UNIVERSITY OF TROMSØ UIT

FACULTY OF HEALTH SCIENCES
DEPARTMENT OF CLINICAL MEDICINE

Targeted *MYCN* suppression and its effect on miRNA in neuroblastoma

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A dissertation for the degree of
Philosophiae Doctor

December 2010

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December 2010

Acknowledgements

This work was conducted both at the Department of Paediatrics, University Hospital of Northern Norway, as well as at the Paediatric Research Laboratory / the neuroblastoma group, Department of Clinical Medicine, University of Tromsø. The financial support was from the Northern Norway Regional Health Authority (the Gene Therapy Program), The University of Tromsø, The Norwegian Cancer Society, Ragnvarda F. Sørvik and Håkon Starheims Foundation and the Erna and Olav Aakre Foundation for Cancer Research.

Team work is essential for the completion of a PhD, and many colleagues and friends deserve my gratitude for achieving this degree.

First I want to offer my thanks to my supervisors for guiding me through this process. To my main supervisor Trond: Thank you for believing in me and offering a never-ending amount of optimism and patience. Whenever I have needed something, you have offered it. To my supervisor Christer: Thank you for finding the intriguing projects that made this thesis possible. You have always been readily available for answering any questions I might have and offering any guidance I might need.

Thank you to my fellow PhD-students at the neuroblastoma group, Jochen and Bjørn Helge. Research has been much more efficient and fun when we have shared not only our experience and knowledge, but also when we have shared our frustrations over results that never seemed to emerge as desired as well as the uninhibited joy when they finally did. Without you I would probably be stuck in 2008-mode forever...

There are large amounts of experimental work behind this thesis. And much of it has been performed by present and former engineers and research assistants at our group. A great thank you goes to Cecilie, Ellen and Martin for offering not only your assistance, but also your pleasant company during countless lunches.

The co-authors not already mentioned have offered large and precious contributions to the papers presented in this thesis. My gratitude for this goes to Baldur Sveinbjörnsson, Tommy Martinsson, Pär Kogner, Dirk Geerts and Rogier Versteeg.

To Elizabeth, Hildegunn, Pauline and Merethe at the biofilm-group: Thank you for being such vital contributors to all shared social arrangements. To such bundles of joy as you I will gladly continue to offer my blood. I especially appreciate the patience shown by Elizabeth when awaiting my arrival at the office for several years, and for her unconditional support when writing this thesis.

At the MH-building there is a fine tradition for giving support, lending reagents and sharing expertise whenever anybody might ask. Thus there are numerous people that have offered small but important contributions during the years this thesis has been under its way. I am truly grateful to you all.

I also want to thank my family and friends for their support. You have all been essential for my well-being whenever I have felt the need to focus on other areas of life than my research.

Last but possibly most important I will express my gratefulness to Marte Beate. Thank you for offering your continuous and unrestricted support, affection and patience when I have been working on this thesis. I truly admire you for leaving and living alone in Spitsbergen for the last three years. And I especially respect your endurance when carrying out your entire pregnancy by yourself in exile without complaints. I am however ecstatic about your return, and the fact that we finally can start living together again.

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Comparison of RNAi efficiency mediated by tetracycline-responsive H1 and U6 promoter variants in mammalian cell lines

Henriksen JR, Løkke C, Hammerø M, Geerts D, Versteeg R, Flægstad T, Einvik C.

Nucleic Acids Research, 2007:35:e67

Paper II:

Conditional expression of retrovirally delivered anti-*MYCN* shRNA as an in vitro model system to study neuronal differentiation in *MYCN*-amplified neuroblastoma

Henriksen JR, Haug BH, Buechner J, Løkke C, Flægstad T, Einvik C.

Manuscript accepted pending minor revisions in *BMC Developmental Biology*

Paper III:

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

Buechner J, **Henriksen JR**, Haug BH, Tømte E, Flægstad T, Einvik C.

Differentiation 2010, In press. Doi:10.1016/j.diff.2010.09.184

Paper IV:

***MYCN*-regulated miRNAs inhibit secretion of the tumor suppressor *DICKKOPF-3* (*DKK3*) in neuroblastoma**

Haug BH, **Henriksen JR**, Buechner J, Kogner P, Martinsson T, Flægstad T, Sveinbjörnsson B, Einvik C.

Manuscript submitted.

2. Abbreviations

Ago	Argonaute
BMP	Bone morphogenetic protein
bp	Base pair
Cre	Cyclization recombination
DKK	Dickkopf
DNA	De-oxy ribonucleic acid
Dox	Doxycycline
dsRNA	Double stranded RNA
e.g.	*exempli gratia*
EFS	Event free survival
Endo-siRNA	Endogenous siRNA
Exp5	Exportin 5
FGF	Fibroblast growth factor
I	Intertmediate
i.e.	*id est*
INSS	International Neuroblastoma Staging System
Lef	lymphocyte enhancer factor
LOH	Loss of heterozygosity
Lox	Locus of X over P1
LRP	low-density lipoprotein receptor-related protein
miRNA	Micro RNA
MLV	Moloney Murine Leukemia Virus
MNA	MYCN Amplified
mRNA	Messenger RNA
MVD	Microvessel density
N	Neuroblastic
NGF	Neural growth factor
nt	nucleotide
P-body	Processing body
PNA	Peptide Nucleic Acid
Pre-miRNA	Precursor micro RNA
Pri-miRNA	Primary micro RNA
RIIID	RNase III domain
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
S	Substrate adherent
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
ssRNA	Single stranded RNA
TCF	T-cell factor
Tet	Tetracycline
TetO	Tetracycline Operator
TetR	Tetracycline Receptor
TLR	Toll-like receptor
UTR	Un-transcribed region

3. Introduction

3.1. Small RNAs

3.1.1. The history of RNAi and miRNA – a summary

In 1958, Crick presented the idea of the central dogma of molecular biology, which states that the information from DNA is passed in a one-way direction via RNA to proteins [1]. Since then, one of the most important questions in molecular biology has been how the expression of proteins is regulated. The first time RNA was proposed as a regulator of protein expression was in 1969, when Britten and Davidson proposed a theory in which RNA might regulate the expression of genes using standard Watson-Crick base-pairing rules [2]. However, the idea that RNA could govern the expression profile of each cell type was neglected when transcription factors were discovered, and for decades transcription factors (there is an estimate of ~1500 transcription factors in the human genome [3]) were seen as almost exclusive effectors for regulating the mRNA expression profile and the proteome of eukaryotic cells.

It was not until the early 1990s that hints of expressional regulation of one mRNA caused by another RNA started to emerge. In 1990, it was reported that the overexpression of exogenous DNA encoding an enzyme, producing a purple pigment in petunias, led to white flowers as a consequence of a reduced expression of both endogenously and exogenously introduced enzyme mRNA [4, 5]. The downregulation of mRNA levels by the introduction of antisense RNA was an established technique as early as 1984 [6], but it was shown in 1995 that both antisense and surprisingly the synthetically produced sense RNA induced the silencing of gene expression in *Caenorhabditis elegans* [7]. In 1998, Fire and Mello demonstrated that this silencing was triggered by dsRNA and that the sequence of the dsRNA determined which mRNA was targeted for silencing [8]. Three years later, the same method was also shown to be a functional tool in mammalian cells [9] and that the concept of RNA-based gene silencing is functional in all eukaryotic supergroups (with a few exceptions, including budding yeast, which seems to have lost this feature during evolution) [reviewed in 10]. This mechanism is called RNA interference (RNAi)

At the same time as the mechanism of RNAi was discovered, another small RNA started to make its appearance. In 1993, Ambros *et al.* described *lin-4* as the first micro RNA (miRNA) in the nematode *C. elegans*. Here, it was shown how the smaller RNA *lin-4* seemed to repress the translation of the larger mRNA *lin-14* and that this repression might be a result of multiple RNA-RNA interactions between *lin-4* and the 3' UTR structure of *lin-14* [11]. In 2001, miRNA was established as a large class of gene regulators in several species including humans [12-14], thereby suggesting that miRNA and the proteins involved in its regulation are a part of a conserved pathway.

In 2001, RNAi and miRNA were linked together when it was demonstrated that Dicer, a protein that sliced long dsRNAs into smaller effector RNAs called small interfering RNAs (siRNAs) [15, 16], also converted longer *lin-4* transcripts into smaller mature *lin-4* miRNAs [17-19]. As a result, it was now apparent that both RNAi and miRNA were regulatory tools conserved in most eukaryotes and that they both used the same pathway to exert their regulatory effect on mRNAs.

3.1.2. The miRNA pathway in animals

There are two main differences between miRNA and siRNA in animals:

1. MiRNAs are transcribed endogenously from non-protein-coding separate genes. There are no dedicated genes for siRNAs. Instead, they are degraded from larger dsRNA introduced to the nucleus exogenously (e.g. viral transcripts) or endogenously (e.g. transposons) [20].

2. SiRNAs have a full complementarity towards their targets, while miRNA show a limited complementarity.

The main features of the miRNA pathway are outlined in Figure 1.

MiRNA genes are primarily transcribed by RNA polymerase II to form primary miRNA (pri-miRNA), a structure which is usually several kilobases long, with local stem loop structures [21, 22]. They can be encoded in independent transcription units, in polycistronic clusters or within introns of protein coding or non-coding genes [reviewed in 23]. The pri-miRNA is further processed in the nucleus into a hairpin approximately 60-70 nucleotides long, which is known as the precursor miRNA (pre-miRNA) [24]. This processing is done by the

Ribonuclease III (RNase III) protein Drosha and its essential co-factor DiGerorge syndrome critical region 8 (DGCR8) [25-29]. After processing, the pre-miRNA hairpin contains a 3' 2 nt overhang and a 5' mono-phosphate group [25, 30, 31], a feature that is characteristic of all RNAs cleaved by an RNase III protein [32]. Polycistronic clusters are transcribed as a single transcript, which is processed into all the separate miRNAs within the cluster by Drosha [12, 13].

Pre-miRNA exits the nucleus through nuclear pore complexes since further processing of the pre-miRNA takes place in the cytosol. This transport is executed by the nuclear transport receptor exportin-5 (exp5), which recognises dsRNA hairpins with stems >16bp long [33-35]. The 3' overhang of 2 nt further facilitates this process [36].

Following release into the cytoplasm, the pre-miRNA is recognised by and bound to the RNase III enzyme Dicer, which cuts it to generate a mature miRNA [16-19]. Dicer contains both an RNase III domain (RIIID) responsible for slicing dsRNA and a highly conserved Piwi Argonaute Zwille (PAZ) domain which recognises and binds the 3' dinucleotide overhang on the 3' end of the pre-miRNAs [15, 37]. This suggests that the 3' dinucleotide overhang locks into the PAZ-domain, bringing the pre-miRNA into position for cleavage by the RIIID of Dicer [38]. Human Dicer then cuts the pre-miRNA stem approximately 22 nt from the 3' end docked in the PAZ domain [39]. A crystal structure of Dicer from the parasite *Giardia* reveals that the distance from the PAZ-domain to the RIIID matches the length of the *Giardia* Dicer cleavage products [40], thus suggesting that Dicer itself acts as a molecular ruler which generates products defined in length by the fixed distance between its PAZ domain and RIIID. The pre-miRNA is now reduced to an approximately 22 nt dsRNA, with a 3' dinucleotide overhang and a 5' phosphate group at both ends.

Dicer works together with several other proteins in order to execute its function. Dicer containing the sliced dsRNA is recruited by the trans-activation-response RNA-binding protein (TRBP) to form a structure known as the RNA-induced silencing complex (RISC) loading complex [41, 42]. Here, the mature miRNA is transferred from Dicer to the RISC where it binds to an Argonaute (Ago) protein, which is the catalytic entity of the RISC [43, 44]. Of the two Ago proteins encoded in humans, Ago2 is functioning in the RNAi pathway [43, 44]. Ago2 also contain a PAZ domain, which recognises the presence of a 3' dinucleotide overhang and a 5' phosphate group on dsRNA, both of which are facilitating the incorporation

of the miRNA to the RISC [45-47]. After the transfer of the RNA duplex, it is the strand with its 5' end at the least thermodynamically stable end that is preferentially kept bound to Ago2 [48]. This strand is known as the guide strand, while the complementary passenger strand is cleaved and degraded [49]. When bound to the RISC, the guide strand functions as a sequence-specific template that leads the RISC to complementary targets through base-pairing interactions.

MiRNAs does not usually have a full complementarity with its targets. When bound to RISC, only nucleotides numbered 2-6 of the guide strand (starting from the 5' end) are exposed to such a degree that base-pairing with target RNA is possible [50]. This is in accordance with the observation that animal miRNAs contains a seed region ranging from nt 2-8, which is critical for the specificity of target recognition [51-53]. MiRNAs usually exert their regulatory functions by binding to a complementary seed sequence in the 3'UTR of their target mRNA, although binding to the 5'UTR or the coding regions has also been reported [54]. When the targeted mRNA is bound to the RISC, it can be moved from the cytosol to cytoplasmic complexes called Processing bodies (P-bodies) [55-57]. Here, de-adenylation and de-capping of the mRNAs followed by 5'→3' degradation occurs [56, 58]. In addition, the protein translation of the mRNAs is prevented as they become sequestered from the ribosomes residing in the cytosol. Nevertheless, degradation of mRNA accounts for the vast majority of miRNA induced gene silencing [59].

If there is perfect complementarity between the bound guide strand and its target, the target is not transported to P-bodies. Instead, Ago2 cuts the target RNA between the 10th and 11th nucleotide as measured from the 5' end of the guide strand [46, 47, 60-62].

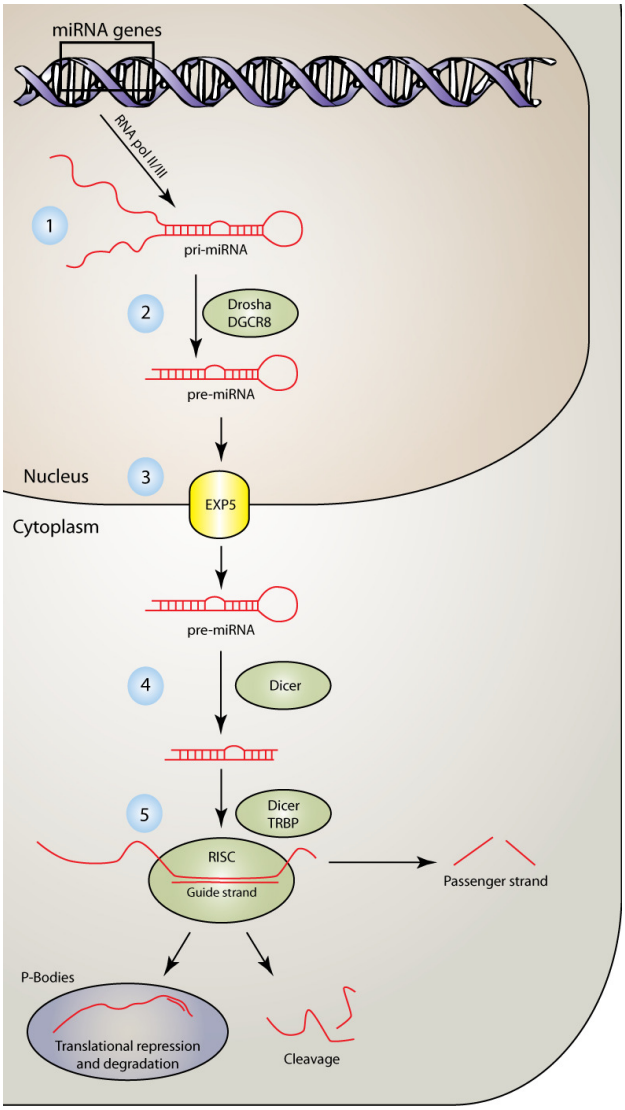

Figure 1: The miRNA pathway. MiRNA genes are transcribed to pri-miRNA often several kilobases long (1). Pri-miRNA is then processed by Drosha to form hairpin structures known as pre-miRNA (2). Exportin 5 recognizes the hairpin structure by its 3' dinucleotide overhang, and exports it from the nucleus to the cytoplasm (3). In the cytoplasm, pre-miRNA is recognized by Dicer, which cuts the pre-miRNA into a mature miRNA ~22 nt long (4). The dsRNA is then transferred into the RISC, where the passenger strand is cleaved and removed. The guide strand is used as a template for binding target mRNA according to base-pairing. If there is full complementarity, the mRNA is cleaved. If there is partial complementarity, the mRNA is transferred to P-bodies where translation is repressed and the mRNA is eventually degraded (5), see text for details. Modified by permission from Macmillan Publishers Ltd: Gonzalez-Alegre, P. and H.L. Paulson July, 2007. "Technology insight: therapeutic RNA interference--how far from the neurology clinic?" Nat. Clin. Pract. Neurol. 3(7):394-404.

3.1.3. The natural functions of RNAi in animals

Since Argonaute-like and Dicer-like proteins are present in all eukaryotic supergroups, it is evident that dsRNA-mediated silencing was already established in the last common ancestor of eukaryotes, but that it was also not required for life since it has been lost several times in various single-cellular organisms [reviewed in 10]. DsRNA-based silencing has probably evolved as a defence mechanism against genomic parasites such as tranposons and viruses [63-68]. Until recently, it has been assumed that defence is the main task of RNAi systems in mammals. Still, the discovery of endogenous siRNA (endo-siRNA) transcribed from L1 transposons in human cultured cells has opened the possibility of additional tasks for siRNA [69]. It has been shown in mouse oocytes that transcribed pseudogenes interact with homologous protein coding mRNAs to form dsRNA being processed to 21 nt siRNAs by Dicer [70, 71].

3.1.4. The natural functions of miRNA in animals

Thus far, miRNAs have only been described in multi-cellular organisms, in which they have evolved to regulators essential for the development of both animals and plants [23, 72-74]. Exactly when miRNAs evolved in evolution is debated, although it seems that the regulation of genes by miRNAs could be a requirement for the emergence of multi-cellular organisms since both plants and animals have evolved miRNA systems out of a separate evolutionary origin [10, 75]. From its discovery, it has taken miRNA merely a decade to achieve status as a regulator of most biological processes in animals and plants, including cell cycle, differentiation, development and metabolism [13, 76-78]. The number of mature miRNA products in the human genome has exceeded 1000 (mirBase version 16.0), while it is estimated that more than 60% of human mRNAs contain conserved miRNA target sites in their 3' UTR [79]. This means that on average each miRNA can target a large amount of mRNAs, e.g. the *miR-15a/16-1* cluster, which is predicted to potentially control 14% of all genes in the human genome [80]. To further add to this complexity, in most cases mRNAs harbour target sites for several different miRNAs [81-83]. Given the immense complexity and biological importance of miRNAs, it is of no surprise that their dysregulation is implicated in a vast number of human diseases, e.g. cancer, diabetes, muscular disorders and even psychiatric diseases [84-87]. MiRNAs were shown to potentially act as both oncogenes and

tumour suppressors in 2005 [88-91], and the dysregulation of miRNAs is now regarded as a vital contributor to the pathogenesis of many tumours [92, 93].

3.1.5. Targeted gene regulation using exogenous RNA

Previously, antisense technology was the most feasible approach for reducing the expression of specific genes, and both antisense DNA and RNA have been used for the modulation of biological processes [reviewed in 94]. However, since the discovery of RNAi, these approaches have been superseded, given the higher efficiency of RNAi compared to antisense technologies [95-97]. Scientists can now exploit RNAi to suppress virtually any gene simply by introducing siRNAs that are perfectly complementary to the target of interest. The miRNA biogenesis pathway contains three distinct RNA intermediates: the pri-miRNA transcript, the pre-miRNA hairpin and the miRNA duplex. All of these intermediates can be exploited as entry points for RNAi (Figure 2).

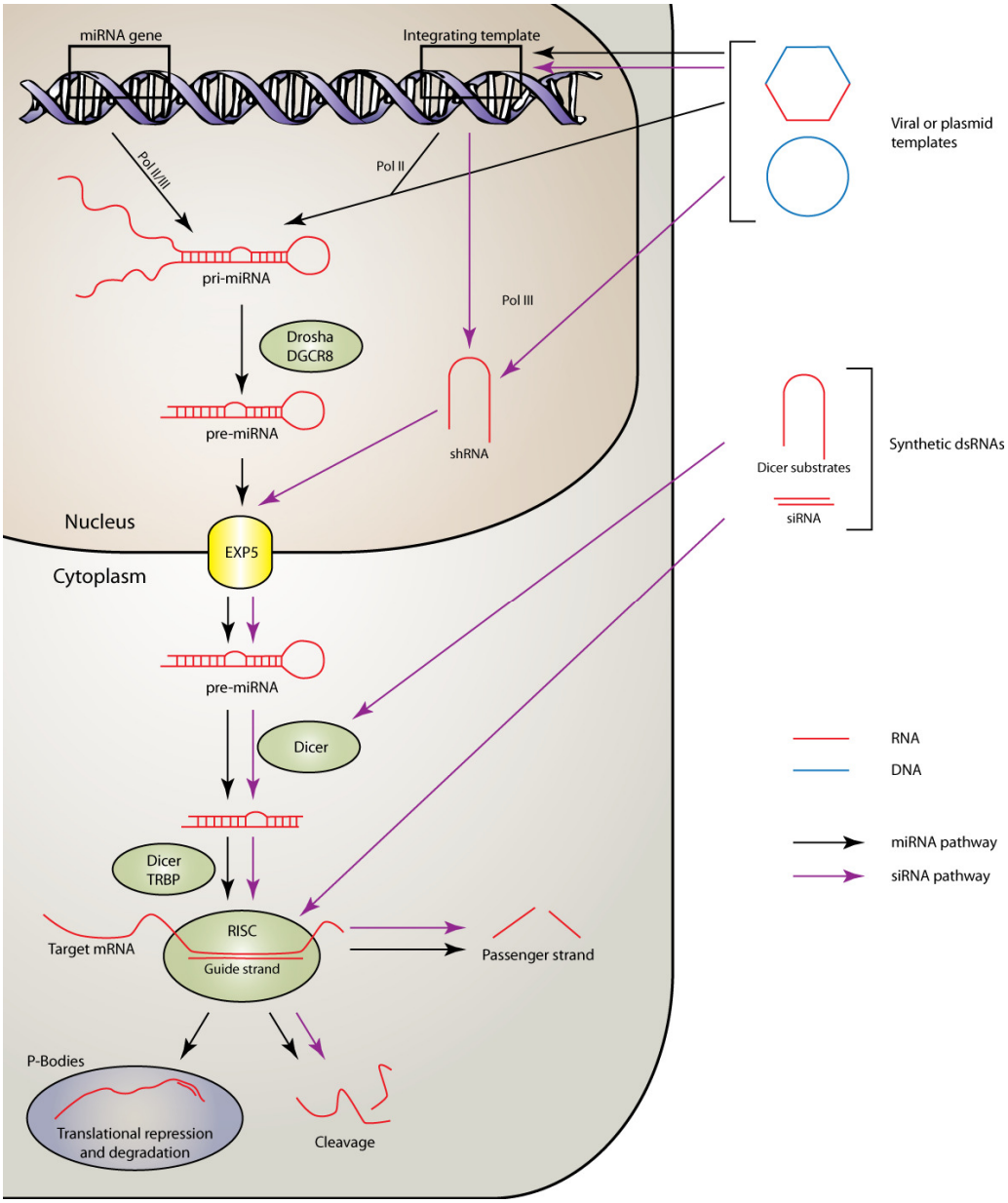

Figure 2: The process of RNAi and its manipulation. For a description of the miRNA pathway, confer with Figure 1. RNAi can be initiated by introducing siRNA or shRNA stems at all steps in the miRNA pathway, see text for details (Chapter 3.1.5). Modified by permission from Macmillan Publishers Ltd: Gonzalez-Alegre, P. and H.L.Paulson July 2007. "Technology insight: therapeutic RNA interference--how far from the neurology clinic?" Nat. Clin. Pract. Neurol. 3(7):394-404.

Imitating the miRNA duplex: siRNA

Synthetic siRNAs are small RNA duplexes designed to imitate the mature miRNA duplex in order to obtain RNAi-mediated gene suppression. In the first study, in which RNAi were shown to mediate effective silencing, dsRNA fragments several hundred nucleotides long were used [8]. Even so, the presence of long dsRNA in the cytoplasm often triggers the non-

specific interferon response pathway in mammalian cells, thereby leading to a broad inhibition of protein synthesis, transcriptional activation of cytokines and ultimately cell death [reviewed in 98]. It is generally believed that dsRNA molecules less than 30 bp long are not able to induce the interferon response [99]. Nonetheless, this is debated, and it has been demonstrated that the triggering of the interferon response pathway by dsRNAs >23 nt long is cell-type dependent [100]. To prevent activation of the interferon pathway, researchers have generally used shorter siRNAs (19-23 bp) that imitate the products of Dicer [46]. To further mimic natural Dicer products, siRNAs are also designed with a dinucleotide 3' overhang in both ends for more efficient loading [47]. It is the strand with the least thermodynamically stable 5' end which is preferred as the guide strand [48]. To ensure that the desired strand is loaded onto RISC, siRNAs are constructed with accordingly GC bp asymmetry.

Imitating the pre-miRNA: Synthetic Dicer products and shRNA

Synthetic Dicer products are 25-30 bp RNA duplexes designed to interact directly with Dicer [101]. They contain only one 3' dinucleotide overhang to ensure that Dicer cuts the RNA at the intended end, thereby producing identical mature siRNAs with the predicted sequence [102]. As a result, synthetic Dicer products mimic cytoplasmic pre-miRNA.

While siRNAs and Dicer products are introduced as RNA directly to the cytoplasm, short hairpin RNAs (shRNAs) are transcribed from DNA in the nucleus of the cell before they are exported by exp5 to the cytoplasm for further processing by Dicer and loading into the RISC [103]. ShRNAs are generally transcribed from RNA polIII into hairpins, thus mimicking nuclear pre-miRNA. In the appendix, we show that cloning of vectors expressing shRNAs are an easy and inexpensive technique for achieving efficient knockdown of a desired target gene.

The RNA polIII promoter is located directly upstream of the gene it is transcribing. It has a well defined starting point, and terminates when transcribing 4-5 consecutive thymidines [reviewed in 104]. When transcribed in the nucleus, the shRNAs folds into hairpins with a 3' dinucleotide overhang in one end and a loop in the other. The stem of the hairpin usually ranges from 23 to 29 bp. This configuration is similar to pre-miRNA constructs, and is therefore recognised as substrates by exp5 and Dicer [105-110]. Regardless of the initial length of the hairpin, Dicer cuts the shRNA into ~22 nt effector dsRNAs before incorporation to the RISC [39].

Imitating the pri-miRNA: shRNA-mir

RNA transcribed from PolII promoters includes elements such as 5' end caps and 3' polyA tails. These elements must be removed by Drosha before the transcript can interact with exp5 and the remaining miRNA pathway [22]. For this reason, polII promoters were initially avoided for transcription of shRNAs. However, shRNAs can also be expressed from RNA PolII promoters by inserting the hairpin within the backbone of a miRNA (usually that of *miR-30*), resulting in a primary transcript with extensive length and folding compared to standard shRNAs [111, 112]. Consequently, this shRNA-mir transcript is a target for processing by Drosha, and utilises the full miRNA pathway instead of accessing it in downstream entry points.

3.1.6. Efficiency of RNAi

All of the above described forms of RNAi exploit the miRNA pathway to exert their effect, and they can all be transiently transfected for short-term suppression of a desired gene. To evaluate the efficiency of the various constructs, one important aspect to consider is how much mRNA the construct is able to suppress on a numerical basis.

SiRNAs enter the miRNA pathway directly into the RISC, which means that they are not bound or processed by Dicer. The main function of Dicer is to cleave long dsRNAs into shorter products, but it is also a vital part of the RISC loading complex, which strongly facilitates the transfer of the guide strand into the RISC. Since siRNAs <23 bp are loaded directly onto the RISC, they are not able to take advantage of this feature [101, 113]. Dicer products are designed to interact with Dicer, and have been shown to be more efficient than siRNAs targeting the same target sequence [39, 101, 114].

There are three promoters used to express shRNAs: the H1, U6 and tRNA promoter [115]. Of these, the U6 and H1 promoters are generally being favoured, and it has been debated as to which of these promoters is the most efficient. Some groups find that U6 is the most effective in terms of expressed shRNA and the duration of gene suppression [115, 116], while others groups find no significant difference [117]. Our results reveal that the efficiency may vary between cell-lines but that the U6 promoter usually demonstrated a marginally higher efficiency (Paper I, [118]). This suggests discrepancies depending on different variables such as type of target cells, delivery method and shRNA sequence and that the most efficient

promoter should be decided on a case-by-case basis. Since shRNAs are processed by Dicer, they are also inserted into the RISC with higher efficiency than siRNAs. It has been shown that shRNAs expressed from a U6 promoter are more than 100 fold more effective on a numerical basis than siRNAs containing the same target sequence [115]. Nonetheless, a vector containing the shRNA is often 100 fold larger than naked siRNA. In addition, the vector needs to enter the nucleus in order to be transcribed, which is shown to be one of the biggest challenges when transfecting [119]. For that reason, the number of shRNA transcripts actually being produced is much lower than the number of siRNAs being introduced to the cytoplasm when the same amount of nucleic acid by weight is transfected. As a result of this, the practical gene suppression efficiency ranges from similar to slightly better for siRNAs compared to shRNAs [105, 115, 120].

By expressing the shRNA placed within the backbone of a miRNA, the resulting shRNA-mir is processed by Drosha in the same manner as the miRNA itself. MiRNAs are almost exclusively expressed from RNA polII promoters, as opposed to traditional shRNAs which are generally expressed from polIII promoters. The suppression efficiency of identical shRNAs expressed from either PolII or PolIII promoters has been compared with conflicting results. It has been shown that shRNA-mir expressed from a CMV PolII promoter slightly outperformed the shRNA transcribed from a U6 RNA polIII promoter [121]. Boden *et al.* reported that the transient expression of shRNAs from PolII promoters with miRNA backbones significantly outperformed conventional shRNAs [122], while it has also been reported that conventional shRNAs are more efficient than shRNA-mir [123]. Boudreau *et al.* however have stated that the above reports could be flawed as a result of missing 3' dinucleotide overhangs in addition to the failure to consider the effect of GC asymmetry in the stem when the conventional shRNAs were designed. When these important features are under control, it has been shown that conventional shRNAs are more potent than shRNA-mirs for three different target sequences, both *in vitro* and *in vivo* [124]. It is not clear whether this is a result of increased expression or higher stability of the transcribed shRNA in comparison to shRNA-mir.

3.1.7. Duration of RNAi-based gene suppression

It is only possible to introduce synthetic siRNAs and Dicer products to cells in a transient manner, which means that all gene suppression resulting from these synthetic RNAs is only

temporary. Bartlett and Davis found that it seems as if the duration of siRNA-mediated knockdown is dependent on how fast the harbouring cells are dividing [125]. Here, it was demonstrated that the levels of the targeted genes returned from optimal knockdown at 24-48 hours to background levels within six days in the dividing cells, and similar dynamics have been reported by other groups [114, 126]. However, it has also been reported that the maximum amount of introduced siRNA molecules peaks at approximately 24 hrs and diminishes within 48 hrs, which indicates a high degradation and turnover [127]. It has been shown that transiently transfected vector-based shRNAs give more durable gene suppression than the transfection of siRNA since shRNAs can be continuously transcribed by the host cell as long as the vector remains in the nucleus [115]. There are two main concerns when considering gene suppression using RNAi with transiently transfected effectors. First, all transient expressions by definition are temporal, and suffer from dilution effects as a result of cell division and RISC turnover. Because of this, it is not possible to maintain gene suppression for more than a week (our unpublished results). Second, transfection efficiency is rarely 100% and might vary considerably between cell lines. As a result, there will always be a background of cells without gene suppression, and this untransfected fraction could vary considerably between different cell lines (our results). Vector-based RNAi has the advantage of being able to produce stable gene suppression through the insertion of the expression cassette into the genome of cells, which is an approach that solves both concerns mentioned above.

3.1.8. Strategies for achieving stable shRNA expression

When an expression cassette is inserted into the genome of a cell, it will not be lost and will be inherited to all offspring. Therefore, the expression of shRNA will remain in the entire cell population. Generally speaking, an expression cassette being genomically inserted is coded on the same plasmid as a construct expressing an enzyme, making the stably transfected cell resistant towards a selection marker. When this selection marker is later added to cell media, untransfected cells will die, leaving behind a population in which all cells express the desired shRNA. Thus, stable transfection will generate populations in which all cells are transfected, and gene suppression does not diminish over time.

There are several strategies for the stable delivery of exogenous DNA to cells [reviewed in 128]. The ones most used in RNAi strategies are random plasmid integration [105, 106] and

viral delivery [129-131]. In random plasmid integration, cells are transfected by any conventional method [reviewed in 128]. On rare occasions, transfected DNA is integrated into the genome of the cell in a random manner. These relatively rare cells will then be able to produce resistance towards a selective drug on a permanent basis, and therefore can be sorted out from the majority of cells not being stably transfected. As a consequence, the expression of the inserted transgene may vary between clones. Transgenes inserted in high density chromatin areas generally show a low expression, whereas insertion in low density areas suggests a higher expression. The plasmid might have been linearised in a manner that resulted in expression of only the resistance gene and no functional version of the transgene. Additionally, the number of copies being inserted may vary [reviewed in 128], which means that every clone should be controlled for plasmid integrity and expression.

Retroviral vectors have become an important tool for stable gene transfer both *in vitro* and *in vivo*. Retroviruses are constituted of RNA packed into a capsid and a membranous envelope. When the virus infects a cell, it transfers the RNA into the target cell, where it is reverse transcribed in the cytoplasm and integrated randomly into the genome [reviewed in 132]. The frequently used Moloney murine leukemia virus (MLV) retroviruses are not able to cross the nuclear membrane, and are thereby only able to integrate into the genome when the nucleus is disassembled during mitosis [133]. For this reason, MLV retroviruses are only able to infect dividing cells. Lentiviruses are a complex class of retroviruses which are able to also transduce non-dividing cells [reviewed in 134]. Both classes of retroviruses are mainly adapted in the same manner for transductions in laboratories. Genes encoding proteins that are necessary for the assembly of the envelope are transcribed from a packaging cell line, which is transiently transfected with the remainder of the viral genome containing an inserted transgene and a resistance gene. This leads to the release of functional viruses which codes for the insert, but not for the envelop proteins into the media. The media containing viral particles can then be used to transduce other cells. The lack of genes encoding the envelope proteins, as well as additional modifications in the viral genome, abolishes the possibility of transduced cells being able to produce viable viruses [reviewed in 132].

All of the strategies mentioned above have both positive and negative properties. Random plasmid integration is cheap and does not require any safety precautions, but is very ineffective. MLV retroviruses have a vastly increased efficiency and are easy to produce, although they are not able to transduce non-dividing cells, while lentiviral vectors can also

infect non-dividing cells [reviewed in 132]. Since viruses released from the packaging cells are theoretically capable of infecting all human cells, including those of the person performing the transduction, there are safety issues involved when transducing cells.

3.1.9. Conditional shRNA expression

The constitutive and ubiquitous expression of shRNAs suffers from limitations when it comes to the study of gene functions involving cell survival, growth and development. The reason for this is that the selection of transduced cells into a pure cell population typically takes several days from the time of the transduction. Within this time frame, the effects of the shRNA will have begun. This has prompted the construction of inducible gene silencing systems based on conditional RNAi expression. In such systems, the expression of shRNA will remain shut off until some sort of signal turns the expression on. In some systems, this expression is reversible.

There are several approaches for achieving conditional expression of shRNA.

Recombination

In the Cre/lox (Cyclization recombination/locus of X over P1) system, the shRNA is generally expressed from a polIII promoter. The complete shRNA, however, is separated from the promoter by a gene sequence flanked by two lox recombination sites. This site is recognised by the recombinase enzyme Cre, which will excise the DNA between the two lox sites. This recombination will result in transcription of the intact shRNA [135-138]. The advantage of this system is that there is no background expression of shRNA until Cre is present and that Cre only needs to be present for a short period to achieve permanent shRNA transcription. The Cre/lox system can also be used to shut down the expression of shRNA. Here, the promoter is placed between two lox sites, which will then be removed by recombination by the addition of Cre [138]. A similar system uses the yeast-derived recombinase FLP and its recognition site FRT [139]. The main disadvantage of both systems is that once the recombination has taken place, it is impossible to reverse it.

The Tet-inducible system

The most commonly used system for achieving conditional regulation of shRNA expression is based on Tetracycline (Tet) -inducible systems. There are three components necessary for the

functioning of this system: The constitutive expression of a Tet-repressor (TetR), a plasmid allowing the inducible expression of RNA through a promoter containing a TetR-binding sequence named Tet-operator (TetO) and the inducer Tet or one of its derivates, usually Doxycycline (Dox). TetR has a high affinity to the TetO, and thus binds tightly to DNA strands containing its sequence. The Tet-inducible system was originally developed in polII promoters and later adapted to polIII promoters in order to control the expression of shRNA [140]. An inducible polII promoter is minimal, meaning that sequences binding enhancers/activators necessary for transcription initiation are replaced. Instead, a Tet-Responsive element (TRE), which contains seven TetO sequences linked to a short stretch of sequences containing the PolII transcriptional start site of the CMV promoter, is added [141]. TetR is then fused to an activator, which recruits RNA polII to a minimal polII promoter and initiates transcription. In the Tet-Off system, the addition of Dox releases TetR and its activator from the TRE, turning the transcription off [141]. In Tet-On systems, the activator has four amino acids in the TetR moiety that are reversed, giving it the reverse phenotype. Here, Dox is required for the binding of TetR and the addition of Dox recruits the activator complex, thus turning gene expression on [142]. Of the two, Tet-Off is regarded as the most effective system [143].

Since PolIII promoters produce RNAs without 3' polyA tails and 5' end caps that are able to interact directly with exp5 and Dicer, they were the first promoters used for the stable expression of shRNAs. It is the U6 and H1 promoters which have been utilised for inducible expression via an adapted Tet-off system. Both the U6 and H1 promoters are extremely compact, and have three essential domains: the distal sequence element (DSE), the proximal sequence element (PSE) and the TATA box. The PSE and the TATA box are binding sites for the RNA polymerase itself, while the DSE binds the transactivators necessary for activation of the polymerase [144]. The activity of the promoters relies on a correct spacing between these elements and the transcription start, while the sequence between them is of lesser importance [145]. These intermediate sequences can be adapted to possess a TetO, one upstream and one downstream of the TATA box. TetO sequences placed upstream of the PSE and in the proximity of the DSE, severely impair transcription from the promoter [146]. When the inducer is absent, the TetR will bind to the TetO, thereby sterically preventing binding of the polIII. The addition of the inducer leads to a conformational change of TetR into a configuration not able to bind to the TetO. This allows polIII to bind and transcription to take place (Figure 3). There are two TetOs that have been used extensively in PolIII-inducible

promoters, the TetO1 and TetO2 [147]. Several combinations of the two TetOs in US, DS or both positions have been described for both the H1 and U6 promoters [140, 148-151]. The general conclusion is that one operator alone is not sufficient for achieving tight regulation of the promoter in an uninduced state. In addition, it seems as though the TetO2 operator is the most effective in terms of suppression [149]. In Paper I, we compared the tightness and efficiency in a number of previously described inducible U6 and H1 promoters in several different human cell lines, in addition to describing an H1 promoter containing two TetO2s for the first time [118]. It was concluded that overall the newly designed promoter performed better than the remaining promoters, including the similar U6 promoter containing two type 2 TetOs. The H12O2 promoter presented a very low background in an uninduced state and up to 90% gene suppression after the addition of the inducer Dox.

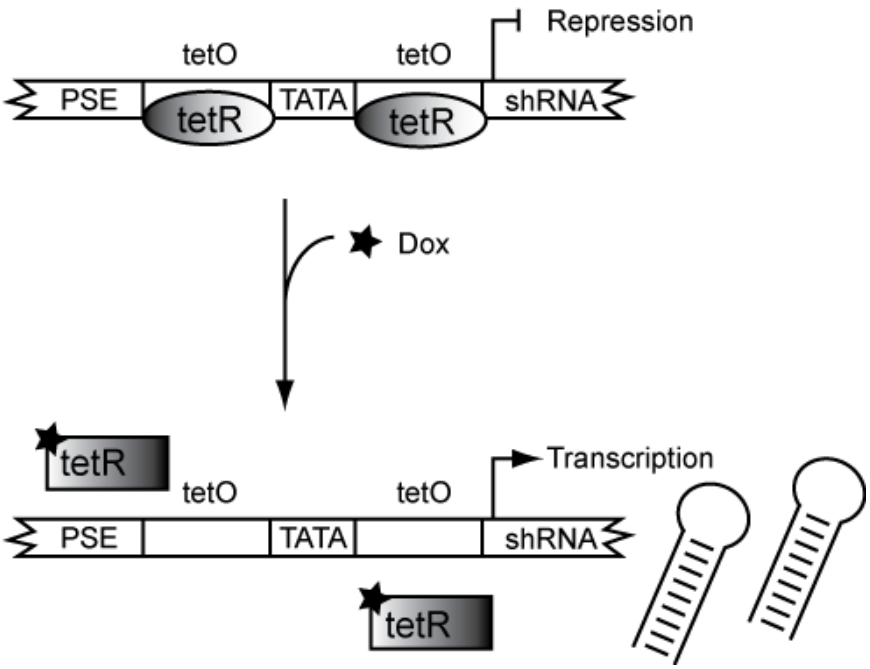

Figure 3: Conditional expression of shRNA from a PolIII promoter. When the inducer Dox is absent, TetR is bound to the TetO, thereby sterically obstructing the binding of polIII to the promoter. Dox induces a conformational change of the TetR into a form with a low affinity to the TetO. This leads to the dissociation of TetR from the promoter, allowing PolIII to transcribe the shRNA.

The U6 RNA polIII promoter has also been modified to an inducible version following the same principles as polII promoters. Here, the DSE was replaced by a TRE, and TetR fused to the activator normally binding to the DSE [152, 153].

Following the discovery of shRNA-mir, which are transcribed by polII, the original Tet-inducible polII promoters returned to the field of RNAi [112, 154]. Here, it was shown that these vectors presented tight regulation, high penetrance and high efficiency, even in single copy levels of the inserted cassette. Although the Tet-Off system has been previously regarded as superior to the Tet-On system, the transcriptional activator originating from the Tet-On system has been improved using viral evolution as a tool to better adapt it from its original host, *E. coli*, to mammalian systems [155]. The resulting activator has been utilised in inducible shRNA systems to yield improved gene suppression [156]. Two shRNAs targeting different mRNAs from the same promoter were also expressed, thereby acquiring the ability to knockdown two genes simultaneously.

Ecdysone

The ecdysone system is similar to the Tet system in its principle of function. The ecdysone receptor is fused to an activator, and binds to the Gal4-binding sequences. It uses ecdysone-analogs as inducers, and the addition of the inducer releases the receptor along with its activator from the minimal promoter, thereby turning transcription off. The system is functional in both polII and polIII promoters [157, 158].The vector integration site strongly influences expression, which requires careful selection of effective clones. Additionally, ecdysone is a steroid prohormone, and activation of its receptor could trigger endogenous gene expression in target cells [reviewed in 159].

3.1.10. Off-target effects

Off-target effects of RNAi are defined as consequences that arise from any effect other than the intended gene suppression. The off-target effects are divided into two main categories: specific and non-specific.

Specific off-target effects

Specific off-target effects arise as a result of a full or partial complementarity between the passenger or guide strand towards any unintended target. In order to achieve target cleavage through the action of Ago, it is necessary to have perfect complementarity extending through at least 13 nt of the strand mounted in the RISC [160]. A BLAST search of the transcriptome of the species investigated will quickly expose siRNAs harbouring unspecific complementarity of this degree, allowing for those siRNAs to be discarded. As a consequence

of this, there is more concern regarding partial mismatches, particularly when including nt 2 – 8 counted from the 5' end of the guide strand. These nucleotides form the seed sequence that allows the guide strand of the siRNA to function as a miRNA [61]. It is has been shown that the off-target effects of siRNAs are strongly biased to occur from ~7 nt complementarity between the "seed sequence" of the guide strand and the 3' UTR of the unintended targets and that the unintended target sequences are often conserved in several mRNAs [161]. The magnitude of the regulation of transcripts targeted as siRNA off-targets is generally less than twofold, which is similar to that of miRNAs [161, 162]. This suggests that siRNAs are prone to target unintended miRNA seed sequences of ~7 nt. Because of this, there could be a discussion as to whether the less than twofold regulation realised by off-target siRNAs has any biological relevance. Adverse unintended phenotypes arising from such off-target effects have been described [161, 163]. There is no algorithm that can significantly eliminate 7-8 nt matches in the transcriptome of a species; therefore, some off-target effects are likely to occur when using any form of RNAi-mediated gene silencing, and there are developed algorithms that try to minimize off-target effects [164-166].

Non-specific target effects

Non-specific target effects are effects that do not result from direct interaction between an RNAi construct and an mRNA target. This includes immune-responses as a result of defence mechanisms triggered against exogenous RNA, any effect related to the delivery vehicle in addition to any effects arising from saturation of the miRNA pathway.

Initially, it was thought that RNA duplexes shorter than 30 nt were small enough to evade stimulation of the interferon response [9]. However, this assumption has been questioned, as activation of immune responses resulting from the introduction of small siRNAs has been described [167, 168]. The 13 Toll-like receptors (TLRs) are a class of proteins that recognises signs of infection and activates the innate immune system. Of these, the ones most relevant for activation as a result of RNAi are TLR3, which recognises the duplex form of siRNA, and TLRs 7 and 8, both of which are activated either by the duplex or its corresponding single strand [169-171]. TLR receptors are often concentrated in endosomes, thus transfection methods utilising cationic lipids enhance the immune response [171]. In contrast, ShRNAs are less likely to induce an immune response since they are presented from a DNA plasmid, thereby avoiding the dsRNA activation of TLR3. In addition, the 5' ends of shRNA, which

are produced endogenously, seem to be less immunogenic than corresponding 5' ends of exogenous siRNAs [101, 172]. Chemical modifications of siRNAs can reduce their immunogenicity [reviewed in 173].

RNAi uses the miRNA pathway in order to exert its effect. As a result, any exogenous RNAi effector competes with endogenous miRNAs for access to the miRNA machinery. ShRNAs are entering the miRNA pathway at a higher level than siRNAs (Figure 2), and are therefore competing with miRNAs in more steps than siRNAs. The most rate-limiting step in the miRNA pathway seems to be the export of pre-miRNA from the nucleus to the cytoplasm by exp5, and shRNA expression is shown to interfere with endogenous miRNAs as a result of saturation from this step [174]. Also, the sustained expression of shRNAs stably delivered to liver cells in adult mice has been revealed to severely downregulate the expression of endogenous miRNA [175]. This was demonstrated to be a result of saturation due to Exportin5 activity as well as the activity of Ago2 in RISC [176]. By expressing the shRNAs from a miR backbone, the toxicity of shRNAs has been shown to be diminished [177]. One group has proposed that siRNAs are unable to saturate the miRNA pathway by showing that the expression of three different miRNAs in liver cells did not change when siRNA-mediated gene suppression was utilised [178]. Nonetheless, it has also been shown that both shRNAs and siRNAs compete not only with each other, but with endogenous miRNAs for incorporation to RISC [179]. In addition, it has been proposed that transfected small RNAs have a global effect on genes under the control of endogenous miRNAs [180]. Here, the results from 151 published experiments based on the transfection of either siRNA or miRNA into cells in culture were analysed. A statistical analysis of the published mRNA profiling or protein mass spectrometry revealed that in the majority of the experiments, endogenous mRNAs containing targets of miRNAs with high endogenous expression were upregulated as a result of competition from exogenous mi/siRNAs.

3.2. Neuroblastoma

3.2.1. Embryonal development and neoplasms

The development of an embryo begins with a single fertilised egg. Countless cell divisions of this perfect stem cell eventually lead to the generation of organs and every other component of the organism. After the first few cell divisions of the zygote, it is already apparent that cell-cell interactions start to decide the fate of the daughter cells [181]. From this point on, the development of the embryo is a process comprised of strictly controlled proliferation and differentiation signals. In the early embryo, there is a majority of strong mitogenic signals that limit the ability of cells to exit the cell cycle. During embryonal development, changes in the concentration of key regulatory signals promote the exit of the cell cycle and the onset of differentiation for the targeted cells. In normal development, the signals indicating proliferation or differentiation are under tight control, though in tumour growth this control is lost. If a strong mitogenic signal expressed transiently during development achieves constitutive expression, it acts as an oncogene. Alternatively, if a protein involved in signalling pathways leading to cell cycle exit and differentiation loses its function, it may no longer act as a tumour suppressor. Tumours originating from tissues that normally proliferate only in a developing embryo are known as embryonal tumours. If the tumour arises from primitive precursor cells, it is given the suffix blastoma. These tumours can arise in various parts of the body and include medulloblastoma in the brain, neuroblastoma in the sympathetic nervous system, retinoblastoma in the eye, Wilms' tumour (nephroblastoma) in the kidney, hepatoblastoma in the liver and embryonal rhabdomyosarcoma in soft tissue. These tumours are very rare after childhood, and most commonly occur during the first years of life.

3.2.2. Development of the sympathetic nervous system

On day 19 of the embryo, the neural plate starts to form on the ectoderm of the embryo. The edge of the neural plate is defined by neural crest precursors, and as the neural plate folds in on itself, the neural crest precursors from each outer edge join and form the dorsal part of the newly developed neural tube. The neural tube is developing to form the central nervous system, while the now mature neural crest cells start to migrate from the dorsal part of the neural tube into several areas of the embryo [reviewed in 182]. Neural crest cells are the origin of several cell lineages: cranial (e.g. forming facial bones), vagal and sacral (e.g. parasympathetic neurons), cardiac (contributing to the development of the heart) and trunk

neural crest-derived cells (e.g. melanocytes and the sympathetic nervous system, including the adrenal medulla) [183].

Trunk neural cells destined to form the sympathetic neural system are of the sympathoadrenal lineage. These migrating sympathetic neuroblasts arrange themselves alongside both sides of the neural tube before they start forming chains of sympathetic ganglia. As internal organs are being developed, sympathetic fibres formed by axonal outgrowth from the ganglia reach out to and connect them to the sympathetic nervous system in a process known as innervation [183].

The differentiation from neural crest precursors at the neural plate to non-dividing neural cells in the sympathetic nervous system requires involvement of a large amount of signalling pathways. The formation of both the neural plate and neural crest precursors are dependent on expression levels of the bone morphogenetic protein (BMP) pathway, the fibroblast growth factor (FGF) pathway and the Wnt signalling pathway [reviewed in 184]. As the neural tube is formed, the neural crest precursors mature to neural crest cells before going through an epithelial to mesenchymal transition, in which they shed from the neural tube and start migrating. This process is largely governed by Wnt signalling, and leads to expression of a set of genes known as neural crest specifiers. As the migrating neural crest cells reach their final target, they start differentiating, while many of the neural crest specifiers are downregulated [reviewed in 185]. Neural crest cells destined for the sympathoadrenal lineage demonstrate increased BMP signalling [186, 187]. As they further mature, they start expressing enzymes required for the synthesis of noradrenalin, e.g. tyrosine hydroxylase [188, 189], and as they acquire a neuronal fate they start expressing neurofilaments, neuron-specific tubulin and other neuronal markers [187, 190, 191]. After the acquisition of neuronal traits, the sympathoadrenal cells undergo a second migration step away from the dorsal aorta to form the secondary sympathetic ganglia, the prevertebral ganglia and the adrenal medulla in a process that probably involves FGF and NGF signalling [192]. This differentiation process also involves a myriad of transcription factors required to be expressed at the correct time and in the correct amount [reviewed in 192]. Among these are *MYCN*, which is expressed in migrating neural crest cells [193]. It seems as if *MYCN* is necessary for keeping the cell in a migrating state, in addition to being an important signal for committing the neural crest cells where it is expressed towards the sympathoadrenal lineage [193]. Like many of the neural

crest specifiers, *MYCN* expression is turned off as the neural crest cells reach their final destination and differentiate towards their ultimate phenotype [194].

As the neural crest cells differentiate and spread throughout the embryo in order to form the sympathetic nervous system, the number of cells in each differential stage must be under strict control. Developmental apoptosis is crucially important in this matter, and progenitor cells of all stages are prone to enter apoptosis if given the appropriate signals [reviewed in 195]. For instance, it has been proposed that during normal development neuronal progenitor cells compete with each other for access to NGF, and as NGF becomes limited, the losers will enter apoptosis via a pathway that includes the tumour suppressor KIF1Bβ [196].

3.2.3. From neuroblasts to neuroblastic tumours

Being an embryonal tumour, neuroblastoma is regarded as a consequence of the disordered normal development of cells from the sympathoadrenal lineage of neural crest cells [197]. As described above, the cells of the sympathetic nervous system originate from neural crest cells, going through an epithelial to mesenchymal transition, before migrating as single cells until they reach their destination. A second migration step is then initiated in order to complete the sympathetic nervous system. Migrating mesenchymal cell types are often related to cancer [reviewed in 198], and it is not difficult to imagine that the failure of neuroblasts to exit the mesenchymal mode and alternatively returning to it, could result in the development of malignant neoplasms.

Neuroblastic tumours (i.e. neoplasms of the sympathoadrenal lineage) can arise anywhere in the sympathetic nervous system, although the majority of primary tumours appear in the abdomen, with a major site being the adrenal medulla. Other common sites include the neck, chest and pelvis [199]. Neuroblastic tumours can be divided into three categories based on their morphologic features: Ganglioneuroma, ganglioneuroblastoma and neuroblastoma [200]. Ganglioneuromas appear as clusters of mature neurons surrounded by a stroma of Schwann cells, while neuroblastoma cells appear as undifferentiated tumours consisting of small, round neuroblasts. Ganglioneuroblastomas are the intermediate of the two [200]. Ganglioneuromas are well encapsulated benign tumours not capable of invading or metastasizing. Ganglioneuroblastomas are generally benign, while neuroblastoma often appear as a very aggressive cancer which commonly metastasizes [200].

3.2.4. Neuroblastoma – the disease

Neuroblastoma is not a common disease. In Sweden, the incidence over a period of 27 years was 1 case/100,000 children below the age of 15 years [201]. Despite being rare, neuroblastoma accounts for 7-10% of all diagnosed childhood cancers and 15% of all childhood cancer deaths [199, 202]. Neuroblastoma is divided into risk groups based on criteria such as age of the patient at diagnosis, International Neuroblastoma Risk Group (INRG) tumour stage and MYCN copy number (Table 1) [203, 204]. Patients in the low and intermediate risk group show fairly good prognosis, while the event-free survival (EFS) rate for patients diagnosed with high risk neuroblastoma is less than 50% [203].

Table 1: International Neuroblastoma Risk Group (INRG) Consensus Pretreatment Classification schema. Pretreatment risk group H has two entries. Blank field = "any"; DI, diploid (DNA index ≤ 1.0); HDI, hyperdiploid (DNA index > 1.0 and includes near-triploid and near-tetraploid tumours); very low risk (A-C, 5-year EFS > 85%); low risk (D-F, 5-year EFS > 75% to ≤ 85%); intermediate (intermed) risk (G-J, 5-year EFS ≥ 50% to ≤ 75%); high risk (K-R, 5-year EFS < 50%). GN, ganglioneuroma; GNB, ganglioneuroblastoma; Amp, amplified; NA, not amplified; L1, localised tumour confined to one body compartment and with absence of image-defined risk factors (IDRFs); L2, locoregional tumour with presence of one or more IDRFs; M, distant metastatic disease (except stage MS); MS, metastatic disease confined to skin, liver and/or bone marrow in children < 18 months of age; EFS, event-free survival [adapted from 203].

INRG	*Age (Months)*	*Histologic Category*	*Grade of Tumour Differentiation*	*MYCN*	*11q Aberration*	*Ploidy*	*Pretreatment Risk Group*
L1/L2		GN maturing; GNB intermixed					A Very low
L1		Any, except GN maturing or GNB intermixed		NA			B Very Low
				Amp			K High
L2	< 18	Any, except GN maturing or GNB intermixed		NA	No		D Low
					Yes		G Intermed
	≥ 18	GNB nodular; neuroblastoma	Differentiating	NA	No		E Low
					Yes		H Intermed
			Poorly differentiated or undifferentiated	NA			
				Amp			N High
M	< 18			NA		HDI	F Low
	< 12			NA		DI	I Intermed
	12 to < 18			NA		DI	J Intermed
	< 18			Amp			O High
	≥ 18						P High
MS	< 18			NA	No		C Very low
					Yes		Q High
				Amp			R High

Neuroblastoma has a high rate of spontaneous regression. The total amount of neuroblastomas that are detected clinically and regress without any treatment is approximately 5-10% [202]. This clinical phenotype (INRG MS stage, risk group C) is mainly seen in infants below the age of 18 months, and is presented with small localised primary tumours with metastases in the liver, skin or bone marrow. It is assumed that the cause of these naturally regressing tumours is the delayed activation of differentiation or apoptosis pathways [202]. In fact, it is shown that microscopic neuroblastic nodules occur uniformly in all fetuses, peaking between 17 and 20 weeks of gestation, and then gradually regressing by the time of birth [205, 206]. Additionally, neuroblastomas within INRG C rarely, if ever, evolve into any of its malignant forms, which indicates that the malignant forms of neuroblastoma affecting children older than 18 months are of a distinct type [202]. Neuroblastoma often contains a range of genomic

aberrations. Neuroblastomas that are prone to spontaneous regression are often characterised by a mitotic dysfunction, resulting in a hyperploid or near triploid phenotype with few structural abnormalities [207, 208]. On the other hand, neuroblastomas in patients one year or older are often characterised by a near-diploid or near-tetraploid karyotype in addition to numeral structural abnormalities [207, 208].

The most frequent aberration in neuroblastoma is gain of chromosome 17q, which is found to be present in a majority of tumours [209, 210]. In neuroblastoma, it seems as if chromosome 17q gain is a result of translocation from several other chromosomes [211]. A frequent aberration is an 11q loss of heterozygosity (LOH), which is a prognostic marker for poor outcome in neuroblastoma without *MYCN* amplification [212, 213]. Another frequent allelic loss occurs on chromosome 1p [214]. Deletions in chromosome 1 are also found more frequently in patients with advanced disease, but LOH on 11q and 1p rarely occurs in the same tumours [202, 215]. Many tumours with 1p LOH also contain an amplification of the 2p24 locus, which is the site of the proto-oncogene *MYCN* [216, 217]. *MYCN* amplification rarely exists without 1p LOH, whereas not all cases of 1p LOH contain MYCN amplification, an observation that suggests that 1p LOH is a prerequisite for *MYCN* amplification [202]. Patients with 1p LOH and eventual *MYCN* amplification are typically 1-5 years old with advanced stage, rapidly progressive and often fatal neuroblastoma, while patients with 11q LOH neuroblastoma are often older, with an advanced stage of disease that slowly progresses and is often fatal [202].

3.2.5. Expression of MYCN in neuroblastoma

MYCN is a member of the MYC gene family of transcription factors, all of which initiate transcription in similar ways. The MYCN transcription factors form an active complex when heterodimerised with its partner MAX [218, 219]. Both MYCN and MAX contain DNA binding motifs, and the active protein complex can initiate transcription when bound to its DNA binding sites. The most frequently targeted DNA sequence is the E-box motif (5'-CAC(A/G)TG) [220-222]. In addition, H4-K3 methylation of a promoter region has been shown to be an indicator for MYCN/MAX binding [223]. MAX can also dimerise with MAD, and the resulting complex also has an affinity towards E-boxes, but functions as a transcriptional repressor instead of an activator [224-226]. MYCN is also shown to be involved in transcriptional repression in neuroblastoma [227]. Here, MYCN has been

demonstrated to act as a bridge between the DNA binding protein Sp1 and the repressor histone deacetylase (HDAC), resulting in transcriptional repression of tissue transglutaminase (TG2).

The amplification of a gene is a result of chromosomal rearrangements leading to an increased copy number of the amplified gene. Amplification of *MYCN* was first observed in 1983, when it was shown that *MYCN* could be amplified up to 140 fold [228]. It seems as if all copies of the amplified gene contribute to the expression of *MYCN* and that the increased gene number is the reason for the high *MYCN* mRNA expression in *MYCN* amplified (MNA) neuroblastoma cells [217, 229]. Generally speaking, it seems that levels of mRNA resulting from *MYCN* amplifications reach a 40-60 fold increase in comparison to single copy cell lines with a low expression of *MYCN*, and that the increase in mRNA level is not always proportional to the number of gene copies [230].

As described above, *MYCN* is naturally expressed in neural crest cells of the sympathoadrenal lineage, which is assumed to be important for maintaining the cells in a migrating state [193]. In cancer terminology, mesenchymal cells that are able to migrate are closely related to a malignant phenotype, so it is therefore not surprising that a protein devoted to maintaining this phenotype is a potent oncogene. Indeed, *MYCN* amplification is one of the most prominent prognostic indicators for a bad outcome in neuroblastoma [203]. *MYCN* has been shown to be causally involved in tumourigenesis and tumour progression, as transgene mice expressing *MYCN* in the neuroectoderm develop neuroblastoma several months after birth [231].

3.2.6. MYCN targets

The exact mechanism *MYCN* uses to mediate its oncogenic effect is still largely unknown, but some pathways are emerging as likely candidates for executing the malignant potential of *MYCN*. Here follows a brief description of a few direct targets of MYCN:

MYCN expression has been shown to increase proliferation by shortening the time used to progress through the cell cycle [232]. E2F1-3 are transcription factors which mainly regulate the expression of numerous genes necessary for the S-phase of the cell cycle such as *thymidine kinase*, *dihydrofolate reductase* (*DHFR*), *DNA Pol*α and *cell division cycle 6* (*cdc6*). Thus, active E2F1-3 pushes the cell into the S-phase [reviewed in 233].When E2F1-3

are bound by hypophosphorylated retinoblastoma protein (RB), it is inactive, and the cell remains in the G1 phase. Phosphorylation of RB through the cyclin D/CDK4/6 complex removes RB from the RB/E2F complex, thereby releasing active E2F. Several possible MYCN targets engaged in the cell cycle progression are probably functioning through regulation of the E2F1-3 transcription factors [reviewed in 234]. *E2F1* has been demonstrated to be a transcriptional target of MYCC, and there are indications that MYCN also regulates *E2F1* expression, while at the same time E2F1-3 activates the expression of *MYCN* itself [reviewed in 234].

MYCN is known to increase the susceptibility of cells entering apoptosis following cellular stress such as DNA damage, survival factor withdrawal, substrate detachment and hypoxia [reviewed in 235]. p53 is known as the guardian of the genome, and is involved in DNA repair and/or initiation of apoptosis as a result of extensive DNA damage. It has recently been reported that p53 is a direct transcriptional target of MYCN [236], and this finding suggests a mechanism for the *MYCN*-driven p53-dependent apoptosis necessary for achieving control of rapidly dividing neuroblasts in normal development. However, MYCN is also a transcriptional activator of *MDM2*, which is a negative regulator of p53 [237].

Bmi1 is a transcriptional repressor required for the self-renewal of stem cells of the central- and peripheral nervous system [238], and are highly expressed in a vast majority of primary neuroblastoma tumours regardless of *MYCN* status [239]. Ochiai *et al.* have shown that its promoter contains E-box sequences and that as a result, *Bmi1* is a direct transcriptional target of MYCN [240]. In addition, they have shown that Bmi1 directly downregulates several tumour suppressors in neuroblastoma, among them *KIF1Bβ* and *TSLC1*, both of which have been correlated to a bad prognosis when downregulated [241, 242]. KIF1Bβ is an important signal for apoptosis of neural progenitor cells when NGF access is reduced [196]. The exact function of TSCL1 in neuroblastoma is not known, but it is suspected that it demonstrates an antiproliferative and/or proapoptotic activity [241].

3.2.7. Targeted MYCN downregulation

Given that *MYCN* is a strong oncogene being expressed naturally primarily in embryonal life only, it has long been regarded as an ideal candidate for targeted therapy [243]. The first approaches for a targeted reduction of *MYCN* expression were conducted by using standard antisense technology [244]. Here, it was found that repression of *MYCN* expression leads to

reduced proliferation and differentiation towards a more neuronal phenotype in a MNA neuroblastoma cell line. Peptide nucleic acids (PNA) are nucleic acid analogues in which the sugar backbone is replaced by a synthetic peptide backbone. The resulting mimic is uncharged, and thus binds with greater affinity to complementary nucleic acids (both DNA and RNA) than nucleic acids. Because there are no naturally occurring PNAs, they are not targets of enzymatic degradation [reviewed in 245]. PNAs have successfully been used for targeted downregulation of both *MYCN* mRNA [246, 247] and the *MYCN* gene at the DNA level [248]. Although fairly effective and selective, PNAs are expensive to design and synthesise. As RNAi has evolved as a superior tool for targeted gene suppression, transient siRNA-based *MYCN*-knockdown has also been used to show reduced cell growth, induced differentiation and induced apoptosis in MNA neuroblastoma cells [249, 250].

The SHEP Tet21N system has been evolved for mainly studying *MYCN*-related cell cycle effects [232]. Neuroblastoma cell lines established in culture appear to be heterogenous, and can be divided into three different subgroups: neuroblastic (N), substrate adherent (S) and intermediate (I) cell types. N-type cells appear as being small, rounded and loosely attached, with numerous neurite-like processes. The S-type is larger, flatter and more strongly substrate adherent, and appears to be fibroblastic/epithelial and do not express any neuronal markers. The I-type appears as a morphological intermediate between the N- and S- types, and can be induced to differentiate towards both subgroups [251, 252]. In the SHEP Tet21N system, a *MYCN* gene is stably transfected into an S-type clone derived from the I-type SKNSH neuroblastoma cell line [232]. In this Tet-Off system, the expression of *MYCN* can be turned off by adding the inducer tet (confer with Chapter 3.1.9 above).

3.2.8. MiRNA in neuroblastoma

MiRNAs are regarded as vital contributors to the pathogenesis of a wide array of tumours [92, 93]. The first expression profiling study indicated that miRNAs were differentially expressed in various genomic subtypes of neuroblastoma [253]. It has now been established that MNA and other chromosomal imbalances lead to vast dysregulation of the miRNA expression in primary neuroblastoma tumours [reviewed in 254]. As mentioned above, MYCN is a transcription factor that mainly induces the transcription of genes with E-boxes in the proximity of their promoter. MiRNAs are also expressed from RNA polII promoters, and MYCN has been shown to regulate the expression of miRNAs [255]. MiRNAs also have the

potential to act as both tumour suppressors and oncogenes in neuroblastoma [reviewed in 254].

An example of a miRNA acting as a tumour suppressor is *miR-34a*, which is frequently downregulated in primary neuroblastomas [256]. *MiR-34a* is located in a region of chromosome 1p36 that is often deleted in neuroblastoma, and is shown to have an anti-proliferative effect when overexpressed in neuroblastoma cell lines [256]. Oncogenes found to be targets of *miR-34a* are the transcription factor *E2F3* and *MYCN* [256, 257].

The *miR-17* family of miRNAs are expressed from three different miRNA clusters located on three different chromosomes: The *17-5p-92* (*miR 17, -18a, -19a, -20a, -19b1 and -92a1*), the *miR 106a-363* (*miR 106a, -18b, -20b, -19b2, -92a2, -363*) and the *miR 106b-25* (*miR 106b*, *-93, -25*) [258]. The *miR-17* family is shown to be vital for development of the embryo and initiating of the differentiation of pluripotent stem cells [259]. All three clusters harbour E-box motifs in its proximity [260], and all clusters are shown to be directly regulated by MYCN [261, 262]. Several members of the *miR-17* family, especially those of the *17-5p-92* cluster, are shown to act as potent oncogenes in neuroblastoma cells [261, 262]. This is a clear indication that at least parts of the oncogenic effect of *MYCN* are mediated directly through the transcriptional activation of miRNAs. MYCN is primarily a transcriptional activator, and as described above, only rarely acts as a transcriptional repressor. So far, no miRNA has been shown to be directly downregulated by MYCN. However, miRNAs can be inversely correlated to *MYCN* expression as a result of indirect mechanisms [253].

In paper III, we document for the first time that *miR-92b* might be activated by MYCN. We also suggest that most miRNAs inversely correlated to MYCN are probably involved in differentiation.

3.2.9. *DKK3* and neuroblastoma

In neural crest development, the BMP-, FGF- and Wnt pathways are the main regulators of neurulation and the subsequent formation of neural crest cells [184]. The disturbance of these early developmental pathways has been shown to be involved in several other forms of cancers [263-265], and these pathways are therefore interesting to study in neuroblastoma as well. In the canonical Wnt pathway, a Wnt protein binds to its trans-membranous receptor frizzled and the co-receptor low-density lipoprotein receptor-related protein 5 (LRP5)/LRP6.

This leads to activation of an intracellular pathway that ultimately leads to the accumulation of the protein β-catenin in the nucleus where it activates the T-cell factor/lymphocyte enhancer factor (TCF/Lef) family of transcription factors. Activation of the canonical Wnt pathway regulates a wide array of biological effects, including activation of cell cycle progression and proliferation, inhibition of apoptosis, regulation of embryonic development, cell differentiation, cell growth and cell migration [reviewed in 266]. Wnt signalling can also activate independent non-canonical pathways, the two most described being: 1. The Wnt/Ca^{2+} pathway, which activates the protein kinase C and the Ca^{2+}- calmodulin dependent protein kinase II, 2. the cytoskeleton pathway, which regulates the organisation and formation of the cytoskeleton and planar cell polarity [reviewed in 267]. There are also several other less described and characterised non-canonical Wnt pathways [268].

The family of dickkopf proteins (DKK1-4 and Soggy) is a group of secreted glycoproteins primarily regarded as inhibitors of the Wnt pathway [269]. While DKK1 and DKK2 inactivate Wnt signalling by obstructing the binding between LRP5/6 and Wnt ligands, DKK3 is not able to interact with LRPR6 [270]. DKK3 has been revealed to have distinct roles in the modulation of the Wnt pathway, depending on the cell types being studied. DKK3 increases Wnt signalling in mouse glia cells and HEK293 [271], but inhibits Wnt signalling in pheochromocytoma cells from rat (PC12) [272] and osteocarcinoma (Saos-2) cells [273]. Since DKK3 does not bind to the LRPR5/6 receptors, little is known about the molecular basis for DKK3-dependent Wnt inhibition. DKK3 does however bind to the membrane bound Wnt inhibitor Kremen, and Nakamura and Hackam have proposed that DKK3 potentiates Wnt signalling by facilitating a relocation of Kremen from the cell membrane by endocytosis [270].

DKK3 is an established tumour suppressor shown to be downregulated in a range of tumour-derived cells, e.g. Saos-2, hepatoblastoma, acute lymphoblastic leukaemia and non-small-cell lung cancer [reviewed in 269]. The downregulation of *DKK3* is often a result of hypermethylation of the *DKK3* promoter [274, 275], whereas the overexpression of *DKK3* has been demonstrated to suppress tumour growth of, e.g. Saos-2 [273], prostate cancer [274, 276] and neuroblastoma [277].

It has been shown that there is an inverse correlation between the expression of *MYCN* and the Wnt antagonists *DKK1* and *DKK3* in neuroblastoma [278, 279]. In particular, the

correlation between *DKK3* and *MYCN* has been proven to be a strong one by Koppen *et al.* [277]. Here, it was documented that *DKK3* is a marker for neuroblastic tumour maturation and that it is indirectly downregulated by MYCN. In paper IV, we show that *DKK3* is repressed by *miR-92a*, *miR-92b* and *let-7e*, all of which are MYCN–regulated miRNAs [261, Paper III].

4. Aims

Neuroblastoma with *MYCN* amplification represents the most aggressive form of this disease. Although *MYCN* has been regarded as the most prominent prognostic indicator for bad outcomes, the role of *MYCN* in neuroblastoma tumourigenesis remains largely unknown. The silencing of *MYCN* and its downstream targets are attractive goals for targeted therapy, but very little is known about the long-term effects of *MYCN* silencing of MNA neuroblastomas. The main aim of this study was to establish an efficient knockdown of *MYCN* in MNA neuroblastoma cell lines in a stable and inducible manner and to use these cell lines to obtain further knowledge of *MYCN* and its downstream targets, including miRNAs.

Paper I

Evaluate available options for inducible expression of shRNA. Design and establish an inducible promoter system that is tight when uninduced, and which has a high expression of shRNA when induced. In addition, the vector system should allow easy incorporation of any desired shRNA.

Paper II

Establish MNA cell lines with stably integrated shRNA expression targeting *MYCN* under inducible control using the promoter system designed and developed in Paper I.

Paper III

Use shRNA to silence *MYCN* and study the differential expression of miRNA expression both before and after knockdown. Investigate the biological effect of the miRNA with the most pronounced change in expression.

Paper IV

Previous data published by others has suggested that the possible tumour suppressor *DKK3* was regulated by MYCN in an indirect manner. We aimed to use the miRNA profiling data obtained in Paper III to search for MYCN-regulated miRNAs with predicted targets in the *DKK3* 3' UTR, as well as investigating whether MYCN regulates *DKK3* through miRNAs.

5. Discussion

5.1. Papers I and II

5.1.1. Brief description of the studies

In Paper I, we compared the properties of six polIII promoters with tet-inducible expression. Four of the promoters were previously described by others [140, 148, 149, 280], while two versions of the H1 promoter were novel designs. The tightness and expression efficiency of the promoters in an induced and uninduced state were compared using a luciferase reporter system. Here, cells containing a stable expression of TetR were transiently cotransfected with three plasmids: a plasmid with a constitutive expression of the firefly luciferase reporter, a plasmid with a constitutive expression of β-galactosidase for normalisation and a plasmid expressing an anti-luciferase shRNA expressed from the promoter being evaluated. Luciferase activities were measured and normalised against β-galactosidase expression. The experiments were performed with the absence of the inducer Dox for an evaluation of tightness, as well as in the presence of Dox for an evaluation of transcription efficiency.

For both U6 and H1 promoters, constructs containing only one tet operator in the promoter were unacceptably leaky. Overall, we found the novel H12O2 promoter to perform slightly better than the similar U62O2 promoter when directly compared.

In Paper II, we introduced a shRNA (aMN-1658) that specifically downregulated the MYCN protein expression ~90% without any observations of off-target effects. This shRNA was inserted into the H12O2 US/DS inducible promoter cassette designed in Paper I before being stably transduced to the MNA neuroblastoma cell lines Kelly and SK-N-BE(2) using an MLV retroviral delivery system. The resulting RV-1658 cell lines appeared with a similar morphology to control cells in an uninduced state. When induced with 1 µg/ml dox, MYCN expression was efficiently downregulated, the cells differentiated towards neuron-like cells, entered G1 arrest and showed a significantly reduced clonogenic growth.

5.1.2. Discussion

The novel H12O2 promoter designed by us was developed by introducing a second TetO2 downstream of the TATA-box in a commercially available pENTRH1-O2 plasmid

(Invitrogen). The sequence between the TATA-box and the transcription start (25 bp) can be altered without affecting the transcription efficiency of the H1 promoter as long as the spacing between the two remains the same [281]. The TetO2 sequence of 19 bp ends with the nucleotides AGA, which is a part of the BglII restriction site (AGATCT). The design of the H12O2 promoter allows the positioning of a stuffer sequence directly downstream of the transcription start site. This stuffer can be removed by BglII restriction enzymes and replaced by a properly designed shRNA sequence targeting a desired gene.

The conditional expression of shRNAs is feasible from both polIII promoters and polII promoters. As described above, it seems that conditional expression from polII promoters may provide some advantages over polIII promoters such as less off-target effects [177], tighter regulation [112, 154] and the possibility of expressing several shRNAs in a polycistronic manner [156]. Even so, shRNAs expressed from polIII promoters seem more potent than similar shRNAs expressed from polII promoters, thus allowing a higher efficiency when the silencing of highly expressed targets is desired [124]. In MNA neuroblastoma cell lines, the expression of *MYCN* mRNAs is generally 40-60 fold higher than in single copy cells [230]. As a result of this, the potency of the shRNAs is of great importance when *MYCN* silencing in MNA cell lines is considered.

In Paper II, we have described a system for the conditional and stable expression of shRNAs targeting *MYCN* in MNA neuroblastoma cell lines. One of the main reasons for developing this cell line was the desire to specifically study the long-term effect of *MYCN* knockdown in MNA cell lines. The SHEP Tet21N system is perhaps the most widely used cell system for studying the effect of *MYCN* silencing in neuroblastoma [232]. Here, *MYCN* is introduced to an S-type neuroblastoma cell line normally not expressing *MYCN*. When S-type neuroblastoma cell lines are exposed to the vitamin A metabolite retinoic acid (RA), they enter apoptosis, while N-type cells generally differentiate towards a more neuronal phenotype [282]. As a consequence, this system is not suitable for studying the effect of *MYCN* on differentiation in neuroblastoma cell lines. The SHEP Tet21N system however has proven especially valuable for determining the effect of *MYCN* on proliferation and apoptosis.

Traditional antisense technology has previously been used for the stable silencing of *MYCN* in non-MNA neuroblastoma cell lines with a high MYCN expression [283]. Still, antisense technology is not efficient enough to successfully silence *MYCN* in MNA cell lines, as it has

been shown that the antisense:sense RNA ratio must be on the order of 1000:1 for obtaining antisense-sense duplexes of 50% of the target mRNA [284]. Successful antisense-based silencing of *MYCN* in the IMR-32 cell line has been reported, but here the authors used an Epstein-Barr viral approach in which the antisense *MYCN* expression was magnified by episomal replication [285]. Here, the *MYCN* suppression was significant, though no differentiation was observed. Later experiments using siRNAs targeting *MYCN* have demonstrated that IMR-32 differentiates extensively upon *MYCN* silencing [250]. This discrepancy might be a result of off-target effects since it has been shown that *MYCN* antisense RNAs are targets for the interferon pathway in a MNA cell line [286].

The superior efficiency of shRNA compared to antisense approaches is apparent when considering the RV-1658 constructs presented in Paper II. Here, we achieved a specific, efficient and conditional downregulation of *MYCN* in two MNA cell lines as a result of shRNA being expressed from a single genomic insert. In addition to this, no off-target effects resulting from interferon response were detected. When using any type of small RNA, the off-target effects most widespread seem to be those resulting from saturation of the miRNA pathway [180]. We did not observe any signs of such off-target effects in our study, although they cannot be ruled out since these symptoms could be very difficult to detect.

We chose to use a MLV retroviral system for stably delivering the inducible expression cassettes to neuroblastoma cell lines. The inducible promoter described in Paper I can easily be gated into a retroviral expression vector, which was then used to transfect the Hek-293 Phoenix packaging cell line for the synthesis of retroviral particles. The entire process utilises vectors and cell lines easily propagated in any lab with suitable facilities, and thus it is relatively easy and cost efficient to produce any new shRNAs towards any target in any dividing human cell. After induction of the RV-1658 cell lines, it typically took 3-5 days before full silencing of *MYCN* was achieved. As a result of this, the inducible system is not optimal for investigating direct targets of MYCN.

As described in Paper II, we observed minimal amounts of leakage of anti-*MYCN* shRNA in an uninduced state within the time frame the cells were grown in. Nevertheless, some leakage is inevitable when Tet-inducible polIII promoters are being used. In our experience, there was no significant leakage within the first month after transduction. When we tried to isolate single clones of transduced cells, leakage in uninduced cells was evident. After proliferating

for approximately 10 weeks, the cells appeared to be morphologically undifferentiated, but expressed up to 30% less MYCN than the control cells. Yet, induction led the MYCN levels to drop further, which was then followed by differentiation of the cells (data not included in the manuscripts). This suggests that a relatively fast drop of MYCN concentration initiates differentiation and growth arrest, rather than an absolute concentration threshold. This however is an issue that must be further investigated before any conclusions can be drawn.

5.2. Paper III

5.2.1. Brief description of the study

MYCN was downregulated using transient shRNA in the MNA cell lines SKNBE(2) and Kelly. MiRNA expression profiling was performed on two independent experiments, and miRNAs differentially expressed as a result of *MYCN* silencing were identified. We found most members of the three clusters of the miR 17 family to correlate with *MYCN* expression, an observation supported by previous findings. Moreover, we observed a clear correlation between *MYCN* and *miR-92b* and *miR-103*. Several non-clustered miRNAs were found to be negatively correlated by *MYCN*.

The miRNA that was most upregulated following *MYCN* suppression was *miR-21*, a miRNA that has been shown to act as an oncomir in a range of other tumours. The potential function of *miR-21* in neuroblastoma was investigated further, but no effect on proliferation or differentiation was observed. Hence, we were not able to establish any role for *miR-21* expression in differentiating neuroblastoma cells.

5.2.2. Discussion

Apart from the members of the miR 17 family, we observed two miRNAs potentially being upregulated by MYCN: *miR-92b* and *miR-103*, neither of which have been experimentally validated as direct MYCN targets.

MiR-103 expression is linked to mesenchymal stem cells [reviewed in 287], which have been recently argued to be a sub-population of neural crest cells [288]. *MiR-103* is expressed in the majority of human cells, but is generally more expressed in the brain. It is expected to be involved in metabolism. So far, oesophageal carcinoma is the only cancer in which it has a prognostic value [reviewed in 289].

MiR-92b appears to be a miRNA that is primarily expressed in neuronal-specific stem cells, the developing brain or brain tumours [290]. Deep sequencing has shown that *miR-92b* is highly expressed in human embryonic stem cells, but diminishes during differentiation [291]. In stem cells, *miR-92b* has been reported to push cells to a proliferative state by downregulating p57 [292]. Despite the indications of *miR-92b* being an oncomir in neural neoplasms, very little has been done to elucidate its potential role in various cancers.

We also found 11 miRNAs inversely correlated to *MYCN* expression, with many of these being shown to be involved in neuronal differentiation in other cell systems. Theoretically, some of these miRNAs could be tumour suppressors inhibited by MYCN, though MYCN is predominately a transcriptional activator, and has not yet been shown to directly repress any miRNA. Thus, miRNAs being inhibited by MYCN are expected to be regulated in an indirect manner. The miRNA most elevated after *MYCN* silencing was *miR-21*. This miRNA has been shown to be highly expressed in a variety of tumours and acts as an oncomir [293]. In addition, increased *miR-21* expression has been documented in differentiating SH-SY5Y non-MNA neuroblastoma cells [294]. We did not find any effects on neither proliferation nor differentiation when *miR-21* mimics or *miR-21* antagomirs were introduced to the SK-N-BE(2) cell line. Furthermore, *miR-21* antagomir did not inhibit differentiation when MYCN was downregulated. This indicates that *miR-21* does not have a proliferative effect in MNA neuroblastoma and that its increase is a consequence of differentiation. Even so, we were not able to reveal a functional role for *miR-21* during the differentiation of neuroblasotoma cells.

The lack of phenotypic changes observed by *miR-21* mimics and antagomirs are in accordance with data recently published by Mestdagh *et al.* [295]. Here, miRNAs correlating with *MYCN* (i.e. possibly transactivation targets of MYCN) were shown to have strongly predicted target enrichment on mRNAs negatively correlated to these miRNAs (i.e. possible targets for MYCN-activated miRNAs). This was not the case for miRNAs negatively correlated with *MYCN* and their putative targets. These observations suggest that it is primarily MYCN-activated miRNAs that account for the miRNA-mediated regulation of mRNAs.

5.3. Paper IV

5.3.1. Brief description of the study

Bell *et al.* have shown that the transcription of the Wnt antagonist DKK3 is downregulated by MYCN. Koppen *et al.* later revealed that this regulation was in an indirect manner. By conducting an *in silico* analysis we found that both *miR-92a* and *miR-92b*, two of the miRNAs upregulated by MYCN in Paper III, shared a predicted miRNA target site on the DKK3 3' UTR sequence. Additionally, members of the *let-7* family were predicted to bind to another target site. We hypothesised that MYCN regulates *DKK3* expression through miRNAs.

By the use of ELISA, we first demonstrated that DKK3 secretion in the culture medium was increased when *MYCN* was downregulated using the inducible Kelly and SK-N-BE(2) RV-1658 cell lines described in Paper II and the SHEP Tet21N cell line. The *DKK3* 3' UTR sequence was cloned downstream of the luciferase gene, and repression of the luciferase expression was observed when cotransfecting these constructs with mimics of *miR-92a*, *miR-92b* and *let-7e*. Mutation of the target sites led to a complete rescue of luciferase expression for *miR-92a* and *miR-92b*, but not for *let-7e*. The reduction of *miR-92a* and *miR-92b* using antagomirs led to an increased secretion of DKK3 in the MNA cell lines SK-N-BE(2) and Kelly, while transfection of *miR-92a* and *miR-92b* mimics into non-amplified cell lines SK-N-AS and SH-SY-5Y led to downregulation of *DKK3* expression. *Let-7e* mimics only led to a moderate reduction of DKK3 levels compared to those obtained by the *miR-92a* and *miR-92b* mimics. DKK3 measurements were performed by ELISA and qRT-PCR. We also performed methylation-specific PCR on genomic DNA from 10 primary neuroblastoma samples (five MNA, five non-MNA) and five neuroblastoma cell lines (three MNA, two non-MNA), and found no hypermethylation of the *DKK3* promoter.

The immunohistochemistry of 25 primary neuroblastoma tissue samples from various biological subsets showed an inverse correlation between the expression of *MYCN* and *DKK3*. DKK3 was mainly detected in the vasculature of the tumours, illustrated by its co-localisation with the vascular endothelial marker CD31.

5.3.2. Discussion

DKK3 is a secreted antagonist of the Wnt pathway. To establish whether MYCN downregulates DKK3 secretion, we utilised the RV-1658 cell lines described in Paper II as a model system. This system was well suited for these experiments since we could suppress *MYCN* for a longer period, thereby allowing the cells to differentiate for five days before measuring DKK3 levels. Fresh media were then added to the cells 24 hrs prior to the measurement of accumulated DKK3 by enzyme-linked immunosorbent assay (ELISA). To the best of our knowledge, this is the first time changes in the secretion of endogenous DKK3 protein have been measured directly in neuroblastoma. Previous studies have either measured mRNA levels or the expression of ectopic Flag-tagged DKK3 [270, 277, 278]. Nonetheless, we cannot be certain that the observed upregulation of DKK3 following *MYCN* silencing is solely the effect of changes in *miR-92a/b* and *let-7* alone, or whether additional unknown factors are involved.

We have established that *DKK3* expression is regulated by *miR-92a*, *miR-92b* and *let-7*. In Paper III, we showed that *miR-92a* and *92b* are downregulated when *MYCN* expression is repressed. Two separate *miR-92a* genes are found in the human genome, one in the *miR-17-92* cluster and one in the paralogue *miR-106a* cluster, and both clusters are direct transcriptional targets of MYCN [261, 262]. *MiR-92b* is expressed as a single intergenic miRNA on chromosome 1q, and little is known about the promoter of *miR-92b*. An E-box is located upstream of the *miR-92b* gene, but no ChIP analysis has been performed to investigate whether this is a direct binding site for MYCN. The mature sequence of *miR-92b* is identical to that of *miR-92a* apart from three nucleotides, which does not theoretically impair with seed sequence binding. Thus, *miR-92a* and *miR-92b* share the same targets. *MiR-92b* is mainly expressed in neuronal-specific stem cells, the developing brain or brain tumours [290]. The expression of *miR-92b* instead of *miR-92a* in these tissues could be an approach for targeting *miR-92a/b* binding sites, while avoiding expression of the remaining miRNAs of the *miR-17-92* cluster. Since the *miR-17-92* cluster is a direct target of MYCN, the distinct expression of *miR-92b* is probably governed by other mechanisms.

The biological function of *DKK3* is debated. *DKK3* knock-out mice are viable, fertile and show no obvious abnormalities [296]. The lack of a distinct *DKK3* knockout phenotype might be a result of Soggy replacing DKK3 in important pathways. Although DKK3 does not

interact with LRP5/6 [270], DKK3-mediated repression of the canonical Wnt pathway has been reported in some cellular settings [272, 273]. Others have reported Wnt activation as a result of *DKK3* expression [271]. A proposed mechanism for DKK3-mediated activation of the Wnt pathway has been described by Nakamura *et al.* [270], whereas the mechanism of DKK3-mediated inhibition of the canonical Wnt pathway remains unknown. Upon discovery of the inverse correlation between *MYCN* and *DKK3*, Bell *et al.* proposed that MYCN could exert its proliferative effect by allowing high canonical Wnt signalling through downregulation of the Wnt inhibitor *DKK3* [278]. However, Koppen *et al.* later documented that DKK3 reduces proliferation in neuroblastoma cells, but not through the canonical Wnt pathway [277]. Here, it was suggested that DKK3 may exert its effect through the non-canonical Wnt pathways. It was also debated whether the Wnt pathway was a significant contributor to malignancy in MNA neuroblastoma. It has been demonstrated that the canonical Wnt pathway is deregulated in high-risk non-MNA neuroblastoma, but not in MNA neuroblastoma. In high-risk non-MNA neuroblastoma, this was proposed to cause increased proliferation as a result of a higher expression of *MYCC* and *cyclinD* [297].

DKK3 is shown to be necessary for Activin/nodal (members of the transforming growth factor beta (TGF-β) superfamily) signalling in *Xenopus* embryos [298]. Here, DKK3 was shown to exert its function by maintaining normal levels of Smad4, a key downstream mediator of all TGF-β pathways. TGF-β signalling induces inhibition of proliferation and increased differentiation of many neuroblastoma cells, and it has been proposed that the effect of retinoic acid is dependent on establishing and maintaining a negative autocrine growth loop involving TGF-β1 [299]. It was also proposed that failure to establish such a loop might be a reason for the resistance to retinoic acid shown by many neuroblastoma cell lines. These results implicate that the loss of DKK3 could theoretically be a reason for a loss of responsiveness to TGF-β signalling. Another member of the TGF-β family shown to be of relevance in neuroblastoma is *Activin A*. Schramm *et al.* revealed that enhanced expression of Activin A suppresses proliferation and colony formation in MNA neuroblastoma cells. It also inhibits neuroblastoma growth and angiogenesis *in vivo*, and is highly expressed in differentiated, but not undifferentiated neuroblastomas [300, 301]. *Activin A* also seems to be downregulated by MYCN [302]. This potential role of DKK3 in TGF-β signalling is highly speculative, and further investigations are needed in order to elucidate whether DKK3 does interact with TGF-β signalling in neuroblastoma.

We analysed a selection of primary tumours by immunohistochemistry, and found that DKK3 was expressed in the tumour endothelium. The amount of DKK3 in the endothelium was inversely correlated to *MYCN* amplification. Expression of DKK3 in the tumour endothelium rather than the tumour parenchyma has been described in many other types of cancer [303-308]. Untergasser *et al.* used murine melanoma cells with ectopical overexpression of DKK3 to establish xenografts in mice. They observed that DKK3 overexpression resulted in slightly larger tumours with a significantly increased microvessel density (MVD) [304]. MVD is a measure of angiogenesis which is considered a prognostic indicator that correlates with an increased risk of metastasis in various epithelial cancers. These results, and the observation of a high expression of DKK3 in the developing heart and blood vessel system in both mice and chicken embryos, suggests that DKK3 has a conserved role in vascularisation [309]. The observations of DKK3 functioning as an inducer of angiogenesis is contradictory to its established role as a tumour suppressor. Compared to low-risk ganglioneuromas, we observed lower expression of DKK3 in the vasculature of high-risk MNA neuroblastomas, an observation which is supported by others [277].

In summary it seems as if DKK3 has distinct roles in cancer cells and epithelial cells involved in angiogenesis. In pancreatic cells, the pro-angiogenetic effect of DKK3 did not contribute to a poor prognosis [303]. Very little is known of the exact function of DKK3 as a tumour suppressor in neuroblastoma. It does not seem to influence the canonical Wnt pathway, but could be involved in regulation of some of the non-canonical pathways. However, none of the Wnt pathways has so far been shown to play a significant role in contributing to the proliferation in MNA neuroblastoma. There is a theoretical possibility that DKK3 are involved in TGF-β signalling in neuroblastoma, but no research has thus far confirmed this.

6. Conclusions

In this thesis we have focused on the oncogene *MYCN* that is often amplified in neuroblastoma. We have designed and developed a system that allows conditional expression of any shRNA from a Tet-inducible RNA polIII H1 promoter (Paper I). In Paper II we used this Tet-inducible promoter to express an anti-*MYCN* shRNA in two MNA neuroblastoma cell lines. The shRNA was introduced to the genome of the cells by the use of an MLV based retroviral vector system. Efficient suppression of MYCN was observed within 3 days after addition of the inducer Dox to the media.

In Paper III the effect of *MYCN* suppression on miRNA expression was investigated. We found that *MYCN* downregulation resulted in a decrease of several miRNAs, many of which are members of the miR 17 family. In addition we found several miRNAs being upregulated as *MYCN* was suppressed. One of these was *miR-21*, which was investigated further. We could not reveal any function for *miR-21* in differentiation or proliferation in MNA neuroblastoma.

Other miRNAs demonstrated to be affected by *MYCN* expression were *miR-92a*, *miR-92b* and *let-7e*. In Paper IV we showed that these three miRNAs suppresses the expression of *DKK3*, a tumour suppressor frequently downregulated in MNA neuroblastoma.

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ISBN 978-82-7589-285-8
Print: Tromsprodukt • 40 00 72 00