Inhibition of *mir-21*, which is up-regulated during *MYCN* knockdown-mediated differentiation, does not prevent differentiation of neuroblastoma cells

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Abbreviations:

MNA - MYCN-amplified
TPA - 12-O-tetradecanoyl phorbol 13-acetate
RA - Retinoic acid
BDNF - Brain-derived neurotrophic factor
bFGF - Basic fibroblast growth factor
IGF - Insulin-like growth factor
NGF - Nerve growth factor
IFN-γ - Interferon-gamma

Abstract

Background: Neuroblastoma is a malignant childhood tumour arising from precursor cells of the sympathetic nervous system. Genomic amplification of the *MYCN* oncogene is associated with dismal prognosis. For this group of high-risk tumours, the induction of tumour cell differentiation is part of current treatment protocols. MicroRNAs (miRNAs) are small non-coding RNA molecules that effectively reduce the translation of target mRNAs. MiRNAs play an important role in cell proliferation, apoptosis, differentiation and cancer. In this study, we investigated the role of N-myc on miRNA expression in *MYCN*-amplified neuroblastoma. We performed a miRNA profiling study on SK-N-BE (2) cells, and determined differentially expressed miRNAs during differentiation initiated by *MYCN* knockdown, using anti-*MYCN* short-hairpin RNA (shRNA) technology.

Results: Microarray analyses revealed 23 miRNAs differentially expressed during the *MYCN* knockdown-mediated neuronal differentiation of MNA neuroblastoma cells. The expression changes were bidirectional, with 11 and 12 miRNAs being up- and down-regulated, respectively. Among the down-regulated miRNAs, we found several members of the mir-17 family of miRNAs. *Mir-21*, an established oncomir in a variety of cancer types, became strongly up-regulated upon *MYCN* knockdown and the subsequent differentiation. Neither overexpression of *mir-21* in the high-*MYCN* neuroblastoma cells, nor repression of increased *mir-21* levels during *MYCN* knockdown-mediated differentiation had any significant effects on cell differentiation or proliferation.

Conclusions: We describe a subset of miRNAs that were altered during the N-myc deprived differentiation of *MYCN*-amplified neuroblastoma cells. In this context, N-myc acts as both an activator and suppressor of miRNA expression. *Mir-21* was up-regulated during cell differentiation, but inhibition of *mir-21* did not prevent this process. We were unable to establish a role for this miRNA during differentiation and proliferation of the two

1 neuroblastoma cell lines used in this study.

Introduction

Neuroblastoma is a highly malignant embryonic childhood tumour arising from primitive cells of the neural crest [1]. As shown in mass screening studies, localised tumours can frequently be detected in the paediatric population. However, many of these tumours differentiate into more benign histological subtypes or regress spontaneously [2]. By contrast, disseminated disease and certain genetic alterations define high-risk groups of neuroblastoma patients in which long-term survival is still below 40%, despite multi-modality treatment efforts [3].

One of the strongest biological predictors of poor outcome is genomic amplification of the oncogene *MYCN* [4]. The gene product, N-myc protein, is a basic helix-loop-helix (bHLH) transcription factor expressed during neural crest development. It belongs to the Myc/Max/Mad network and plays a key role in the regulation of cell growth, differentiation and apoptosis [5]. Like other members of the myc-family, N-myc can both activate and repress transcription. The direct binding of N-myc/Max heterodimers to specific genomic DNA binding sites (E-box motifs) induces the transcription of target genes. By contrast, the transcriptional repression by N-myc [6-8] is presumably mediated through interaction with other DNA-binding proteins [9].

MicroRNAs (miRNAs) are a class of small (19-22 nt), non-coding RNAs capable of repressing protein expression by binding to sequences in the 3'untranslated region (3'UTR) of respective target mRNAs. Most miRNAs are transcribed as long monocistronic, bicistronic or polycistronic primary transcription units (pri-miRNAs) by RNA polymerase II, and cleaved by a series of cellular processing events to produce mature miRNAs. The degree of complementarity between mature miRNA and its target mRNAs determines the mechanism responsible for blocking protein synthesis. In mammals, miRNAs-mRNA interactions are most often through imperfect base pairing, resulting in translational repression [10].

To understand the mechanisms that control the neuronal differentiation of neuroblastoma cells is crucial since induction of differentiation is one of the treatment strategies for this type of cancer. Most model systems used to study neuroblastoma differentiation *in vitro* are based on the addition of various agents and growth factors to neuroblastoma cell lines without *MYCN*-amplification (reviewed in [11]). Typically, SH-SY-5Y cells are exposed to retinoids, phorbolesters or combinations of growth factors to induce a neuronal-like phenotype characterised by neurite outgrowth. The addition of RA to MYCN-amplified (MNA) neuroblastoma cells has also been shown to induce neuronal differentiation with the subsequent down-regulation of *MYCN* expression [12]. The function of N-myc during RA-induced differentiation of MNA neuroblastomas, however, is contradictory and unclear [13-15].

Another method to induce neuronal differentiation in MNA neuroblastomas is the specific reduction of *MYCN* expression by traditional antisense techniques or short-interfering RNA molecules (siRNA) [16-19]. In addition, we have previously reported an efficient method to down-regulate *MYCN* in MNA neuroblastoma cell lines by the use of vector-based anti-*MYCN* short-hairpin RNA (shRNA) technology [20]. In SK-N-BE (2) cells, the knockdown of *MYCN* resulted in prominent morphological and biochemical neuronal differentiation. During the last few years, several studies have been reported which address miRNA expression during induced neuroblastoma differentiation [21-26]. With the exception of one study using anti-*MYCN* siRNA [22], all reports have focused on non-MNA neuroblastoma cell lines induced to differentiate by TPA or RA alone, or in combination with growth factors. To investigate how the miRNA transcriptome is affected during the *MYCN* knockdown-mediated neuronal differentiation of MNA neuroblastoma cells, we performed a miRNA profiling study on SK-N-BE (2) cells, and determined differentially expressed miRNAs during cell differentiation using anti-*MYCN* shRNA technology.

Materials and Methods

Neuroblastoma cell lines

SK-N-BE (2) cells have a complex karyotype which includes a del(1p), monosomy 17 and unbalanced der(3)t(3;17). Homogeneously stained regions (HSRs) in 6p and 4q are reported sites of *MYCN* amplification [27, 28]. Kelly cells are *MYCN*-amplified at der(17), and the complex near-diploid karyotype includes a del(1)(p34). SK-N-BE (2), Kelly, SMS-KCN, SMS-KCNR, SKNAS and SKNSH cells were grown in RPMI-1640, LAN-5 cells in DMEM medium with 1% NEAA and 2 mM glutamine, all supplemented with 10% heat-inactivated FBS, at 37 °C under 5% CO2.

Short-hairpin RNA vectors and transfection

The design and validation of shRNA molecules targeting human *MYCN* mRNA (sh*MYCN*) were previously reported by us in detail [20]. For the present study we used sh*MYCN* sequence aMN-887, in which the number indicates the first position of the shRNA target recognition site in the *MYCN* cDNA (GeneBank accession NM_005378) sequence. The aMN-887 sequence and an upstream human U6 promoter from plasmid panti*MYCN*-887 [20] were gated into vector pDS_hpCG (ATCC-Nr. 10326383) using Gateway technology (Invitrogen) to generate the aMN-887 shRNA expressing plasmid pDS-anti*MYCN*-887. As a negative control, we designed vector pDS-shSCR, expressing a scrambled shRNA sequence with no complementarity to any known mRNA in the human genome (shSCR sequence available on request). At a 70% confluence, cells were transfected with Lipofectamine2000 (Invitrogen) according to the manufacturer`s standard recommendations, and the transfection efficiency reached 70-80%.

Immunofluorescence confocal laser microscopy

Morphological changes and *in situ* N-myc expression were evaluated by immunostaining and confocal laser microscopy. Cells were cultured on round poly-L-lysine coated glass slides (Hecht Assistant, Germany, No.1014), transfected in six well dishes, and fixated three days after transfection with 4% paraformaldehyde. After permeabilisation with ice-cold MeOH and blocking with BSA, cells were incubated with primary antibodies either against N-Myc (mouse polyclonal antibody, Calbiochem) or Neuronal Class III β-Tubulin (rabbit polyclonal antibody, Covance), and covalent bindings visualised by fluorescent secondary antibodies (Alexa Fluor -546 goat anti-mouse and -633 donkey anti-rabbit conjugated IgG, respectively; both from Molecular Probes/Invitrogen). Cell nuclei were stained with Draq5 (BioStatus, UK). We used a Zeiss LSM500 confocal microscope, the software LSM Image Browser (Zeiss), ImageJ (NIH, USA), and an Adobe Illustrator for image processing and preparation.

MiRNA microarray profiling

MiRNA microarrays: MicroRNA expression in SK-N-BE (2) cells transfected with pDS-shSCR or pDS-anti*MYCN*-887 was measured in two independent miRNA microarray experiments (SK07 and SK08), and the cells were harvested three days after transfection. Total RNA was isolated with the miRVana miRNA isolation kit (Ambion) according to the manufacturer`s instructions. The miRNA microarray assay started with 10 µg total RNA and was performed on µParaflo® Microfluidic Biochips using a service provider (LCSciences). The technical details of the assay, including miRNA enrichment, fluorescent dye labelling and hybridisation conditions, are described elsewhere [29]. Microarrays SK07 and SK08 included all miRNAs listed in the Sanger miRBase Release 9.2 (471 human miRNAs) and Release 10.1 (723 human miRNAs), respectively. The exogenously expressed shRNA (shSCR and aMN-887) molecules and 18 endogenous small housekeeping RNAs were added as custom probes on the SK08 array (Supplementary 1). SK07 and SK08 included seven and four redundant probes for each miRNA, respectively.

Data analysis: The data analysis included subtraction of the background and the normalisation step. The background was determined using a regression-based background mapping method. The regression was performed on 5–25% of the lowest intensity data points excluding blank spots. Raw data matrix was then subtracted by the background matrix.
To be regarded as detectable, a transcript must meet at least two conditions: signal intensity higher than 3 × (background standard deviation) and spot CV < 0.5. CV was calculated by (standard deviation)/(signal intensity). In addition, signals from at least 50% of the repeating probes must be above the detection level.
Normalisation was carried out using a LOWESS (Locally Weighted Regression) method on the background-subtracted data. After normalisation, the p-values of the difference between the two fluorescent signals were calculated. Differentially expressed miRNAs were those with a p-value < 0.01 in at least 50% of the array replicates. Due to a systematic dye bias, mir-377 and mir-542-5p were excluded. In addition, 10 miRNAs from Sanger miRBase Release 9.2 were no longer included in Release 10.1. By making these adjustments, the expression data on 459 identical human miRNAs were comparable on both arrays.

Microarray validation by Taqman miRNA RT-PCR

The microarray data was validated by Taqman quantitative stem-loop real-time RT-PCR (provided by LCSciences) with the same RNA preparations used in the microarray analyses. Sequences of the miRNA specific stem-loop-primers are available at www.lcsciences.com. RNU38B was unaffected by *MYCN* knockdown on the miRNA microarray (Supplementary 1) and was used for qRT-PCR normalisation. All PCR reactions were done in triplets. A two-sided student's t-test was used to calculate the p-values for differential expression.

Overexpression and inhibition of *mir-21*

Premir-21 miRNA Precursor Molecules ("mimics") and anti-mir-21 miRNA Inhibitors (both from Ambion) were used to overexpress and inhibit *mir-21* expression, respectively. Pre-miR Precursor Negative Control and Anti-miR Inhibitors Negative Control (Ambion) were used as negative controls. The cells were transfected with Lipofectamin 2000 according to the manufacturer's instructions. The transfection efficiency of a FAM-labeled miRNA negative control mimic reached 85-90 % as measured by flow cytometry.

pmir-21-luc assay

SK-N-BE(2) and Kelly cells were seeded in 12-well culture plates and co-transfected with 30 pmol anti-mir-21 or anti-NC (negative control antagomir), 1,2 μg,(SK-N-BE(2)) or 0,8 μg (Kelly) pmir-21-luc (kind gift from Anders H. Lund, University of Copenhagen, Denmark) and 20 ng pGL4.75[hRluc/CMV] (expressing Renilla luciferase for normalization) using Lipofectamin 2000. 48 hours after transfection, cells were harvested and luciferase activities were measured using the Dual Luciferase Assay (Promega).

Quantitative real-time RT-PCR

Three days following transfection, RNA was isolated using a Qiagen miRNeasy Mini Kit, and 1 μg total RNA was reverse transcribed using a Qiagen miScript Reverse Transcription Kit according to the manufacturer´s instructions. Power SYBR Green PCR Master Mix (Applied Biosystems) was used to determine the expression of MYCN (F: CACCCTGAGCGATTCAGATGA, R: CCGGGACCCAGGGCT), Neuropeptid Y (NPY) (F: TCCAGCCCAGAGACACTGATT, R: AGGGTCTTCAAGCCGAGTTCT), HPRT1 (F: TGACACTGGCAAAACAATGCA, R: GGTCCTTTTCACCAGCAAGCT) and UBC (F: ATTTGGGTCGCGGTTCTTG, R: TGCCTTGACATTCTCGATGGT). The expression of *miR-21* and SNORD38B were measured using a Qiagen miScript SYBRGreen PCR Kit and

the specific primer sets from Qiagen. HPRT1, UBC and SNORD38B were selected for cDNA normalisation, and the results were analysed using ΔΔCT method in qBase Software [30].

Western immunoblotting

Western immunoblotting was performed as previously described [20]. Primary antibodies were Anti-N-Myc Mouse (CALBIOCHEM), PDCD4 Rabbit (Cell Signaling), PTEN Rabbit (Cell Signaling) and Actin mouse (Santa Cruz).

Cell proliferation assay

SK-N-BE (2) cells were seeded in 24 well plates and transfected in three replicates with Lipofectamine 2000 the following day. Cell proliferation was determined in two replicates by AlamarBlue (Invitrogen) according to the manufacturer´s standard procedure.

Flow cytometric analysis

Cells were harvested using Trypsin-EDTA (Sigma-Aldrich) and washed once in 1 x PBS. The cells were then fixed for 2 hrs in ice-cold 70% EtOH. After fixation, the EtOH was removed by centrifugation and the cells were washed once in 1 x PBS before being stained for 30 min at room temperature in a propidium iodide (PI)-staining solution (PBS with 20 µg/ml PI (Sigma), 60 µg/ml RNase A (Sigma) and 0.1% v/v Triton X-100 (Sigma)). Fluorescence emitted from the PI-DNA complex was analysed by flow cytometry, using a FACS Aria Flow Cytometer (BD Biosciences, San Jose, CA, USA).

To determine the miRNA transfection efficiency, SK-N-BE (2) and Kelly cells were transfected with a FAM-labeled miRNA negative control mimic (GenePharma, Shanghai) and harvested after 24 hours. Cell fluorescence was measured using the FACS Aria Flow Cytometer.

Results

MYCN knockdown in the MNA neuroblastoma cell line SK-N-BE (2)

We have previously described the efficient knockdown of *MYCN* mRNA in a MNA neuroblastoma cell line using anti-*MYCN* shRNAs [20]. In brief, SK-N-BE (2) cells were transiently transfected with plasmid pDS-anti*MYCN*-887, expressing shRNA molecule aMN-887 specifically targeted against *MYCN* mRNA. A real-time RT-PCR analysis demonstrated that *MYCN* mRNA decreased by ~70% (Figure 1a), while Western immunoblotting revealed an almost complete depletion of N-myc protein (Figure 1b) when compared to transfections with a plasmid expressing scrambled control shRNA (shSCR). Morphologically, cells expressing aMN-887 shRNA exhibited multidirectional neurite-like projections, indicating neuronal cellular differentiation (Figure 1c).

Immunocytochemical stainings with antibodies against N-myc and neuronal class III β-tubulin confirmed that the neuronal phenotype was restricted to cells where N-myc was efficiently knocked down by aMN-887 shRNA. Moreover, the relative expression of several other neuronal differentiation markers such as Neuropeptide Y (NPY), Calreticulin (CRT) and Chromogranin B (CHGB) increased [20]. Neither differentiation markers nor several different housekeeping genes were affected by treatment with shSCR (data not shown). We also noticed the complete absence of non-specific stress responses after these treatments using the Interferon Response Detection kit (SBI) (data not shown).

These results clearly demonstrate that SK-N-BE (2) cells transfected with pDS-anti*MYCN*-887 undergo neuronal differentiation as a consequence of highly specific and potent shRNA-mediated inhibition of N-myc expression.

MicroRNA expression profile in MNA SK-N-BE (2) cells

The shSCR transfected control cells were used to generate a miRNA expression profile for the MNA SK-N-BE (2) cell line. Two individual shSCR transfections were analysed on two

separate miRNA microarrays, covering 471 (SK07) and 723 (SK08) known human microRNA genes. Of the 459 miRNAs common to both arrays, 259 miRNAs (56%) were not detectable. Of the remaining 200 detectable miRNAs, we found a high expression of 14 miRNAs located within the distinct miRNA gene clusters mir-17-92 (chromosome 13q31), mir-106a-363 (chromosome Xq26), mir-106b-25 (chromosome 7q22), mir-23b-24 (chromosome 9q22) and mir-15b-16 (chromosome 3q26) (Figure 2). None of the 252 miRNAs that distinguished Sanger miRBase Release 10.1 (SK08) from Release 9.2 (SK07) showed a high expression in SK-N-BE (2).

Differential miRNA expression upon *MYCN* knockdown-mediated differentiation

Two independent *MYCN* knockdown experiments were performed, and each was analysed on a separate miRNA microarray. To determine differentially expressed miRNAs, the expression profile of SK-N-BE (2) cells transfected with pDS-anti*MYCN*-887 (low N-myc and differentiated morphology) was compared to cells treated with pDS-shSCR (high N-myc and undifferentiated morphology). We identified 23 miRNAs with consistent differential expression on both arrays: 11 miRNAs were up-regulated and 12 miRNAs were down-regulated (Figure 3a and Supplementary 2). In the group of up-regulated miRNAs which includes *mir-21, -22, -126, -137, -181d, -218, -663, -671, let-7c, let-7d* and *let-7f*, we observed a 1.6 – 5.3 fold increase in expression. Among the down-regulated miRNAs, 7 of the 12 differentially expressed miRNAs are members of the mir-17 family encoded by three paralogous miRNA clusters: the mir-17-92 cluster, the mir-106a-363 cluster and the mir-106b-25 cluster (Figure 3b). The remaining five down-regulated miRNAs (*mir-24, -92b, -103, -494* and *mir-495)* are single intergenic or intron encoded. The differential expression pattern for 11 miRNAs was confirmed by real-time stem-loop quantitative RT-PCR (Supplementary 3).

Collectively, our data show that the expression level of several miRNAs is altered during the *MYCN* knockdown-mediated differentiation of SK-N-BE (2) cells. We observed both up- and down-regulation in miRNA expression, but were not able to differentiate between expressional changes due to *MYCN* knockdown or the following differentiation process. These data show the differential expression pattern of miRNAs during *MYCN* knockdown-mediated neuronal differentiation of an MNA neuroblastoma cell line.

Increased *mir-21* expression is not sufficient to induce neuronal differentiation in SK-N-BE (2) and Kelly cells

Mir-21, which is expressed from a single gene locus on chromosome 17q23, demonstrated the strongest inverse correlation with N-myc expression in SK-N-BE (2) cells (Figure 3a and Supplementary 2), with a similar observation being made in MNA Kelly cells (Supplementary figure 4). The *MYCN*-knockdown mediated increase in *mir-21* expression coincided early with *MYCN* downregulation (Supplementary Figure 5). By the use of the stem-loop RT-PCR assay for *mir-21* and real-time RT-PCR on *MYCN*, we measured the levels of *mir-21* and *MYCN* mRNA in 7 neuroblastoma cell lines. As shown in Figure 4a, we observed a reverse correlation between the expression of *mir-21* and *MYCN* mRNA. Cell lines with low to moderate *MYCN* mRNA levels expressed significantly higher levels of *mir-21*.

In order to elucidate a function for the substantial increase in *mir-21* expression during *MYCN* knockdown-mediated differentiation, SK-N-BE(2) and Kelly cells were transfected with premir-21 mimics or anti-mir-21 antagomirs. While *MYCN* knockdown increased *mir-21* expression ~2-fold, transfection of the *mir-21* mimic resulted in ~23-fold overexpression. Co-transfection of aMN-887 and anti-mir-21 abolished the aMN-887 induced increase in *mir-21* expression (Figure 4b). To functionally validate the efficiency of the antagomir treatment, SK-N-BE (2) and Kelly cells were co-transfected with anti-mir-21 and a luciferase reporter containing the *mir-21* target sequence in the 3'UTR (pmir-21-luc). The luciferase activity

increased ~ 2-fold compared to a negative control antagomir (anti-NC) demonstrating the specific repression of endogenous *mir-21* by the antimir-21 (Figure 4c). Treatment with premir-21 did not induce a noticeable neurite outgrowth, as was observed in differentiating SK-N-BE (2) and Kelly cells (data not shown). In consistence with a lack of morphological changes, the expression of the early neuronal differentiation marker NPY did not increase during the pre-mir-21 treatment (Figure 4d). Furthermore, decreasing *mir-21* expression with antagomir-21 in differentiating SK-N-BE (2) had no effect on NPY expression. Neither the overexpression of *mir-21* alone nor the repression of *mir-21* in combination with the anti-*MYCN* shRNA treatment had any significant effect on *MYCN* mRNA expression levels as expected (Figure 4e).

These data show that the increase in *mir-21* expression observed upon anti-*MYCN* shRNA treatment alone is not a sufficient stimulus to induce differentiation and might instead be a consequence of *MYCN* knockdown-mediated neuronal differentiation in MNA neuroblastoma. In addition, counterbalancing the increased *mir-21* expression with antagomir-21 during differentiation is not able to reverse the process.

Altered *mir-21* expression has no effect on proliferation during *MYCN* knockdown-induced differentiation

Mir-21 has tumour-promoting properties in a variety of cancers [31]. For that reason, we investigated the proliferative effects of *mir-21* in high-*MYCN* SK-N-BE (2) cells.

Premir-21 mimics were transfected into SK-N-BE (2) and Kelly and cell proliferation was monitored for three consecutive days. As shown in Figures 5a and 5b, premir-21 treatment had no significant effect on the cell proliferation of these cell lines, even when the premir concentration was increased to 80 nM. In contrast, control cells receiving culture media with low serum showed a marked decrease in cell proliferation.

Cell cycle analyses using flow cytometry after overexpression of *mir-21* in SK-N-BE (2) cells showed no significant differences in the fraction of proliferating cells (S-phase) when compared to cells transfected with the mir-NC control. The lack of a sub-G1 phase also indicated that few cells underwent apoptosis due to the treatments (Figure 5c).

Since *mir-21* was up-regulated during *MYCN*-knockdown mediated differentiation, we next investigated if proliferation was altered when the *mir-21* increase was abolished by antagomir treatment. As shown for the SK-N-BE (2) cells in Figure 5d and for the Kelly cells in Figure 5e, antimir-21 did not significantly affect the cell proliferation when co-transfected with MN-887 or sh-SCR at day 2 and 3 after transfection.

Finally, we performed Western blot analyses of SK-N-BE (2) and Kelly cells treated with premir-21 or antimir-21 to investigate if changes in *mir-21* levels affected the expression of the known *mir-21* target genes PDCD4 (Programmed Cell Death 4) or PTEN (Phosphatase and Tensin homolog). Neither overexpression of *mir-21*, nor repression of the observed *MYCN* knockdown-mediated *mir-21* increase by anti-mir-21 treatment resulted in significant changes to PDCD4 or PTEN expression (Supplementary Figure 6). This indicates that these tumour suppressor genes are not targeted by *mir-21* in SK-N-BE (2) and Kelly cells

Discussion

MYCN is amplified in a subgroup of neuroblastomas with highly aggressive behaviour. We have previously established an efficient model system to selectively down-regulate *MYCN* expression in MNA neuroblastoma by specific anti-*MYCN* shRNA molecules [20]. This approach allows us to investigate and compare cellular processes in both high- and low-*MYCN* neuroblastoma cells. In particular, our model system can be used to study neuronal differentiation in MNA neuroblastoma initiated by specific *MYCN* knockdown, as opposed to

induced neuronal differentiation using protocols with RA, TPA or various combinations of growth factors (BDNF, bFGF, IGF, NGF).

Over the past few years, several studies have addressed the role of N-myc on the expression of miRNAs in neuroblastoma [22, 32-39]. These studies were mainly performed by overexpressing *MYCN* in non-MNA neuroblastoma cell lines with or without the capacity to undergo neuronal differentiation (SH-SY-5Y or SHEP, respectively) or by comparing miRNA profiles in MNA vs. non-MNA neuroblastoma tumours. The contribution of miRNAs to the neuronal differentiation processes in neuroblastoma has mainly been investigated in SH-SY-5Y cells induced to differentiate by the addition of TPA or RA alone, or RA in combination with BDNF [21, 26].

In this study, we analysed the expression of 723 known human miRNAs during the differentiation of MNA SK-N-BE (2) cells upon *MYCN* knockdown. By comparing miRNA expression levels in high and low N-myc SK-N-BE (2) cells, we found 23 differentially expressed miRNAs. Twelve miRNAs (*mir-17, -18a, -20, -24, -25, -92a, -92b, -93, -103, -106a, -494 and mir-495)* were down-regulated, and 11 miRNAs (*mir-21, -22, -126, -137, -181d, -218, -663, -671, let-7c, let-7d* and *let-7f*) were up-regulated.

Among the down-regulated miRNAs, most are members of the oncogenic miRNA clusters which constitute the mir-17 family. It is now well established that N-myc is a transcriptional activator by direct binding to the promoter regions of several miRNAs, including the mir-17 family clusters [34, 36, 38]. In addition, miRNAs of the mir-17 family clusters have been shown to be down-regulated in non-MNA neuroblastoma cells in which differentiation was induced by various agents and growth factors [21]. In this study, we show for the first time that most miRNAs belonging to the mir-17 family are down-regulated upon the *MYCN* knockdown-mediated neuronal differentiation of MNA neuroblastoma cells. Interestingly, Lovén et al. recently reported that the stable knockdown of *mir-18a*, but not *mir-19a* (both from the mir-17-92 cluster), resulted in the differentiation of SK-N-BE (2) cells [36].

Similarly, we found *mir-18a*, but not *mir-19a,* down-regulation during the differentiation of SK-N-BE (2) upon N-myc knockdown.

We also observed several miRNAs being up-regulated upon *MYCN* knockdown in SK-N-BE (2) cells, and most have previously been linked to a neuronal phenotype or been shown to induce neuronal differentiation. The *let-7* family of miRNAs was found to be highly represented in miRNA populations in mouse, rat and primate brains [40, 41]. Moreover, the expression of *let-7*, *mir-218* and *mir-137* has been reported to increase during induced neuronal differentiation in mouse embryonic stem cells, mouse and human embryonic carcinoma cells and mouse neuronal stem cells (mNSC) [42-44]. Additionally, the exogenous expression of *mir-137* promoted neuronal-like differentiation in several mouse and human stem cells [43]. In a study using RA or *MYCN* siRNA to induce neuronal differentiation in MNA neuroblastoma cells, Chen and Stallings reported the vast majority of differentially expressed miRNAs to be up-regulated. In accordance with our data, *mir-137*, *mir-181* and *let-7* family members were among the up-regulated miRNAs reported.

In summary, these observations support the idea that miRNAs up-regulated during *MYCN* knockdown-mediated neuroblastoma differentiation are either directly involved in, or are a consequence of, the observed neuronal differentiation process.

The most prominent up-regulated miRNA in our study was *mir-21*. Neuroblastoma cell lines have been reported to express low or undetectable levels of *mir-21* [45]. However, Afanasyeva et al. reported *mir-21* to be among the most frequent miRNAs detected in primary neuroblastoma tumours [46]. We found *mir-21* expressed in all neuroblastoma cell lines investigated in this study. Interestingly, *mir-21* expression was inverse correlated to *MYCN* mRNA expression. However, we cannot exclude that the expression of *mir-21* is also influenced by variations in *mir-21* gene dosages, as *mir-21* is encoded on chromosome 17q which is frequently involved in unbalanced translocations in NB cell lines [28]. *Mir-21* is an miRNA with putative anti-apoptotic and tumour promoting activities, and has previously been

described to be highly expressed in a variety of solid tumours [31]. Experimentally validated *mir-21* targets include several proteins with a tumour suppressor function, eg. PDCD4 and PTEN [31, 47-51]. However, overexpression of *mir-21* in SK-N-BE (2) and Kelly cells did not alter proliferation of these cell lines. In addition, neither PCDC4, nor PTEN expression was changed upon *mir-21* overexpression. This indicates that *mir-21* is not involved in the regulation of these proteins in SK-N-BE (2) and Kelly cells. Similar to our studies, Folini et al. recently reported that changes in *mir-21* expression did not alter proliferation of prostate cancer cell lines [58].

Induced *mir-21* expression has previously been shown in neuronal differentiating neuroblastoma cells [24, 26, 45, 52-54]. In non-MNA SH-SY-5Y cells, RA, TPA and IFN-γ treatments increase *mir-21* expression. These treatments also induce neuronal differentiation in some MNA neuroblastoma cell lines and reduce N-myc expression through both transcriptional repression and the shortening of *MYCN* mRNA half-life [55, 56].

An increased expression of *mir-21* alone did not induce neuronal differentiation in SK-N-BE (2) or Kelly cells, and neither morphological nor biochemical alterations compatible with neuronal differentiation were observed. Using antagomir-21 to reduce the observed *mir-21* increase had no effect on differentiation. These observations indicate that the observed increase in *mir-21* expression does not directly influence the neuronal differentiation process in MNA neuroblastoma cells induced to differentiate by *MYCN* knockdown. We suggest that the increase of *mir-21* is a consequence rather than a cause for this differentiation process.

Recently, *mir-21* was reported to be the most significantly down-regulated miRNA when N-myc expression was induced in Tet21N neuroblastoma cells [36]. These cells (SHEP-based) are derived from the surface-adherent S-type fraction of SK-N-SH and lack the ability to differentiate into neuron-like cells. These results indicate an inverse connection between *MYCN* and *mir-21* that does not involve neuronal differentiation. We did not observe an

altered expression of *MYCN* in SK-N-BE (2) cells transfected with *mir-21* mimics or antagomirs.

From a wider perspective, the TPA-induced differentiation of human promyelocytic leukemia cells and the RA-induced differentiation of mouse embryonic stem cells have been shown to drastically increase *mir-21* expression [52, 53]. Kim et al. have recently demonstrated that the overexpression of *mir-21* enhanced adipogenic diffentiation by targeting *TGFBR2* [57]. Taken together, these reports could indicate that the up-regulation of *mir-21* expression has a more general role in cell differentiation.

In summary, we have found a subset of miRNAs that were altered during the *MYCN* knockdown-mediated differentiation of MNA neuroblastoma cells. We observed both up- and down-regulation of miRNA expression. The majority of down-regulated miRNAs are located in N-myc controlled miRNA gene clusters with established proliferative functions. By contrast, most up-regulated miRNAs have been previously linked to neuronal differentiation processes. *Mir-21* was found to be up-regulated during differentiation. Functional analyses indicate that the observed increase in *mir-21* expression is not a prerequisite to initiate the differentiation process since inhibition of *mir-21* did not prevent differentiation. In SK-N-BE (2) and Kelly cells, we were unable to establish a role for *mir-21* during differentiation and proliferation.

Conflict of Interest Statement

The authors have declared that no conflicts of interests exist.

Authors' contributions

JB and CE designed the research. JB and JRH performed the experimental work. CE supervised the experimental work. JB and CE wrote the manuscript. TF assisted the research design and critically commented on the manuscript. BHH and ET performed experiments for the revised version of the manuscript. The final manuscript was read and approved by all of the authors.

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Figures

Figure 1: shRNA-mediated knockdown of *MYCN* in SK-N-BE (2) cells. (**A**) Bar graph showing the normalized expression of *MYCN* mRNA from qRT-PCR analyses. (**B**) Representative western blot analysis of N-myc and β–actin expression from shSCR and aMN-887 treated SK-N-BE (2) cells. (**C**) Confocal laser microscopy images. Transfected cells appear green due to GFP expression from the transfected plasmids. Nuclei are stained with Draq5 (blue). Merge is an overlay of all 3 pictures. Cells transfected with pDS-anti*MYCN*-887 display a neuronal phenotype with neurite outgrowth and expression of Neuronal Class III β-Tubulin (TUJ1, purple, white arrow). Nuclear N-myc disappears in anti-*MYCN* transfected cells (yellow arrow). In contrast, shSCR-transfected control cells remain undifferentiated and show abundant nuclear N-myc expression.

Figure 2: Highly expressed miRNAs in SK-N-BE (2) cells. MiRNAs with high expression in SK-N-BE (2) are transcribed either from miRNA gene clusters (grey boxes) or monocistronically (non-clustered miRNAs).

Figure 3: Differential miRNA expression in differentiating *MYCN* repressed SK-N-BE (2) cells.

(**A**) Summary of consistently differentially expressed miRNAs ($p<0.01$) represented in a horizontal bar graph as log2 values from microarray SK07. Down-regulated and up-regulated miRNAs are shown as blue and red bars, respectively. (**B**) MiRNAs from the paralogous miRNA clusters mir-17-92, mir-106a-363 and mir-106b-25 were down-regulated upon *MYCN* knockdown-induced differentiation, either on both arrays (indicated as blue boxes) or on one array (hatched boxes).

Figure 4: *Mir-21* is inverse correlated to *MYCN* mRNA expression: (**A**): *MYCN* mRNA (left) and *mir-21* (right) levels in SK-N-SH, SK-N-AS, KCN, KCNR, LAN5, Kelly and SK-N-BE (2) neuroblastoma cell lines. (**B**) Cells transfected with either aMN-887 or premir-21 displayed a ~2-fold and 20-fold increase in *mir-21* expression, respectively. Co-transfection of aMN-887 and anti-mir-21 prevented the *mir-21* increase. (**C**) Inhibition of endogenous *mir-21* by the anti-mir-21 antagomir. The luciferase-reporter plasmid (pmir-21-luc), containing a 3'UTR with the *mir-21* target sequence, was co-transfected into SK-N-BE (2) and Kelly cells along with anti-mir-21 or a negative control antagomir. (**D**) *MYCN*-knockdown with aMN-887 induced strong up-regulation of NPY, a neuronal differentiation marker. In contrast, overexpression of premir-21 alone did not alter NPY. Co-transfection of aMN-887 and anti-mir-21 did not prevent cells from differentiation. (**E**) *MYCN* mRNA levels were reduced in SK-N-BE (2) cells transfected with aMN-887. Overexpression or inhibition of *mir-21* did not alter *MYCN* mRNA levels compared to the respective negative controls.

Figure 5: Proliferation assays. SK-N-BE (2) cells (**A**) and Kelly cells (**B**) were transfected with premir-21 at different concentrations (40, 60 and 80 nM) and monitored for proliferation on 3 consecutive days after transfection. mir-NC= negative control miRNA mimic. (**C**) Flow cytometric data showing that the S-phase of the cell cycle was unaffected by premir-21 treatment of SK-N-BE (2) cells. SK-N-BE (2) cells (**D**) and Kelly cells (**E**) treated with antimir-21 in combination with aMN-887 showed no significant alterations in proliferation during the 2^{nd} and 3^{rd} day post-transfection compared to cells treated with a negative control antagomir (anti-NC).

Additional files

Supplementary 1:

Averaged signal intensity of small endogenous RNA molecules and short-hairpin RNA molecules after pDS-shSCR (high N-myc) and pDS-anti*MYCN*-887 transfection (N-myc knockdown).

Supplementary 2:

Overview of miRNAs with consistent differential expression on both microarrays SK07 and SK08.

Supplementary 3:

Validation of microarray data by quantitative RT-PCR. P-values refer to qRT-PCR data and are calculated by student´s t-test. Down-regulated and up-regulated miRNAs are shown as blue and red bars, respectively. A selection of 11 miRNAs which were consistently differentially expressed on both microarrays was confirmed by qRT-PCR with $p<0.05$. * indicates miRNAs shown to be significantly down-regulated on a miRNA microarray in a similar *MYCN*-knockdown study in Kelly cells ($p<0.01$) (data not shown)

Supplementary 4:

Differential expression of *mir-21* in Kelly cells. Cells were transfected with either sh-SCR or aMN-887. *Mir-21* levels were measured 3 days after transfection using a microRNA microarray and quantitative stem-loop RT-PCR.

Supplementary 5:

Mir-21 and *MYCN* mRNA expression at day 1 after transfection with sh-SCR and aMN-887. The MYCN-knockdown mediated increase in *mir-21* expression coincides early with *MYCN* downregulation.

Supplementary 6:

Western blot analyses of N-myc, PDCD4, PTEN and β-actin expression in SK-N-BE (2) and Kelly cells transfected with aMN-887, premir-21 and a combination of aMN-887 and antimir-21.

Figure 1

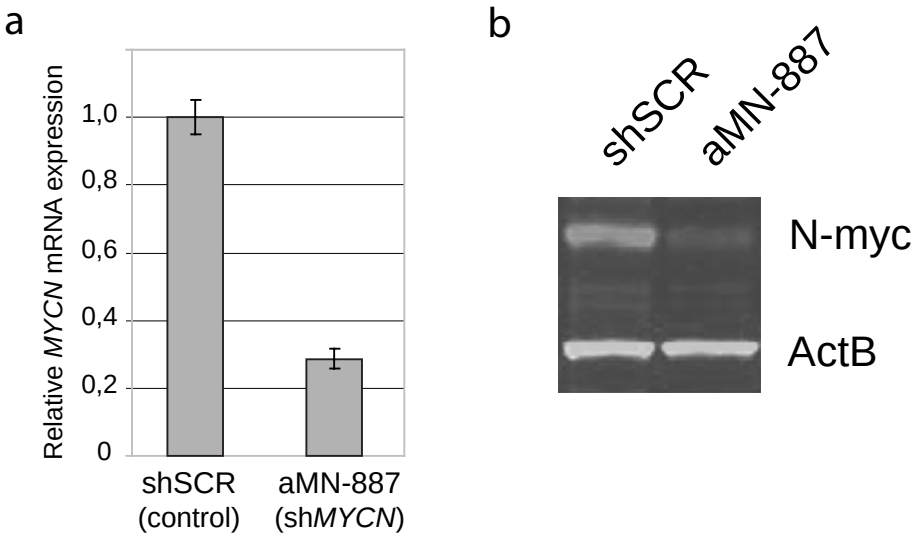
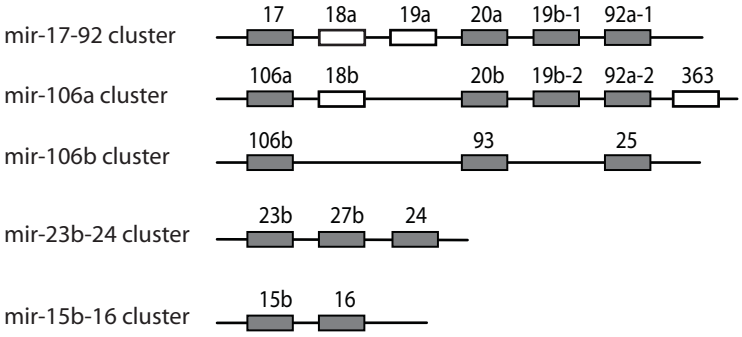

a
Relative MYCN mRNA expression
1,0
0,8
0,6
0,4
0,2
0
shSCR (control)
aMN-887 (shMYCN)
b
shSCR
aMN-887
N-myc
ActB
c
shSCR
aMN-887
Draq5 + TUJ1
GFP
N-myc
Merge
Draq5 + TUJ1
GFP
N-myc
Merge

Figure 2



Non-clustered miR let-7a, mir-23a, mir-26a, mir-92b, mir-103, mir-107, mir-214, mir-218, mir-320, mir-638

Figure 3

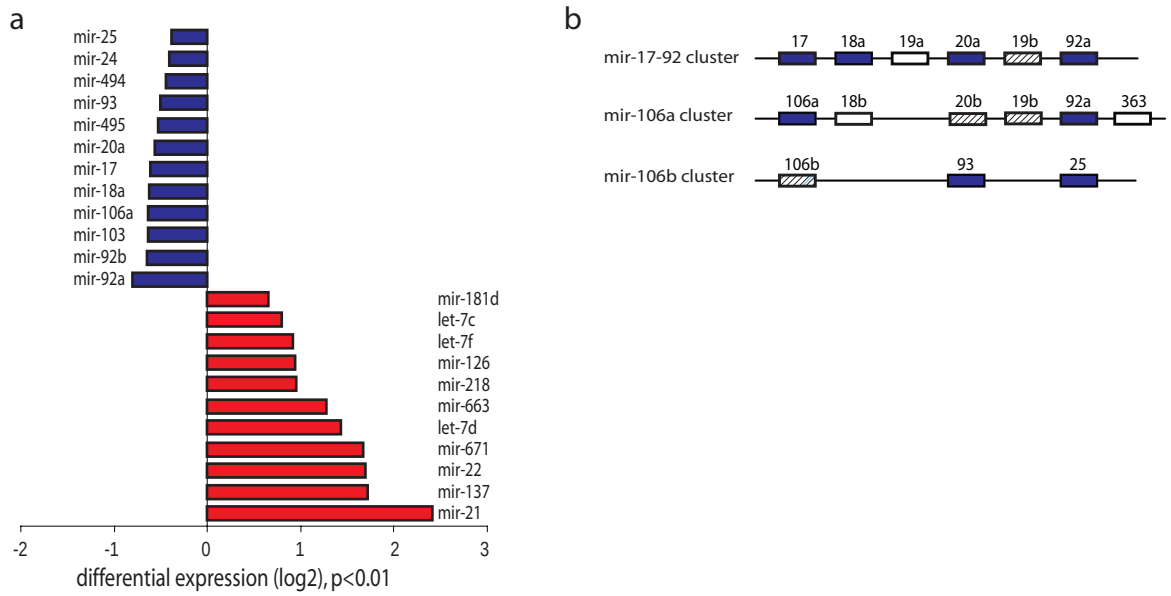

a
mir-25
mir-24
mir-494
mir-93
mir-495
mir-20a
mir-17
mir-18a
mir-106a
mir-103
mir-92b
mir-92a
mir-181d
let-7c
let-7f
mir-126
mir-218
mir-663
let-7d
mir-671
mir-22
mir-137
mir-21
-2
-1
0
1
2
3
differential expression (log2), p<0.01
b
mir-17-92 cluster
17
18a
19a
20a
19b
92a
mir-106a cluster
106a
18b
20b
19b
92a
363
mir-106b cluster
106b
93
25

Figure 4

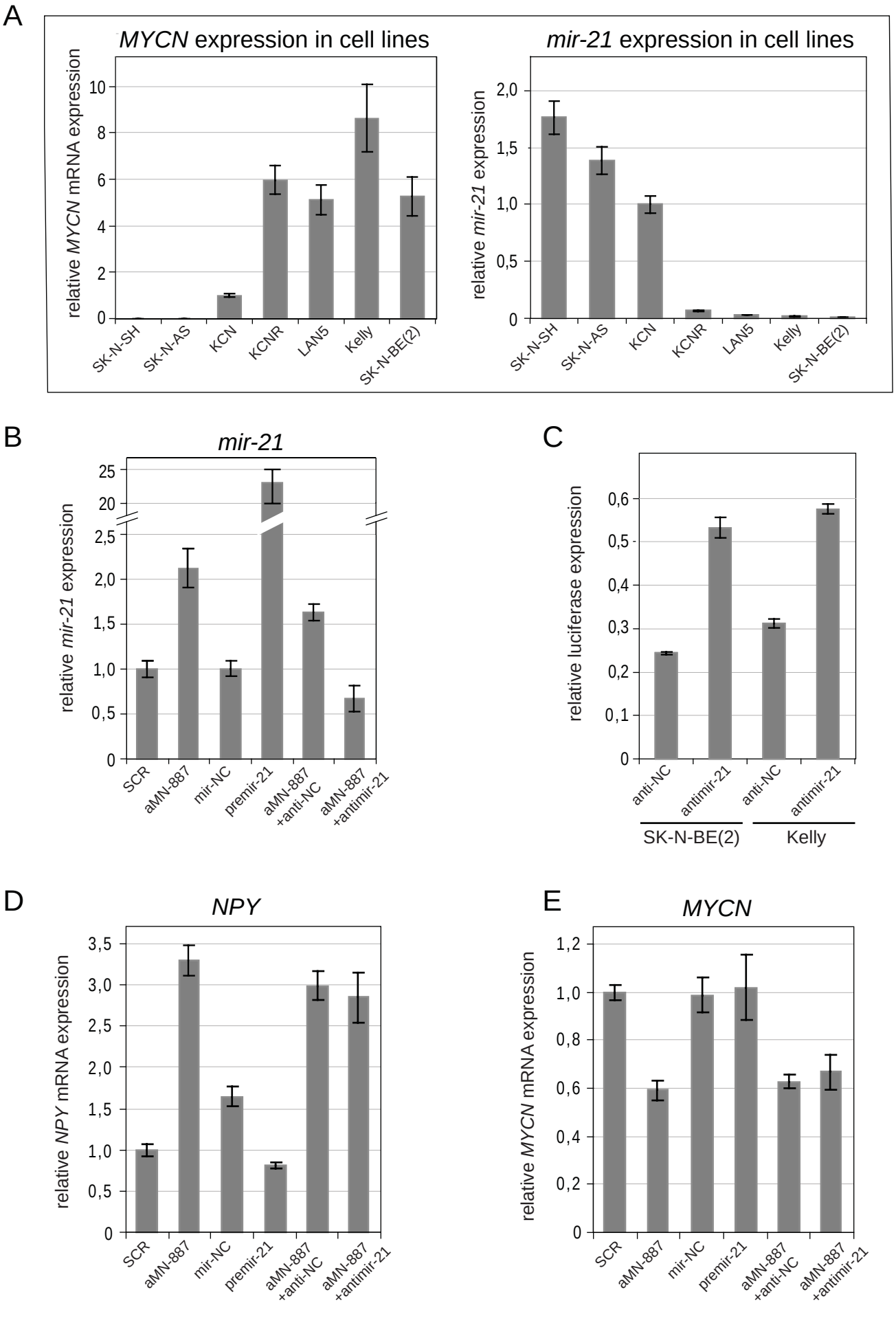
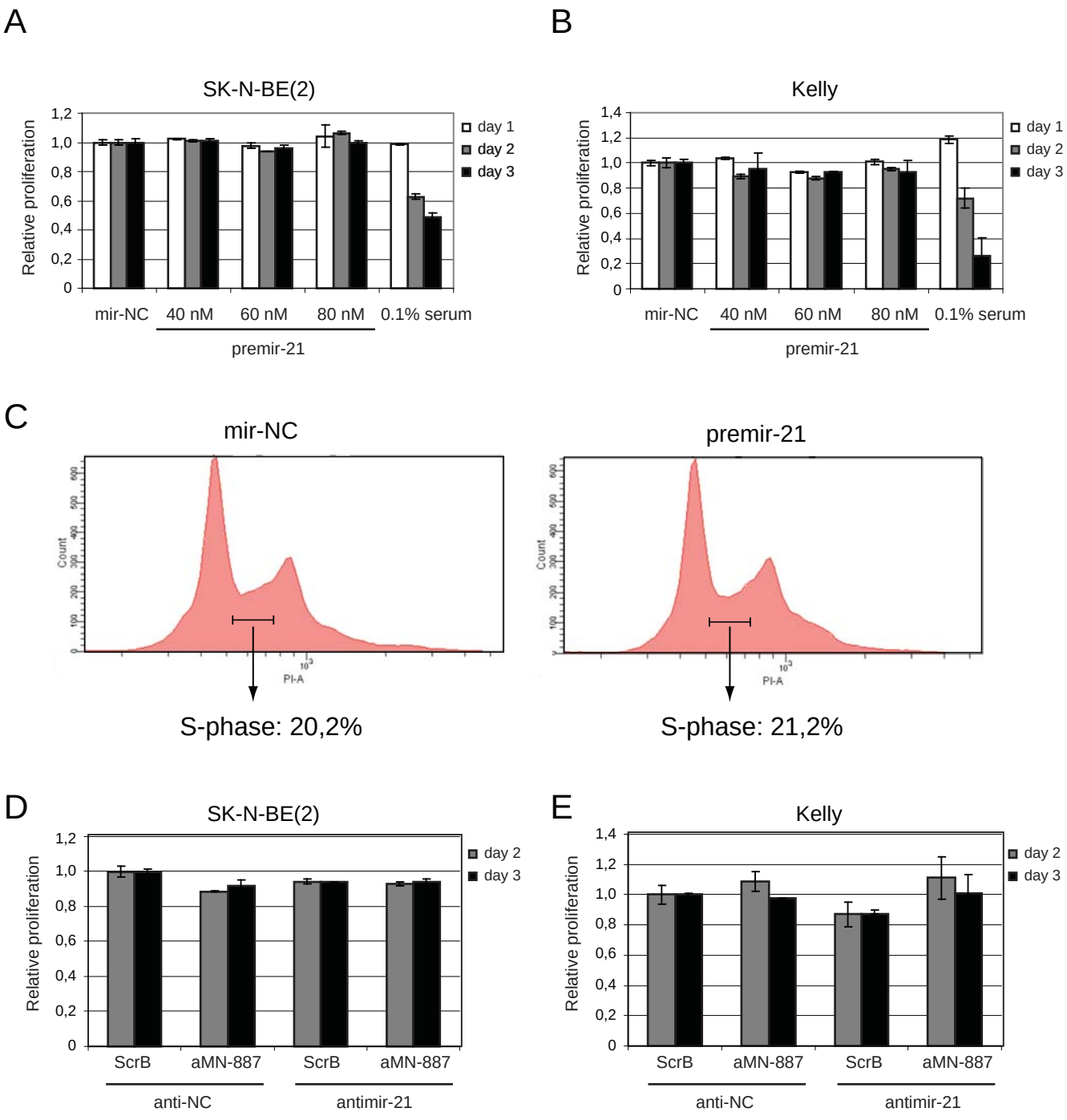

A
MYCN expression in cell lines
relative MYCN mRNA expression
0
2
4
6
8
10
SK-N-SH
SK-N-AS
KCN
KCNR
LAN5
Kelly
SK-N-BE(2)
mir-21 expression in cell lines
relative mir-21 expression
0
0,5
1,0
1,5
2,0
SK-N-SH
SK-N-AS
KCN
KCNR
LAN5
Kelly
SK-N-BE(2)
B
mir-21
relative mir-21 expression
0
0,5
1,0
1,5
2,0
2,5
20
25
SCR
aMN-887
mir-NC
premir-21
aMN-887 +anti-NC
aMN-887 +antimir-21
C
relative luciferase expression
0
0,1
0,2
0,3
0,4
0,5
0,6
anti-NC
antimir-21
anti-NC
antimir-21
SK-N-BE(2)
Kelly
D
NPY
relative NPY mRNA expression
0
0,5
1,0
1,5
2,0
2,5
3,0
3,5
SCR
aMN-887
mir-NC
premir-21
aMN-887 +anti-NC
aMN-887 +antimir-21
E
MYCN
relative MYCN mRNA expression
0
0,2
0,4
0,6
0,8
1,0
1,2
SCR
aMN-887
mir-NC
premir-21
aMN-887 +anti-NC
aMN-887 +antimir-21

Figure 5


A
SK-N-BE(2)
Relative proliferation
1,2
1,0
0,8
0,6
0,4
0,2
0
day 1
day 2
day 3
mir-NC
40 nM
60 nM
80 nM
0.1% serum
premir-21
B
Kelly
Relative proliferation
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0
day 1
day 2
day 3
mir-NC
40 nM
60 nM
80 nM
0.1% serum
premir-21
C
mir-NC
Count
PI-A
S-phase: 20,2%
premir-21
Count
PI-A
S-phase: 21,2%
D
SK-N-BE(2)
Relative proliferation
1,2
1,0
0,8
0,6
0,4
0,2
0
day 2
day 3
ScrB
aMN-887
ScrB
aMN-887
anti-NC
antimir-21
E
Kelly
Relative proliferation
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0
day 2
day 3
ScrB
aMN-887
ScrB
aMN-887
anti-NC
antimir-21

Supplementary 1

small RNA	shSCR-transfected	aMN-887 transfected
RNU19	86,96	81,01
RNU43	80,05	77,51
RNU38B	**198,52**	**181,00**
RNU24	288,89	217,83
U18	39,80	43,72
RPL21	157,04	103,55
HY3	660,38	1 533,30
RNU44	3 439,07	1 701,35
RNU48	1 508,94	954,87
RNU49	1 030,45	585,84
RNU58B	313,48	202,84
RNU66	48,66	72,20
RNU6B	72,06	471,90
U47	536,65	301,00
RNU58A	Not detect.	Not detect.
U54	Not detect.	Not detect.
U75	Not detect.	Not detect.
Z30	Not detect.	Not detect.
shMYCN (aMN-887)	**146,91**	**68 710,01**
shSCR	**45 612,95**	**23,92**

Supplementary 2:

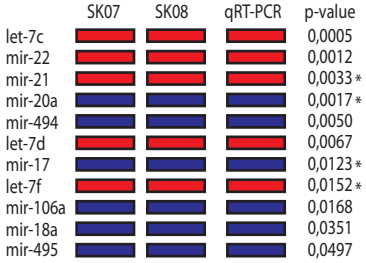
Down-regulated miRNAs

	SK07		SK08	
miRNA	**fold change**	**log2 change**	**fold change**	**log2 change**
mir-103	0,64	-0,64	0,86	-0,21
mir-93	0,70	-0,51	0,78	-0,35
mir-24	0,75	-0,41	0,84	-0,25
mir-25	0,76	-0,39	0,76	-0,39
mir-17	0,66	-0,61	0,73	-0,45
mir-106a	0,64	-0,64	0,68	-0,55
mir-494	0,73	-0,45	0,67	-0,58
mir-495	0,69	-0,53	0,66	-0,61
mir-18a	0,65	-0,62	0,65	-0,62
mir-92a	0,57	-0,80	0,63	-0,66
mir-20a	0,68	-0,56	0,63	-0,67
mir-92b	0,64	-0,65	0,62	-0,68

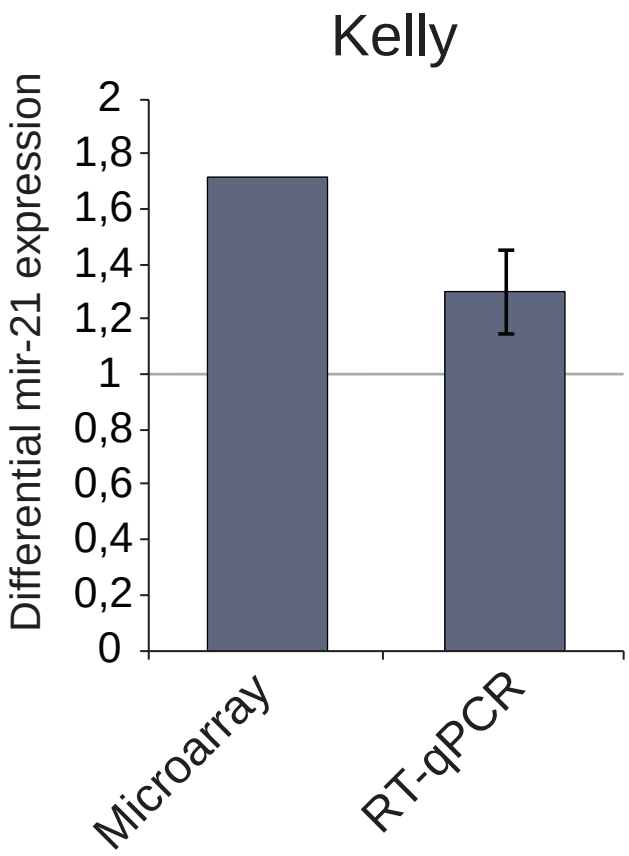
Up-regulated miRNAs

	SK07		SK08	
miRNA	**fold change**	**log2 change**	**fold change**	**log2 change**
mir-671	3,18	1,67	4,82	2,27
mir-663	2,43	1,28	2,66	1,41
mir-181d	1,58	0,66	1,69	0,76
mir-137	3,29	1,72	1,56	0,64
mir-22	3,25	1,70	1,54	0,62
mir-21	5,31	2,41	1,51	0,59
let-7d	2,69	1,43	1,38	0,46
let-7f	1,89	0,92	1,39	0,47
mir-126	1,92	0,94	1,30	0,38
let-7c	1,74	0,80	1,29	0,37
mir-218	1,93	0,95	1,21	0,28

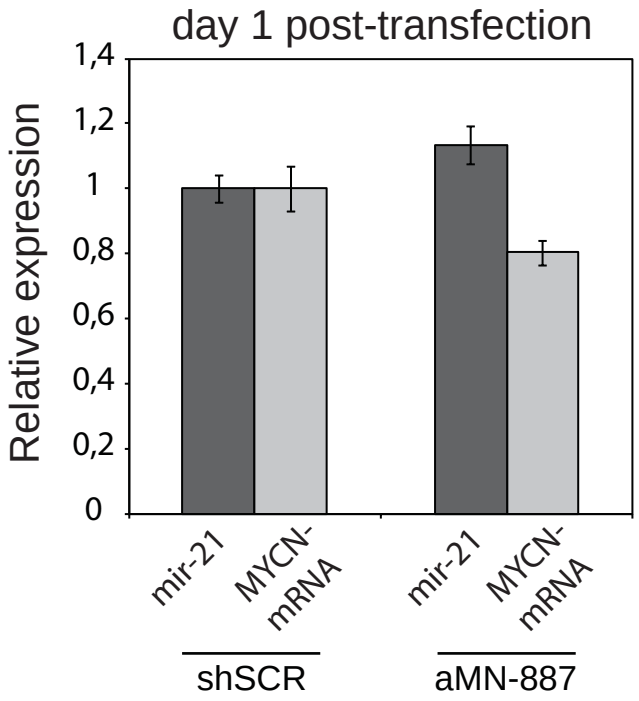
Supplementary 3



Supplementary Figure 4



Supplementary Figure 5



Supplementary Figure 6