal granular layer (IGL). The bodies of several Purkinje cells in each high magnification picture were indicated by a dotted line. (E) Mir-22 expression in p21 and p60 mouse cerebellum sagittal slices.
Figure 2. Effect of Mir-22 over-expression or Mir-22 knock-down on CGNPs proliferation. The percentage of cells labelled with BrdU or PCNA (X) among GFP positive (X/GFP x100) was calculated for each marker. The graphs show the mean ±SD of at least 3 independent experiments. The total number of GFP positive cells counted for each data point is indicated in each bar. (A-C) Freshly isolated CGNPs were transfected with the indicated constructs and cultured for 48h with Shh (3µg/ml). Cell proliferation was studied by PCNA staining or BrdU incorporation. pMICRO (empty vector), Scr22 (scrambled Mir-22), Mir-22, miR-361 (miRNA regulated by DBA), dec22 (Mir-22 decoy) (D) Cell proliferation was measured by BrdU incorporation in CGNPs cultures transfected with Scr22 or dec22 and cultured for 48h in a media containing 3µg/ml of Shh plus 0, 50 or 100 ng/ml of BMP2.

Figure 3. Expression of Mir-22 increases cell-cycle length but does not induce neuronal differentiation of CGNPs. ) Freshly isolated CGNPs were transfected with the indicated constructs and cultured for 48h or longer in a Shh (3µg/ml) containing media to study different aspects of cell cycle progression. The percentage of cells labelled with each specific marker (X) among the GFP positive (X/GFP x100) was calculated for each marker. The bar graphs show the mean ±SD of at least 3 independent experiments. The total number of GFP positive cells counted for each data point is indicated in each bar. pMICRO (empty vector), Scr22 (scrambled Mir-22), Mir-22, dec22 (Mir-22 decoy). (A) Progression through G1 restriction point was studied by anti-Retinoblastoma PS807 labelling. (B) G1-S and G2-M progression was studied with anti-Cdc2 PY15 antibody. (C)
Anti-phospho-Histone3 was used to measure the percentage of cells in M phase. (D)

Representative FACS analysis of GFP/DAPI stained cells. The percentage of cells in each phase is indicated above each bar. A minimum of 2500 GFP positive cells were evaluated in each transfection (E) Apoptosis was studied by cleaved Caspase 3 staining. A B27-deprived culture (Scr-B27), was used as positive control for apoptosis. (F-H) Neuronal differentiation of CGNPs was studied at 48 and 72h by staining with Tuj1 and HuC/D neural markers. BMP2 treatment at 100 ng/ml was included as positive control for neuronal differentiation. (I) The effect of Mir-22 expression on cell cycle duration was studied by "Cumulative BrdU staining". Tendency lines for Mir-22 or Scr22 were fitted with GraphPad Prism statistical program. The Growth Fraction is given by the intersection between the plateau lines and the Y axis (coloured arrow heads). Total cycle duration (Tc) minus the duration of the S-Phase (Ts) is given by the intersection between the tendency lines and the X axis (coloured arrows). The S-Phase duration is calculated from the intersection between the tendency lines and the Y axis. The equation formula and the $R^2$ coefficient of determination are shown in the coloured squares. The number of cells counted for each data point ranged from 483 to 2019 with a mean of 972.

Figure 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 co-stained with anti-Calbindin, a Purkinje cell marker and the nuclear stain DAPI. (A’) Similar P8 mouse cerebellum sections were stained with TO-PRO, a the DNA dye, and with the proliferation marker Ki67 to expose proliferating cells (B) Enlargements of the areas encircled by the white line in panels A and A’. The location
of the different cerebellar layers is indicated by grey 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 followed by Western blot. Anti-Max antibody immunoprecipitated and blotted a single band in CGNPs lysates which was coincident in molecular weight with recombinant human Max. (D) Max protein expression levels were measured by IP/Western blot in cultures transfected with Mir-22 or dec22 (Mir-22 decoy), values were Actin–normalized and refereed 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 semi-quantitative RT-PCR in CGNP cultures transfected for 48h. PCR reaction cycles were optimized for each set of primers to ensure linearity. (F) pLUCMAX (see methods and Fig S3 for construct details) luciferase activity was studied in CGNPs transfected for 48h with either empty vector (pMICRO), Mir-22 or its scrambled control (Scr22). (G) In a similar experiment, the effect of Mir-22 expression on pLUCMAX activity was compared to pLUMAX-Ser, a variant of pLUCMAX where the Mir-22 binding site on MAX3'UTR was scrambled. Graph bars represent the mean ± SD.

**Figure 5. Mir-22 reduces MYC dependent transcription and proliferation.**

The bar graphs show the mean ± SD of at least 3 independent experiments. The total number of cells counted for each data point is indicated in each bar (A) CGNPs electroporated with a 5X E-box driven luciferase reporter vector plus Mir-22, MYCN or their combination where cultured for 24h with Shh (3 μg/ml) and for an additional 24h period without Shh, cells were then lysed and Luciferase activity measured. Empty pMICRO vector was used as control and was also employed to equalize the amount of...
transfected DNA among wells. (B) Transfections were performed as in A but in these experiments the luciferase activity was controlled by the Cyclin D2 promoter (contains 2 natural E-boxes). (C) CGNPs were transfected with Mir-22 or Mir-22 plus a non-targetable form of Max and cultured with Shh (3 µg/ml). Proliferation was studied at 48h 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 48h after transfection. (F,G) In a similar experiment, the effect of Mir-22 expression was evaluated in two cell lines where 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.
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