Differentiation 81 (2011) 25–34

Contents lists available at ScienceDirect

Differentiation

journal homepage: www.elsevier.com/locate/diff

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

Jochen Buechner [a,b], Jørn R. Henriksen [a,b], Bjørn Helge Haug [b], Ellen Tømte [b], Trond Flaegstad [a,b], Christer Einvik [a,*]

[a] Department of Paediatrics, University Hospital of North-Norway, 9038 Tromsø, Norway
[b] Department of Paediatric Research, Institute of Clinical Medicine, University of Tromsø, 9037 Tromsø, Norway

ARTICLE INFO

Article history:
Received 1 April 2010
Received in revised form
2 September 2010
Accepted 30 September 2010

Keywords:
Neuroblastoma
MYCN
microRNA
Neuronal differentiation
Mir-21

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 neuroblastoma cell lines used in this study.

1. Introduction

Neuroblastoma is a highly malignant embryonic childhood tumour arising from primitive cells of the neural crest (van Noesel and Versteeg, 2004). 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 (Hero et al., 2008). 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 (Maris et al., 2007).

One of the strongest biological predictors of poor outcome is genomic amplification of the oncogene *MYCN* (Brodeur and Seeger, 1986). 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 (Henriksson and Luscher, 1996). Like other members of

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

* Corresponding author. Tel.: +47 77644735; fax: +47 77645350.
E-mail addresses: jochen.buechner@unn.no (J. Buechner), Joern.Remi.Henriksen@fagmed.uit.no (J.R. Henriksen), bjorn.haug@gmail.com (B.H. Haug), ellen.tomte@uit.no (E. Tømte), trond.flaegstad@unn.no (T. Flaegstad), christer.einvik@uit.no (C. Einvik).

0301-4681/$ - see front matter © 2010 International Society of Differentiation. Published by Elsevier Ltd. All rights reserved.
Join the International Society for Differentiation (www.isdifferentiation.org)
doi:10.1016/j.diff.2010.09.184

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 (Breit et al., 2000; Li and Kretzner, 2003; Judware and Culp, 1997) is presumably mediated through interaction with other DNA-binding proteins (Peukert et al., 1997).

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 (Bartel, 2009).

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 Edsjo et al., 2007). 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 (Thiele et al., 1985). The function of N-myc during RA-induced differentiation of MNA neuroblastomas, however, is contradictory and unclear (Edsjo et al., 2004; Peverali et al., 1996; Thiele and Israel, 1988).

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) (Bell et al., 2006; Haber et al., 1999; Negroni et al., 1991; Tonelli et al., 2005). 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 (Henriksen et al., in press). 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 (Beveridge et al., 2009; Chen and Stallings, 2007; Evangelisti et al., 2009; Fukuda et al., 2005; Laneve et al., 2007; Le et al., 2009). With the exception of one study using anti-*MYCN* siRNA (Chen and Stallings, 2007), 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.

2. Materials and methods

2.1. 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 (Tweddle et al., 2001; Schleiermacher et al., 2004). 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% CO_2.

2.2. 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 (Henriksen et al., in press). 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 (Henriksen et al., in press) 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 Lipofectamine 2000 (Invitrogen) according to the manufacturer's standard recommendations, and the transfection efficiency reached 70–80%.

2.3. 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.

2.4. MiRNA microarray profiling

2.4.1. 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 (Wang et al., 2008). Microarrays SK07 and SK08 included all miRNAs listed in the Sanger miRBase Release 9.2 (471 human miRNAs) and Release 10.1 (723 human