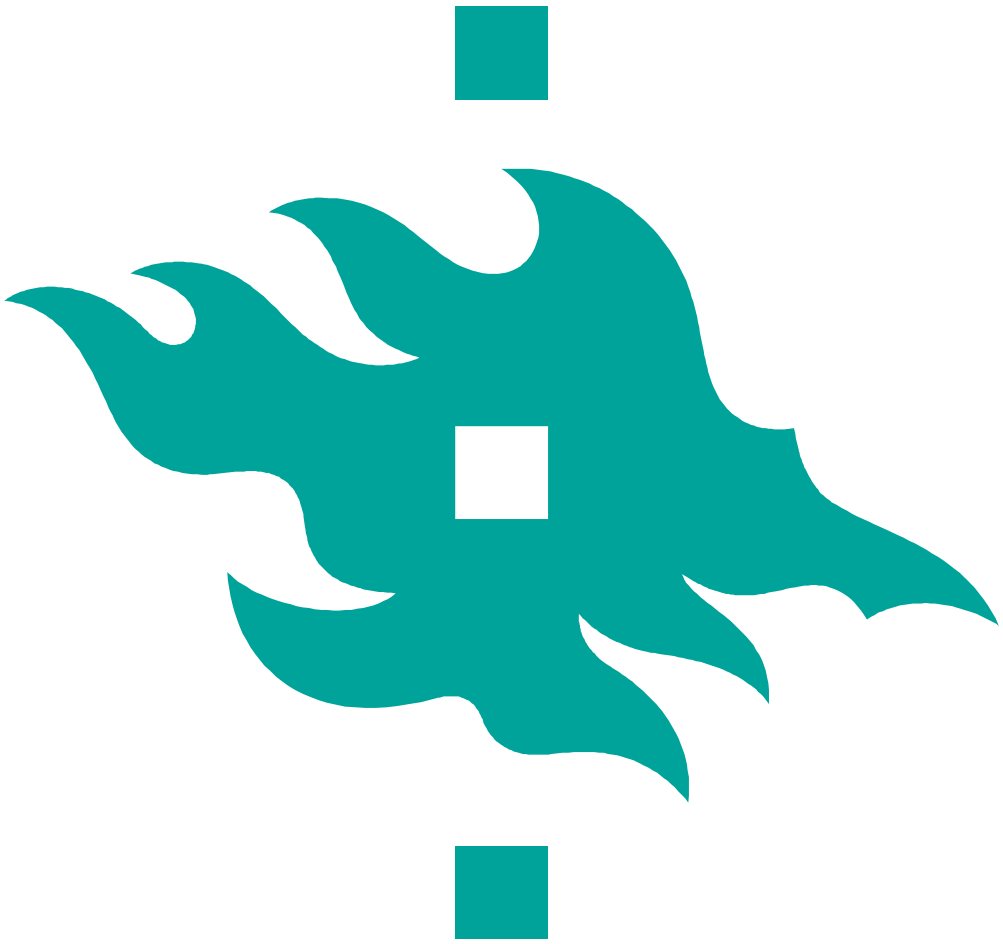

# RATIONAL ENGINEERING OF microRNA-REGULATED VIRUSES FOR CANCER GENE THERAPY

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# Rational engineering of microRNA-regulated viruses for cancer gene therapy

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**ACADEMIC DISSERTATION**

To presented with the permission of the Medical Faculty of the University of Helsinki, for public examination in the Lecture Hall 2, Haartman Institute, Haartmaninkatu 3, on May 31st, 2013, at 12 noon

Helsinki 2013

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ISBN 978-952-10-8700-4 (paperback)
ISBN 978-952-10-8701-1 (PDF)
Picaset Oy
http://ethesis.helsinki.fi
Helsinki 2013

*Dedicated to the loving memory of my little brother*

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**Generation of a conditionally replicating adenovirus based on targeted destruction of E1A mRNA by a cell type-specific MicroRNA.**
J Virol. 2008 Nov;82(22):11009-15.

**II: Ylösmäki E**, Lavilla-Alonso S, Jäämaa S, Vähä-Koskela M, Af Hällström T, Hemminki A, Arola J, Mäkisalo H, Saksela K.
**MicroRNA-Mediated Suppression of Oncolytic Adenovirus Replication in Human Liver.**
PLoS One. 2013;8(1):e54506.

**III: Ylösmäki E***, Martikainen M*, Hinkkanen A, Saksela K.
**Attenuation of Semliki Forest virus neurovirulence by microRNA-mediated detargeting.**
J Virol. 2013 Jan;87(1):335-44.

***** equal contribution

## ABSTRACT

MicroRNAs (miRNAs) are small noncoding RNA molecules that have important regulatory roles in a wide range of biological processes. miRNAs are often expressed in a tissue- and/or differentiation state-specific patterns, and it is estimated that miRNAs can regulate the expression of more than 50% of all human genes. We have exploited these tissue-specific miRNA expression patterns in the modification of viral replicative tropism. In order to engineer the replicative tropism of oncolytic adenoviruses, we developed a recombinant adenovirus that in the 3' UTR of the critical E1A gene contains sequences complementary to the liver-specific miRNA miR122. This allowed us to generate a novel recombinant adenovirus that was severely attenuated in human liver, but replicated to high titres in colorectal cancer. Systemic injection of miR122-targeted adenovirus into mice did not induce liver toxicity. In a human lung cancer xenograft mouse model this miR122-targeted adenovirus showed potent antitumour activity.

We also studied the possibility to exploit neuron-specific miRNA expression patterns in the modification of tissue tropism of an alphavirus Semliki Forest virus (SFV). We engineered SFV genome to contain sequences complementary to the neuron-specific miRNA miR124. *In vitro* characterization of this novel virus showed that the modification of the SFV genome *per se* did not affect polyprotein processing or oncolytic potency. Intraperitoneally administered miR124-targeted SFV displayed an attenuated spread into the central nervous system (CNS) and increased survival of infected mice. Also, mice pre-infected with miR124-targeted SFV elicited strong protective immunity against otherwise lethal challenge with a highly virulent wild-type SFV strain.

In conclusion, these results show that miRNA-targeting is a potent new strategy to engineer viral tropism in development of safer and more efficient reagents for virotherapy applications.

## ABBREVIATIONS

| | |
|---|---|
| Ago2 | argonaute 2 |
| ALAT | alanine aminotransferase |
| APC | antigen-presenting cell |
| BCL2 | B-cell lymphoma 2 protein |
| BLV | bovine leukaemia virus |
| bp | base pair |
| CAR | cocsackievirus B and adenovirus receptor |
| CLL | B-cell chronic lymphocytic leukaemia |
| CNS | central nervous system |
| CTCF | CCCTC-binding factor |
| CVA21 | Coxsackievirus A21 |
| DCP2 | decapping protein 2 |
| DGCR8 | DiGeorge syndrome critical region 8 |
| dsRNA | double-stranded RNA |
| EBV | Epstein-Barr virus |
| eIF4F | eukaryotic initiation factor 4F |
| FGF | fibroblast growth factor |
| FIX | coagulation factor IX |
| FMR1 | fragile X mental retardation-related protein 1 |
| GDP | guanosine diphosphate |
| GFP | green fluorescent protein |
| GTP | guanosine triphosphate |
| HCC | hepatocellular carcinoma |
| hCMV | human cytomegalovirus |
| HCV | hepatitis C virus |
| HIV-1 | human immunodeficiency virus 1 |
| HPV | Human papillomavirus |
| HVT | herpesvirus of turkeys |
| ILTV | infectious laryngotracheitis virus |
| Imp8 | importin 8 |
| KSHV | Kaposi's sarcoma-associated herpesvirus |
| LETF | liver-enriched transcription factor |
| mCMV | murine cytomegalovirus |
| MDV | Marek's disease virus |
| Mef2 | myocyte enhancer factor-2 |
| MHV | murine herpesvirus |
| miRISC | microRNA-induced silencing complex |
| miRNA | microRNA |
| MLP | major late promoter |

| | |
|---|---|
| MOI | multiplicity of infection |
| mRNA | messenger RNA |
| NMD | non-sense mediated decay |
| nt | nucleotide |
| PABPC | poly-A binding protein, cytoplasmic |
| PAZ | PIWI, Argonaute and Zwille domain |
| PDCD4 | Programmed cell death 4 protein |
| PFU | plaque forming unit |
| pre-miRNA | precursor microRNA |
| pri-miRNA | primary microRNA |
| PTBP1 | polypyrimidine track binding protein 1 |
| PTEN | Phosphatase and Tensin homolog |
| rLCV | rhesus lymphocryptovirus |
| RNA | ribonucleid acid |
| RRV | rhesus monkey rhadinovirus |
| SCP1 | synaptonemal complex protein 1 |
| SFV | Semliki Forest virus |
| SFV | Semliki Forest virus |
| ssRNA | single-stranded RNA |
| $TCID_{50}$ | a 50% tissue culture infectious dose |
| TGF-β | transforming growth factor β |
| TNFα | tumour necrosis factor alpha |
| TRBP | human immunodeficiency virus trans-activating response RNA-binding protein |
| TSP | tumour/tissue specific promoter |
| UTR | untranslated region |
| VA1 | viral associated RNA 1 |
| VEEV | Venezuelan equine encephalitis virus |
| VEGF | vascular endothelial growth factor |
| VSV | vesicular stomatitis virus |

# 1 REVIEW OF THE LITERATURE

## 1.1 Introduction

MicroRNAs (miRNAs) are endogenous small, approximately 22 nucleotides long RNA molecules that have important regulatory roles in animals and plants. MiRNAs act as posttranscriptional suppressors by binding usually to the 3'untranslated region (UTR) of their target genes thus inhibiting their target gene expression by translation inhibition or messenger RNA (mRNA) degradation (Lee, Feinbaum et al. 1993; Reinhart, Slack et al. 2000; Lagos-Quintana, Rauhut et al. 2001; Bartel 2004). They were first discovered in 1993 by Lee et al. who reported that *lin-4*, the essential gene controlling the timing of *C.elegans* larval development does not encode for a protein but instead produces two small RNA transcripts of approximately 22 and 61 nucleotides in length. These two RNA transcripts were found to contain sequences complementary to a repeated sequence element in the 3'UTR of *lin-14* mRNA, a gene known to be down regulated by *lin-4*. These findings led Lee et al. to propose that *lin-4* regulates *lin-14* translation via a novel antisense RNA-sense RNA interaction (Lee, Feinbaum et al. 1993). At the same time Wightman et al. showed that the repeated sequence element in the 3'UTR of the *lin-14* gene was sufficient for its regulation and that the regulation is at the level of protein translation and mediated by *lin-4* gene product (Wightman, Burglin et al. 1991; Wightman, Ha et al. 1993). These findings were the first example of regulation of an important developmental process by short RNA molecules, microRNAs. Seven years after the discovery of *Lin-4*, another essential miRNA gene controlling the timing of *C.elegans* larval development called *Let-7* was identified (Reinhart, Slack et al. 2000). Soon after the discovery of *let-7* in *C.elegans*, homologs of *let-7* were identified in a wide range of animal species including vertebrate, ascidian, hemicordate, mollusc, annelid and arthropod (Pasquinelli, Reinhart et al. 2000). To date, there are over 25000 miRNAs identified in 193 species including over 2000 miRNAs identified in the human genome (www.mirbase.org) (Griffiths-Jones, Saini et al. 2008).

## 1.2 MiRNA genes

The majority of miRNA genes identified from *C.elegans* were found to be encoded by independent transcription units that do not contain an open reading frame and which are located quite distant from previously annotated genes (Lau, Lim et al. 2001). Profiling of *Drosophila melanogaster* miRNA genes revealed that majority of the genes were in close proximity to each other forming larger gene clusters containing on average three different miRNA genes and the longest cluster contained eight different miRNA genes (Aravin, Lagos-Quintana et al. 2003). More than half of the mammalian miRNA genes are located within the introns of either protein-coding transcription units (messenger RNAs, mRNAs) or noncoding transcription units. Approximately 10% of miRNA genes are encoded by exons of long nonprotein-coding transcripts (mRNA-like

noncoding RNAs). MiRNA genes can also locate either in exon or intron depending on alternative splicing of the host transcript (Rodriguez, Griffiths-Jones et al. 2004). Also clusters of mammalian miRNA genes can overlap with a single host transcript. In those cases, the majority of these miRNA genes are located in the same intron or exon of the host transcript (Rodriguez, Griffiths-Jones et al. 2004).

## 1.3 MiRNA biogenesis

### 1.3.1 MiRNA transcription

MiRNAs are transcribed as parts of longer primary transcripts (pri-miRNAs) that are generated by RNA polymerase II (Lee, Jeon et al. 2002; Lee, Kim et al. 2004). Like other RNA polymerase II transcripts, also pri-miRNAs possess 5' 7-methyl guanosine cap and a 3' poly-A-tail. Approximately 40 % of all miRNAs are found within the introns of protein-coding genes and in these cases the spliced forms of pri-miRNAs can function as mRNAs (Cai, Hagedorn et al. 2004; Rodriguez, Griffiths-Jones et al. 2004). The transcribed pri-miRNAs form stem-loop structures, which contain the mature miRNA or miRNAs as a part of an imperfectly paired double stranded stem connected with a terminal loop (Lee, Ahn et al. 2003; Gregory, Yan et al. 2004).

### 1.3.2 Canonical miRNA processing: Pri-miRNA processing by Microprocessor complex

In the canonical miRNA processing pathway, the stem-loop structures are recognised and further processed in the nucleus by Microprocessor complex, a multiprotein complex including the essential components RNase III Drosha and a double-stranded-RNA-binding protein DGCR8 (Lee, Ahn et al. 2003; Gregory, Yan et al. 2004). Pri-miRNA processing consists of two sequential processing steps. First, the DGCR8 recognizes the single stranded RNA-double stranded RNA (ssRNA-dsRNA) junction at the root of the stem-loop structure and anchors the Microprocessor complex at the correct location. Second, Drosha transiently interacts with the substrate RNA for catalytic reaction to occur (Han, Lee et al. 2006). Drosha cleaves the dsRNA stem at ~11 base pair (bp) from the root of the stem loop-structure generating a two nucleotide (nt) overhang at the 3' end (Han, Lee et al. 2006). The catalytic activity of Drosha results in a hairpin-shaped dsRNA molecule of 70-100 bp in length called miRNA precursor or pre-miRNA.

### 1.3.3 Noncanonical miRNA processing: The miRtron pathway

MiRNA processing pathways that bypass the processing by Drosha/DGCR8 are collectively called noncanonical miRNA processing pathways. An example of such a pathway is the miRtron pathway. MiRtrons are pre-miRNA hairpins located in short introns, characterized by the precise mapping of the mature miRNA strand and the complementary passenger strand, the miRNA* strand to the 5' and 3' ends of the introns (Okamura 2012). Majority of the miRtron bearing introns are sliced and the intron lariat is debranched to generate a pre-miRNAs that can then be exported to the

cytoplasm. However, a subset of these sliced and debranced miRtrons (tailed miRtrons) have 5' or 3' tails that require RNA exosome mediated trimming of the 3' tail or removal of the 5' tail by a yet unknown mechanism for efficient export to the cytoplasm (Yeo and Chong 2011; Okamura 2012) (see figure 1. for schematic presentation of miRNA processing pathways).

### 1.3.4 Nuclear export of pre-miRNAs

Pre-miRNAs processed by either canonical or noncanonical processing pathways are then exported to the cytoplasm by the nuclear export receptor Exportin 5 (Exp5) together with its cofactor Ran-guanosine triphosphate (Ran-GTP). Exp5/Ran-GTP complex recognize the pre-miRNA stem and the 2 nt 3'overhang generated by Drosha and export the complex bound pre-miRNA to the cytoplasm where the hydrolysis of the Ran-GTP to Ran-GDP releases the pre-miRNA for further processing (Yi, Qin et al. 2003; Bohnsack, Czaplinski et al. 2004; Lund, Guttinger et al. 2004).

### 1.3.5 Pre-miRNA processing by Dicer

The cytoplasmic pre-miRNAs are further processed by a member of the RNAse III family of nucleases called Dicer. Dicer is a multidomain enzyme including a PAZ (PIWI, Argonaute and Zwille) domain, two RNaseIII domains (RNase IIIa and RNase IIIb) and a dsRBD (double-stranded RNA-binding) domain. The PAZ domain contributes to the pre-miRNA substrate recognition by interacting with the two nucleotide overhang at the 3' end of the pre-miRNAs and by positioning the double-stranded stem correctly for the cleavage. After substrate recognition and its correct positioning by PAZ domain, the pre-miRNA is then cleaved at a set distance of approximately 21-nt away from the end of the molecule in the catalytic centre formed by intramolecular dimerization of the two RNAse III domains (Bernstein, Caudy et al. 2001; Zhang, Kolb et al. 2004; Macrae, Zhou et al. 2006). A miRNA duplex intermediate of ~21-nt in length having 2-nt 3'overhangs on both ends is generated as a result of Dicer processing.

### 1.3.6 Dicer independent pre-miRNA processing

To this date, there is only one known example of Dicer independent miRNA, miR451 (Cheloufi, Dos Santos et al. 2010; Cifuentes, Xue et al. 2010). The processing of miR451 begins with canonical processing by microprocessor complex to form pre-mirR451, but the subsequent processing step is unique to this miRNA. Pre-miR451 is loaded into Ago2 (Argonaute 2) and cleaved by the Ago2 catalytic centre to generate an intermediate 3'end, which is then further trimmed by a mechanism yet unidentified (Cheloufi, Dos Santos et al. 2010).

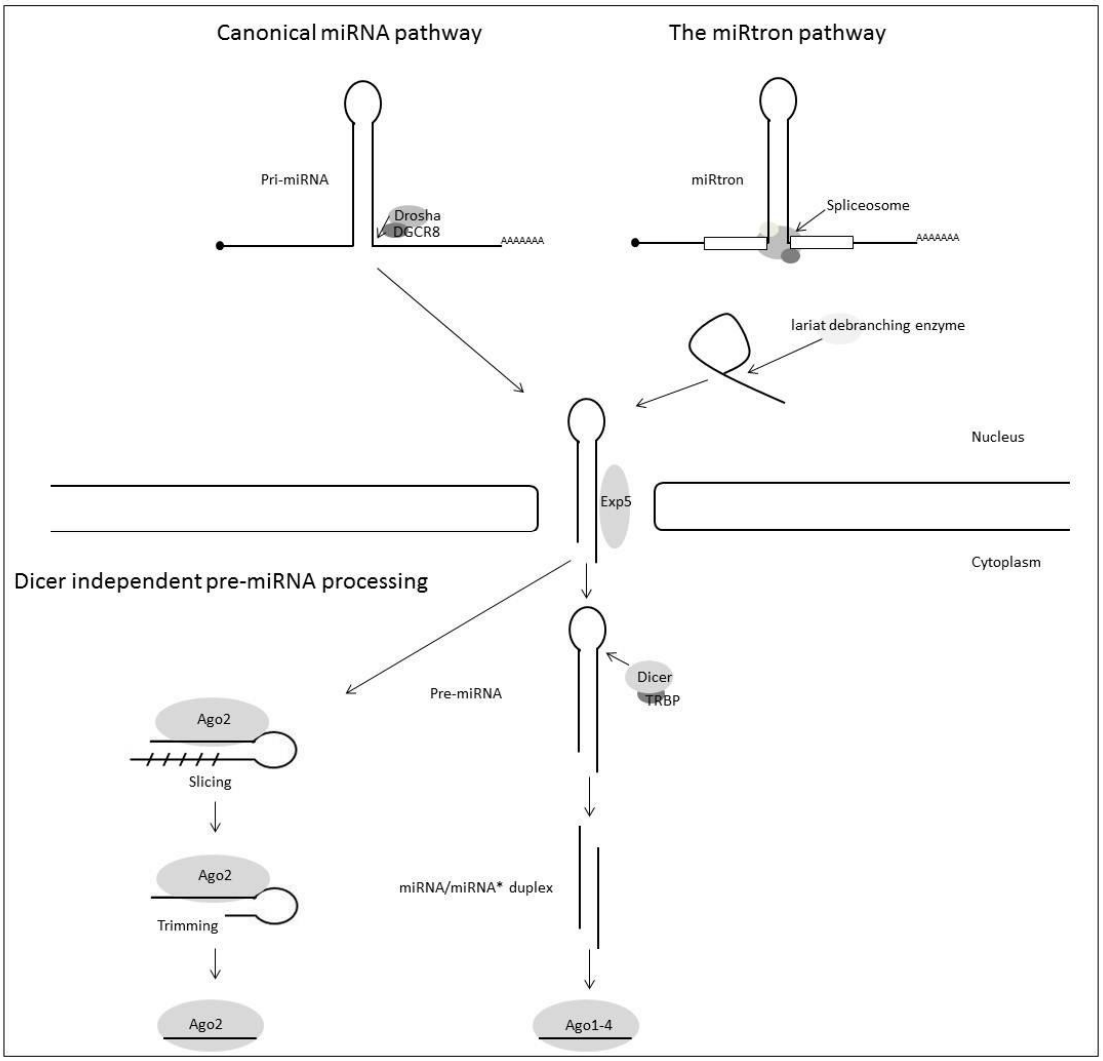


**Figure 1. Canonical miRNA pathway, the miRtron pathway and Dicer independent pre-miRNA processing of miR451.**

## 1.4 MiRNA-induced silencing complex (miRISC) assembly

The Dicer/miRNA intermediate complex is associated with TRBP (human immunodeficiency virus trans-activating response RNA-binding protein) which recruits the complex to Ago2 protein of the miRISC complex (Chendrimada, Gregory et al. 2005; Gregory, Chendrimada et al. 2005). One strand of the miRNA duplex (the mature miRNA) is loaded into Ago2 and the other strand (miRNA*) is degraded. It has been shown that the miRNA duplexes are thermodynamically asymmetric and the strand with less stable base pairing at the 5' end is loaded into Ago2 and thus serves as the mature miRNA (Schwarz, Hutvagner et al. 2003). Dicer in association with TRBP can sense this thermodynamic asymmetry by repositioning the intermediate duplex into the helicase domain of Dicer where the less stable end of the duplex is bound by Dicer and the more stable end is bound by TRBP. This positions the 3' end of the mature miRNA strand in close proximity to the Ago2 PAZ domain and finally leads to loading of the RNA duplex into Ago2 (Noland, Ma et al. 2011). The N domain of Ago2 then unwinds the duplex and removes the miRNA* strand through a slicer-independent

unwinding, forming core of the miRISC complex (Kwak and Tomari 2012). In addition to Ago2, another key component of the miRISC complex is the GW182 protein family. Members of this family can directly interact with Ago2. In vertebrates there are three different GW182 paralogues named TNRC6A, -B and –C. The amino-terminal domain of GW182 proteins with multiple glycine-tryptophan repeats (GW-repeats) is responsible for Ago2 binding. In the carboxy-terminal part of the GW182 is a silencing domain (SD) that is an important effector domain and required for miRNA-induced repression (Fabian, Sonenberg et al. 2010; Huntzinger and Izaurralde 2011). Several additional proteins interact with miRISC complex including fragile X mental retardation protein (FMRP), RNA helicase RCK/p54, Importing 8 (Imp8) and TRIM32. All these proteins may function as regulatory factors that modulate miRNA function (Fabian, Sonenberg et al. 2010; Huntzinger and Izaurralde 2011).

## 1.5 MiRNA target recognition

MiRNAs interact with their target mRNAs most commonly via base-pairing at the 3'UTR of mRNAs, although some miRNAs have also been shown to base-pair with regions in the 5'UTR of mRNAs or open reading frames (ORFs) (Duursma, Kedde et al. 2008; Orom, Nielsen et al. 2008). Bioinformatics as well as experimental analyses have revealed that the most stringent requirement for miRNA target recognition is a contiguous and perfect Watson-Crick base-pairing of the miRNA 5' nucleotides 2-7, a so called seed region nucleating the interaction with the target mRNA, although very recent data suggest that a substantial fraction of miRNA target sites do not have this seed region base-pairing (Lewis, Shih et al. 2003; Bartel 2009; Khorshid, Hausser et al. 2013). In addition to the seed region of perfect complementarity, it has been found that A residue in the mRNA opposite to position 1 of the miRNA (this nucleotide does not need to base-pair with the miRNA nucleotides) and an additional Watson-Crick base-pairing at position 8 improve miRNA activity (Lewis, Burge et al. 2005). Bioinformatic analyses have shown a lack of conservation between miRNAs and their target mRNAs beyond the residues immediately flanking the conserved seed region suggesting that a majority of miRNA-target interactions are mediated primarily by seed matches and the 3' portion of miRNAs has little role in target recognition. Some atypical sites with additional Watson-Crick base-pairing, usually centering on miRNA nucleotides 13-16, have also been found (Lewis, Burge et al. 2005; Bartel 2009) (see figure 2. for various types of miRNA target recognition).

## 1.6 Alternative mode of miRNA target recognition

Recently, Chi et al. identified an alternative binding mode used by miRNAs (Chi, Hannon et al. 2012). By analysing the genome-wide use of atypical miRNA binding sites in Ago-interacting mRNAs, they found that Ago binds to a large number of bulged sites *in vivo.* This finding led them to propose a transitional nucleation model in which the nucleotide in position 6 of the miRNA, called the "pivot", interacts with target bulge

site serving as a general means of enabling a transitional nucleation state (by stabilising nucleation base pairing positions 2-6), allowing subsequent bulge formation and propagation of the seed interaction (see figure 2. for example of alternative mode of miRNA target recoqnition).

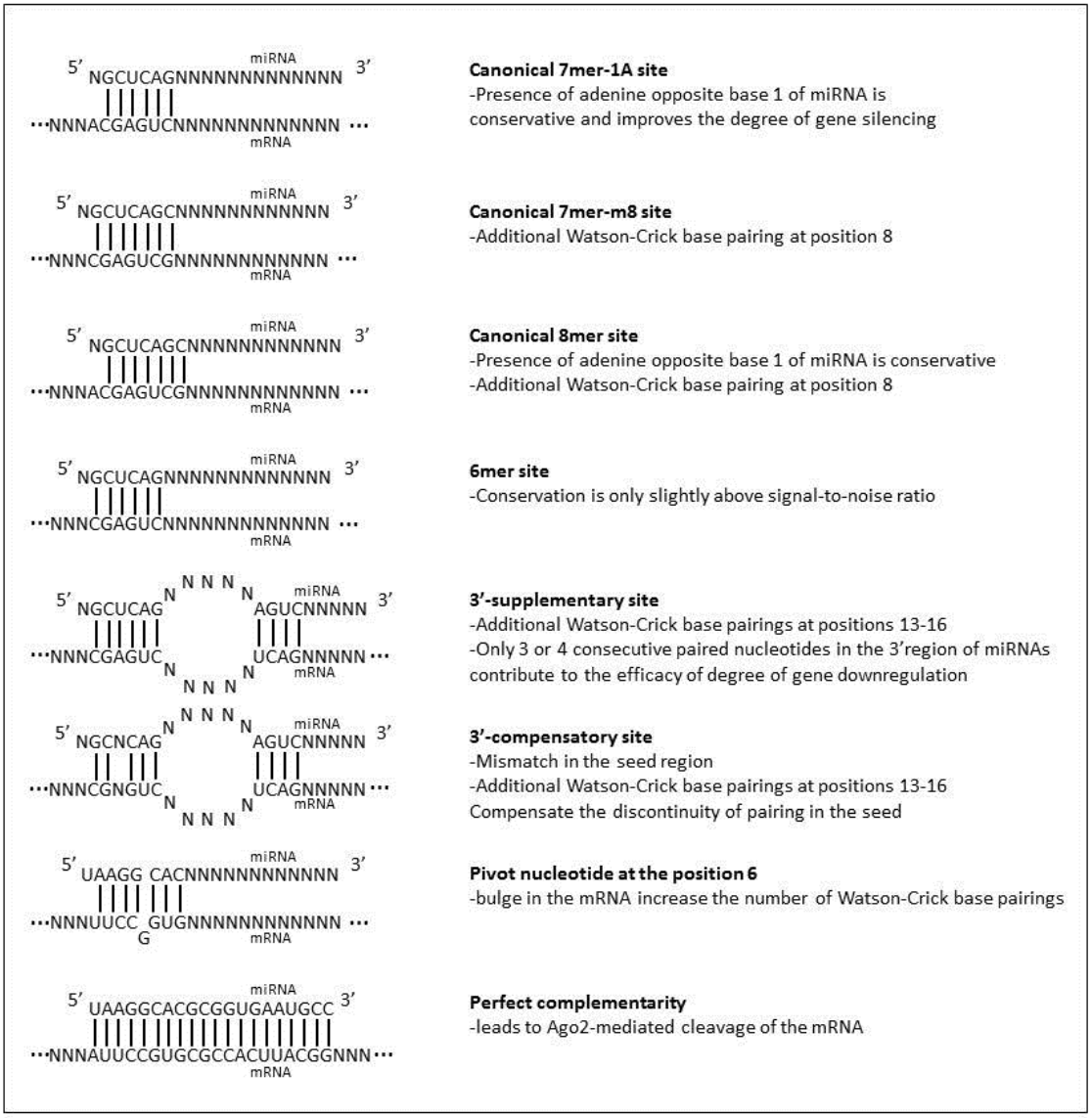


**Figure 2. Different types of miRNA-mRNA interactions.**

## 1.7 Mechanisms of gene silencing

After target recognition miRNAs can regulate their target gene expression through multiple pathways involving translational repression and/or target degradation. These pathways can influence each other and act synergistically, but are also capable of functioning independently. The contribution of an individual pathway to the overall degree of gene silencing might differ depending on cell type, cell condition, the context of target sites and the tissue level differences on the expression of critical components of the miRNA-machinery.

A mRNA that is competent for translation possesses 5’ 7-methyl guanosine cap structure and a 3’ poly-A-tail. The cytoplasmic poly-A-binding protein (PABPC) which is associated with the 3’ poly-A-tail interacts with eukaryotic initiation factor complex 4F (eIF4F; comprising the cap-binding protein eIF4E, an ATP-dependent RNA helicase eIF4A and a scaffold protein eIF4G) which in turn is associated with the 5’ cap structure bringing the 5’ and 3’ ends of the mRNA to a close proximity for translation initiation and protection from degradation (Derry, Yanagiya et al. 2006; Jackson, Hellen et al. 2010).

### 1.7.1 GW182-dependent deadenylation and mRNA destabilization

MiRNAs can direct their target mRNAs to the cellular 5’-to 3’ mRNA decay pathway where the target mRNAs are first deadenylated by the CRR4-NOT or PAN2/PAN3 deadenylase complexes and then decapped by the decapping enzyme DCP2. This miRNA-mediated mRNA decay requires several components of the miRISC complex; The target recognition is accomplished by AGO proteins which are also acting as a scaffold to recruit GW182 to the mRNA. The subsequent deadenylation is coordinated by GW182 that is interacting directly with the CRR4-NOT deadenylase complex containing the deadenylases CCR4/NOT6 and CAF1/CNOT7. GW182 can also indirectly interact with the PAN2/PAN3 deadenylase complex established by PABPC, an adaptor protein that interacts with both GW182 and PAN2/PAN3 deadenylase complex.

The subsequent decapping involves decapping enzyme DCP2 together with several decapping activators including DCP1, Ge-1, EDC3 and RCK/p54 (Behm-Ansmant, Rehwinkel et al. 2006; Wu, Fan et al. 2006; Eulalio, Huntzinger et al. 2009; Fabian, Mathonnet et al. 2009; Zekri, Huntzinger et al. 2009; Fukaya and Tomari 2012). The remaining body of the RNA is then degraded by Xrn1, a 5’-3’ exonuclease of the mRNA decay pathway (Fabian, Sonenberg et al. 2010). Recent studies aimed at determining the contribution of translational repression and mRNA degradation to the effect of miRNAs on a genome-wide level suggest that the predominant mode of miRNA-induced gene silencing is indeed through target mRNA destabilization (Baek, Villen et al. 2008; Selbach, Schwanhausser et al. 2008; Hendrickson, Hogan et al. 2009; Guo, Ingolia et al. 2010).

### 1.7.2 GW182-independent repression and GW182-dependent repression of early translation initiation

Gene silencing can also be induced by the miRISC-mediated inhibition of translation initiation by blocking the interaction of the eIF4F complex with the 5’ cap structure (Humphreys, Westman et al. 2005; Pillai, Bhattacharyya et al. 2005; Fukaya and Tomari 2012). This blocking of early translation initiation step can be divided into GW182-independent and GW182-dependent repression (Fukaya and Tomari 2012). Fukaya et al. showed that when knocking down GW182 protein to a level invisible in Western blots, the Ago1-RISC complex was still able to repress translation of a reporter gene independently of GW182 and thus independently of deadenylation in Drosophila S2

cells. They also showed that tethering of GW182 directly to the reporter mRNA and simultaneously knocking down CAF1/CNOT7 of the CRR4-NOT deadenylase complex abolished GW182-mediated deadenylation but not translational repression. Thus, GW182-dependent translational repression seems to occur independently of the deadenylase activity of GW182. Both of these repression mechanisms block formation of 48S and 80S ribosomal complexes, strongly indicating that the mode of repression is blocking the interaction of the eIF4F complex with the 5' cap structure (Fukaya and Tomari 2012). Very recently, it has been shown that also human Ago2 associates with the eIF4F complex via eIF4G (Ryu, Park et al. 2013). This Ago2/eIF4F association facilitates miRNA-mediated gene silencing, thus, possibly being the underlying mechanism of GW182-independent repression.

### 1.7.3 Endonucleolytic cleavage of mRNA by minimal miRISC complex

There are also some reports where miRNAs have been shown to direct endonucleolytic cleavage of perfectly or near perfectly complementary targets (Yekta, Shih et al. 2004; Davis, Caiment et al. 2005). In this mode of gene silencing only minimal miRISC complex is required comprising Ago2 bound to the miRNA guide strand. The perfectly complementary target is degraded by the endonucleolytic action of Ago2 PIWI domain that cleaves the phosphodiester linkage between the target nucleotides that are base paired to the guide strand residues 10 and 11. The cleavage of the phosphodiester linkage generates products with 5' -monophosphate and 3' -hydroxyl termini that are further degraded by the cellular exonucleases (Hutvagner and Zamore 2002; Liu, Carmell et al. 2004; Carthew and Sontheimer 2009). After the initial cleavage, the target dissociates from the minimal miRISC complex enabling the complex to direct multiple rounds of target cleavage (Hutvagner and Zamore 2002).

### 1.7.4 MiRNA-mediated gene activation

Interestingly, miR369-3 has been shown to upregulate tumour necrosis factor α (TNFα) expression in certain cell conditions (Vasudevan, Tong et al. 2007; Steitz and Vasudevan 2009). TNFα mRNA contains a conserved AU-rich element (ARE) in its 3'UTR that exerts post-transcriptional control over the expression of TNFα. In conditions of cell growth arrest (upon serum starvation), TNFα translational efficiency is increased by the recruitment of Ago2 and fragile X mental retardation-related protein 1 (FMR1) to the ARE. MiR369-3 directs the association of these proteins with ARE to activate translation in these conditions (Vasudevan and Steitz 2007). The same miRNA was shown to repress translation under normal conditions, so at least some miRNAs can oscillate between repression and activation in coordination with the cell cycle (Vasudevan, Tong et al. 2008) (see figure 3 for schematic presentation of different mechanism of gene silencing).

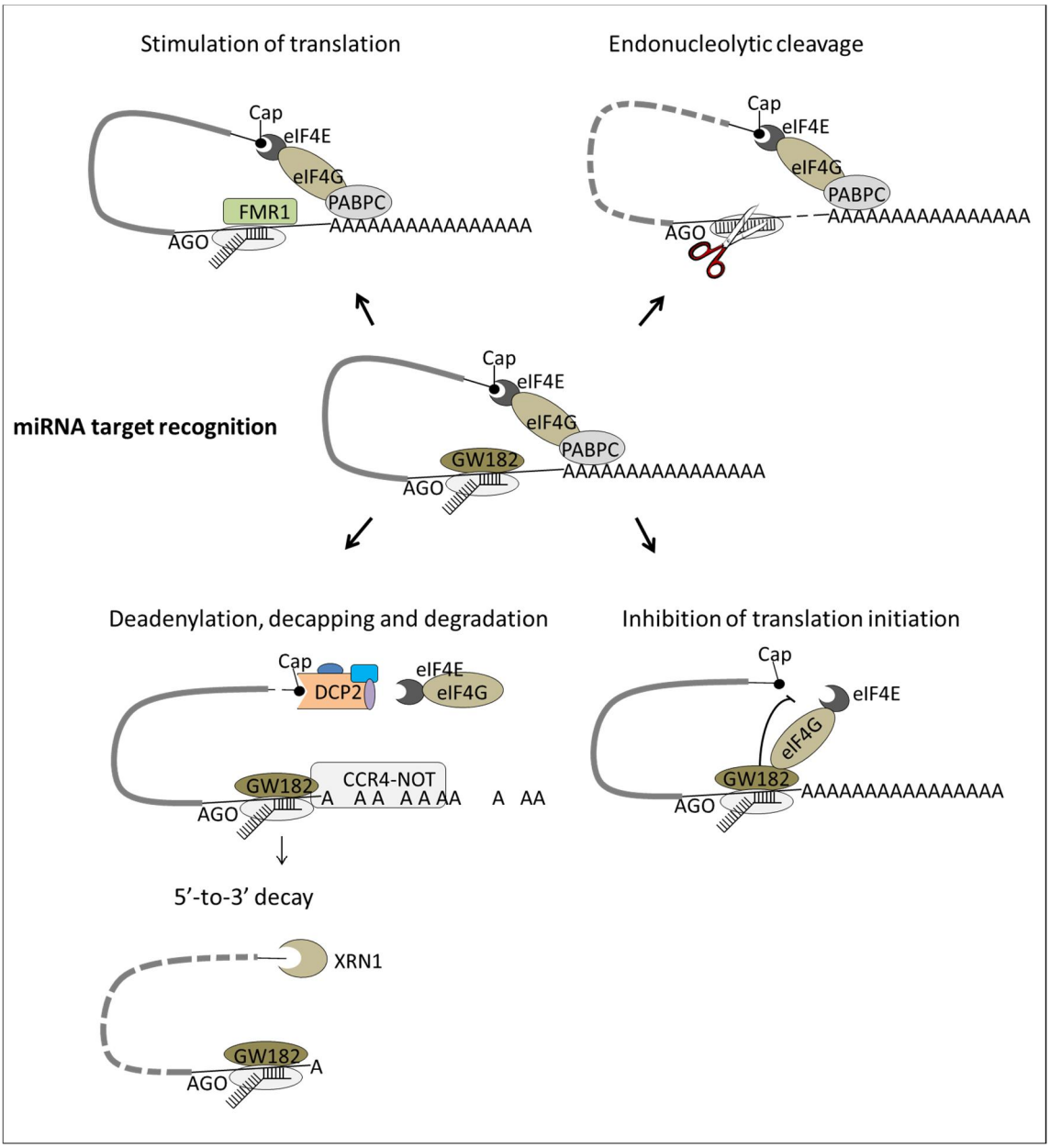


**Figure 3. Mechanisms of miRNA-mediated gene silencing and stimulation of translation.**

## 1.8 The role of P bodies in miRNA-mediated gene silencing

Cytoplasmic processing bodies (P bodies) are non-membranous aggregates of 300-500 nm in diameter that contain proteins involved in diverse posttranscriptional processes including mRNA storage and degradation, nonsense- mediated mRNA decay (NMD), translational repression of mRNAs and miRNA-mediated gene silencing (Eulalio, Behm-Ansmant et al. 2007; Ernoult-Lange, Benard et al. 2012). A number of findings strongly indicate an important role for P bodies in miRNA-mediated gene silencing. First, the majority of molecules involved in miRNA-mediated gene silencing have been detected in P bodies including all Argonaute proteins, GW182, CRR4-NOT deadenylase complex, DCP2-DCP1 decapping complex and Xrn1 exonuclease as well as miRNAs and their

targets. Second, the inhibition of miRNA biogenesis or the depletion of GW182 proteins causes the disappearance of P bodies. And finally, a positive correlation occurs between miRNA-mediated gene silencing and the accumulation of their target mRNAs in P bodies (Eulalio, Behm-Ansmant et al. 2007). Recent studies aiming at defining the role of P bodies in miRNA-mediated gene silencing have shown that P bodies (at least those microscopically visible) are not required for function, suggesting that P-body formation is a consequence and not the cause of miRNA-mediated gene silencing (Pauley, Eystathioy et al. 2006; Eulalio, Behm-Ansmant et al. 2007).

## 1.9 The regulation of miRNA biogenesis

Since a large fraction of protein-coding genes are under the regulation of miRNAs, it is critically important that the timing and extend of miRNA expression and their activity is also tightly regulated (Krol, Loedige et al. 2010; Finnegan and Pasquinelli 2012; Treiber, Treiber et al. 2012). The regulation can occur at each step of the miRNA biogenesis (see table 1).

**Table 1. Factors affecting the regulation of miRNA biogenesis. Adapted from (Finnegan and Pasquinelli 2012), (Treiber, Treiber et al. 2012) and (Hansen, Jensen et al. 2013).**

| Regulation of miRNA biogenesis | Factor | Effect on miRNA biogenesis |
|---|---|---|
| **Regulation of miRNA gene transcription** | Transcription factors (eg. p53, PITX3) | Regulation of transcription, miRNA/TF feedback loops |
| **Regulation of Drosha processing** | SMAD proteins | Stimulation of Pri-miRNA processing |
| | p53 | Stimulation of Pri-miRNA processing |
| | p68/DDX | Facilitation of Pri-miRNA processing |
| | KHSRP | Stimulation of Pri-or Pre-miRNA processing |
| | ERα | Inhibits the cleavage of specific miRNAs |
| | Lin-28b | Repression of Let7 Pri-miRNA processing |
| **Regulation of Dicer processing** | Lin-28 | Repression of Let7 Pre-miRNA processing |
| | TUT4 | Reduction of Let7 Pre-miRNA stability |
| | BCRA1 | Stimulation of Pre-miRNA processing |
| | TRBP | Facilitation of Pre-miRNA processing |
| | PACT | Facilitation of Pre-miRNA processing |
| **Regulation of miRISC stability** | Lin-48 | Attenuation of miRNA-guided gene silencing |
| **Regulation of miRNA abundance** | circular RNAs (circRNAs) | Loss of miRNA function |

## 1.10 MiRNA function

MiRNAs have a crucial role in regulating developmental morphogenesis e.g. by participating in the regulation of developmental timing, cell death, cell proliferation

and patterning of the nervous system (Bartel 2004; Shruti, Shrey et al. 2011). Here are examples of miRNAs that have been shown to have a key regulatory role in the control of organ development.

### 1.10.1 MiR126 in angiogenesis

Angiogenic growth factors, vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) modulate endothelial cell proliferation, migration and adhesion through activation of MAP kinase pathway, which in turn enhances the expression of genes required for angiogenesis and vascular integrity (Cross and Claesson-Welsh 2001). MiR126 was found to be specifically expressed in endothelial cells and thus enriched in tissues with a high vascular component such as heart and lung (Wienholds, Kloosterman et al. 2005; Harris, Yamakuchi et al. 2008; Wang, Aurora et al. 2008). Using a miR126 knockout mouse model, Wang et al. showed that miR126 modulates angiogenesis by repressing the expression of Spred-1, a negative regulator of MAP kinase signalling. The miR126 knockout mice showed vascular leakage, haemorrhaging and embryonic lethality in a subset of mice. They concluded that miR126 functions as an endothelial cell-specific regulator of angiogenic signalling (Wang, Aurora et al. 2008).

### 1.10.2 MiR1 in cardiogenesis

Several miRNA expression profiling studies have identified miR1 as a cardiac- and skeletal muscle specific miRNA (Lagos-Quintana, Rauhut et al. 2002; Zhao, Samal et al. 2005; Li, Song et al. 2010). It has been shown that miR1 is a direct transcriptional target for several muscle differentiation regulators including serum response factor (SRF), MyoD, myocyte enhancer factor-2 (Mef2) (Wang 2013). MiR1 itself targets the transcription factor Hand2 that promotes ventricular cardiomyocyte expansion (Zhao, Samal et al. 2005). The expression of miR1 coincides with the cardiac myocyte differentiation and the artificial overexpression of miR1 in the developing mouse heart results in developmental arrest at embryonic stage E13.5, secondary to thin-walled ventricles and heart failure associated with reduced myocyte proliferation, thus demonstrating the essential role of miR1 in heart development and homeostasis (Zhao, Samal et al. 2005).

### 1.10.3 MiR124 in neurogenesis

MiR124 is the most abundant miRNA of the brain, specifically expressed in neuronal cells in developing and adult nervous system (Lagos-Quintana, Rauhut et al. 2002; Smirnova, Grafe et al. 2005; Deo, Yu et al. 2006; Maiorano and Mallamaci 2009; Hua, Mo et al. 2012). MiR124 has been shown to have various important roles during neural development. The expression of miR124 mediates the repression of non-neural genes in neurons as well as the downregulation of genes expressed in neural progenitors (Lim, Lau et al. 2005; Cao, Pfaff et al. 2007). MiR124 promotes neuronal differentiation by triggering brain-specific alternative pre-mRNA splicing by suppressing the global repressor of alternative pre-mRNA splicing factor named polypyrimidine track binding protein 1 (PTBP1). In non-neuronal cells PTBP1 inhibits the alternative splicing of a

critical cassette exon in the pre-mRNA of polypyrimidine track binding protein 2 (PTBP2) exposing it to nonsense-mediated decay. In neuronal cells PTBP1 is downregulated resulting in an alternative splicing of pre-mRNA of PTBP2 and increase in its protein amount (Makeyev, Zhang et al. 2007). Also, miR124 enhances neurogenesis by suppressing the expression of small C-terminal domain phosphatase 1 (SCP1), a phosphatase that has anti-neural functions (Visvanathan, Lee et al. 2007).

### 1.10.4 MiR122 in liver development

MiR122 is the most abundant miRNA in the liver, accounting for more than 70% of the total miRNA population (Lagos-Quintana, Rauhut et al. 2002). It is one of the most tissue-specific miRNAs, expressed virtually exclusively in the liver (Lagos-Quintana, Rauhut et al. 2002; Thomson, Parker et al. 2004; Landgraf, Rusu et al. 2007; Liang, Ridzon et al. 2007). Also, the expression of miR122 is strongly upregulated in mouse liver during embryonic development. Its expression is undetectable at the earliest stages of liver development at embryonic stage E9.5 but is detected starting at E12.5 reaching to almost maximum levels at birth (Chang, Nicolas et al. 2004). MiR122 has been shown to be positively regulated by at least six different liver-enriched transcription factors (LETFs); hepatocyte nuclear factor (HNF) 1α, HNF3β, HNF4α, HNF6, Onecut2 (OC2) and CCAAT/enhancer-binding protein (C/EBP) α (Xu, He et al. 2010; Li, Xi et al. 2011; Laudadio, Manfroid et al. 2012). miR122 itself is shown to be involved in the regulation of several key regulators of development, such as transcription factors CUTL1 and CCCTC-binding factor (CTCF) and protein kinases MAP3K3 and MAP3K12 (Xu, He et al. 2010). Thus, it is suggested that miR122 functions as an effector of LETFs and contributes to liver development by regulating the balance between hepatocyte proliferation and differentiation by targeting various important developmental regulators (Xu, He et al. 2010).

## 1.11 The role of miRNAs in cancer

As miRNAs have emerged as key regulators of fundamental biological processes, it is not surprising that miRNAs can also have a profound role in pathogenesis of cancers. Approximately 50% of known human miRNAs are located in fragile sites of the genome, that is, areas that associate with cancer, thus indicating that miRNAs might have a crucial function in cancer progression (Calin, Sevignani et al. 2004). In principle, miRNAs can function as tumour suppressors or oncogenes, but there are yet several other possible mechanisms through which miRNAs can affect tumourigenesis (Esquela-Kerscher and Slack 2006; Ventura and Jacks 2009) (see table 2). The first indication of miRNA acting as a tumour suppressor came from the studies of Calin et al. who found that majority of patients diagnosed with B-cell chronic lymphocytic leukaemia (CLL) had deletions or downregulation of two clustered miRNA genes, miR15a and miR16-1 (Calin, Dumitru et al. 2002). MiR15a and miR16-1 have been shown to negatively regulate B-

cell lymphoma 2 (BCL2), an anti-apoptotic gene that is overexpressed in many types of human cancers including leukaemias and lymphomas (Cimmino, Calin et al. 2005). Thus, the lowered or complete lack of expression of miR15a and miR16-1 in CLL results in increased expression of BCL2 promoting leukaemogenesis and lymphomagenesis in haematopoietic cells (Esquela-Kerscher and Slack 2006).

MiR21 is an example of an oncogene whose expression is upregulated in almost all classes of cancer, including advanced human breast cancer, cervical cancer, ovarian cancer, hepatocellular cancer, esophageal cancer, head and neck cancer, pancreas cancer, prostate cancer, lung cancer, colon carcinoma, gliomas and B-cell lymphoma (Selcuklu, Donoghue et al. 2009; Jazbutyte and Thum 2010; Kumarswamy, Volkmann et al. 2011). MiR21 has been shown to function as an anti-apoptotic and pro-survival factor targeting many tumour suppressor proteins and pro-survival proteins such as Phosphatase and Tensin homolog (PTEN), BCL2, Programmed cell death 4 (PDCD4), Tropomyosin, Sprouty 1 and 2, Jagged 1 and BTG2. Deregulation of these proteins by miR21 overexpression can lead to development of cancer (Jazbutyte and Thum 2010).

**Table 2. Possible mechanism of miRNA-mediated tumourigenesis. Adapted from (Esquela-Kerscher and Slack 2006) and (Ventura and Jacks 2009).**

| Potential oncogenic and epigenetic changes | Predicted functional consequence | Examples |
|---|---|---|
| Deletion of miRNA | Derepression of oncogene | miR-15a-16-1 |
| Mutations affecting miRNA-mRNA interaction | Reduced affinity for oncogene | No known examples |
| | Increased affinity for tumour suppressor gene | No known examples |
| Mutations affecting miRNA biogenesis | Reduced/increased processing efficiency | miR-15a-16-1 |
| Genomic amplification of miRNA locus | Increased repression of tumour suppressor gene | miR-17 -92, miR-21 |
| Point mutation in tumour suppressor gene | Novel or increased affinity for miRNA | No known examples |
| Point mutation in oncogene | Decreased or abolished affinity for miRNA | No known examples |
| Rearrangement of 3' UTR of mRNA | Gain of miRNA-mediated suppression | No known examples |
| | Loss of miRNA-mediated repression | HMGA-2 |

### 1.11.1 MiRNAs as diagnostic tools in cancer classification

Since miRNAs clearly have role in cancer development, attempts has been made to establish whether miRNA profiling could be used for tumour classification (Iorio and Croce 2012). Genome-wide profiling of miRNA expression signature (miRNome) has showed that different types of cancers can be identified with surprisingly high accuracy and the tissue of origin of poorly differentiated tumours can be identified (Lu, Getz et

al. 2005; Volinia, Calin et al. 2006). In the first report aimed at investigating the miRnome across large set of different cancers (total amount of samples were 334, including various human cancers), Lu et al. showed by using bead-based flow cytometric miRNA expression profiling that tumours originating from different developmental lineages and differentiation states could be identified with high accuracy. As an example, they examined 73 bone marrow samples obtained from patients with acute lymphoblastic leukaemia and were able to hierarchically cluster the samples into three major branches: one containing all five BCR/ABL-positive samples and 10 out of 11 TEL/AML1 samples; a second branch containing 15 out of 19 T-cell ALL samples; and a third branch containing five out of six of the samples with *MLL* gene rearrangement. The authors were also able to identify with high accuracy the identity of poorly differentiated tumours of unknown origin by using miRNA profiling: 12 out of 17 samples were correctly diagnosed by miRNA profiling as opposed to mRNA profiling of the same samples that was highly inaccurate, diagnosing correctly only one out of 17 samples (Lu, Getz et al. 2005). Recently, miRNA expression profiling of 11 normal breast tissue samples, 17 non-invasive and 152 invasive breast carcinoma samples obtained using Solexa deep sequencing showed that differentiation of tumour subtypes by miRNA expression profiling is possible, albeit the accuracy was only similar to that of mRNA expression profiling of the same set of samples (Farazi, Horlings et al. 2011).

### 1.11.2 MiRNAs as cancer biomarkers

As miRNAs can be used to distinguish tumour types and tissues were they originated from, can miRNA expression profiling also be used as a tool for predicting cancer outcome or response to therapy? First, evidence that tumour specific miRNA signature is associated with prognostic factors and disease progression came from miRNA expression profiling study of 94 samples of CLL cells were it was shown that altered expression signature of 13 miRNAs had clear prognostic significance and was also able to differentiate patients with a short interval from diagnosis to therapy from patients with significantly longer interval (Calin, Ferracin et al. 2005). Subsequent studies further established that various cancers have cancer-specific miRNA signature with clear prognostic value including those of breast cancer (Yanaihara, Caplen et al. 2006), colon adenocarcinoma (Schetter, Leung et al. 2008), malignant melanoma (Caramuta, Egyhazi et al. 2010), gastric cancer (Li, Zhang et al. 2010) and pancreatic cancer (Lee, Gusev et al. 2007). In addition to using miRNA signature to predict cancer outcome, it can also be used to predict patient's response to therapy. For example, aberrant expression of miR21 has been linked to resistance to chemotherapeutic agents VM-26 in glioblastoma cells (Li, Li et al. 2009), gemcitabine in pancreatic cancer (Giovannetti, Funel et al. 2010) and fluorourasil based agents in adenocarcinoma (Schetter, Leung et al. 2008).

## 1.12 MiRNAs and viruses

### 1.12.1 Virally encoded miRNAs

The first viral miRNAs were discovered in 2004 in Epstein-Barr virus (EBV), a human γ-gammaherpesvirus belonging to the family of herpesviruses (Pfeffer, Zavolan et al. 2004). Since then, multiple viral miRNAs have been discovered in members of all three herpesvirus subfamilies. From the α-herpesvirus subfamily, herpes simplex virus 1 (HSV-1), HSV-2, Marek's disease virus 1 (MDV-1), MDV-2, herpesvirus of turkeys (HVT) and infectious laryngotracheitis virus (ILTV) have been shown to express miRNAs; from the β-herpesvirus subfamily the human cytomegalovirus (hCMV) and mouse cytomegalovirus (mCMV) have been shown to express miRNAs; from the γ-herpesvirus subfamily Kaposi's sarcoma-associated herpesvirus (KSHV), mouse γ-herpesvirus, rhesus lymphocryptovirus (rLCV), rhesus monkey rhadinovirus (RRV), EBV and murine herpesvirus-68 (MHV-68) have been shown to express miRNAs (Skalsky and Cullen 2010). Although the majority of miRNAs found in viruses to date are from herpesviruses, some members of other DNA virus families, namely polyomavirus, ascovirus, baculovirus, iridovirus and adenovirus families, also express miRNAs (Cullen 2011; Kincaid and Sullivan 2012). Apart from bovine leukaemia virus (BLV), a member of the *Retroviridae* family, RNA viruses do not seem to contain miRNAs (Skalsky and Cullen 2010; Libri, Miesen et al. 2013). Two possible explanations why RNA viruses may not encode miRNAs are: First, majority of RNA viruses replicate in the cytoplasm and thus cannot access the host nuclear factors such as Drosha and DGCR8 required for miRNA biogenesis as opposite to miRNA-expressing double-stranded DNA viruses that replicate in the host nucleus. Second, processing of miRNAs from nuclear RNA viruses such as human immunodeficiency virus 1 (HIV-1) would mean the cleavage and degradation of the viral genomic RNA (Skalsky and Cullen 2010). A recently found exception of these rules is the bovine leukaemia virus, a retrovirus that replicates in the nucleus and has been shown to express 10 noncanonical RNA-polymerase III transcribed miRNAs (Kincaid, Burke et al. 2012; Rosewick, Momont et al. 2013). Since the noncanonical processing of these miRNAs does not involve Drosha or DGCR8, the viral RNA genome and viral mRNAs transcribed by RNA polymerase III escape cleavage and degradation involved in canonical miRNA biogenesis (Kincaid, Burke et al. 2012).

### 1.12.2 Functions of virally encoded miRNAs

Viral miRNAs can target both host mRNAs and viral mRNAs and are involved, together with other viral factors, in cellular reprogramming including: i) regulation of the latent-lytic switch of infection ii) modulation of immune responses iii) supporting viral replication by promoting cell survival, proliferation and/or differentiation (Skalsky and Cullen 2010; Takane and Kanai 2011). For example, infectious laryngotracheitis virus miRNA iltv-miR-I5 has been shown to participate in the regulation of latency by targeting the major transcriptional regulator ICP4 gene that is essential for viral growth and is repressed during latency (Waidner, Burnside et al. 2011). The iltv-miR-I5 lies

antisense to and is 100% complementary to its target ICP4 and regulates the ICP4 mRNA expression by direct endonucleolytic cleavage (Waidner, Burnside et al. 2011). Similar miRNA-driven regulation of the latent-lytic switch of infection has been also shown in other herpesviruses; EBV miR-BART2 regulates viral DNA polymerase BALF4 expression inhibiting the transition from latent to lytic infection (Barth, Pfuhl et al. 2008) and HSV-1 miR-H2-3p and miR-H6 regulate viral immediate early transcription activator ICP0 and HSV-1 ICP4, respectively, stabilizing viral latency (Umbach, Kramer et al. 2008). Viral miRNAs can also affect host gene expression in order to modulate the host cell environment into one more suitable for virus replication. For example, KSHV encodes four miRNAs that are strong suppressors of a particular host gene, thrombospondin 1 (THBS1) (Samols, Skalsky et al. 2007). THBS1 is a potent tumour suppressor gene and anti-angiogenic factor that functions via activating the latent form of transforming growth factor β (TGF-β). Thus, KSHV-encoded miRNAs contribute to the pathogenesis by decreasing TGF-β activity via down-regulation of THBS1 (Samols, Skalsky et al. 2007). KSHV also encodes an ortholog of human miR155, a critically important regulator in B-cell development and an oncogene which expression is frequently found to be up-regulated in lymphomas (Skalsky, Samols et al. 2007). KSHV-miR-K12-11 shares 100% seed sequence with human miR155 and they regulate common set of cellular mRNAs. It is thought that sustained KSHV-miR-K12-11 expression in KSHV infected B-cells regulates a set of miR155 targets in a developmental stage where miR155 is not normally expressed and thereby directly contributes to deregulated non-mature B-cell proliferation, potentially leading to lymphomagenesis (Skalsky, Samols et al. 2007).

### 1.12.3 Interplay between host cell-encoded miRNAs and viruses

Cellular miRNA expression is profoundly altered upon viral infection due to the alteration of cellular environment by viral factors as well as host antiviral defences (Skalsky and Cullen 2010). Cellular miRNAs have been shown to promote viral replication, also, viral factors have been shown to suppress individual cellular miRNAs as well as to suppress the global miRNA biogenesis, thereby indicating intricate interplay between cellular miRNAs and viral factors affecting replication (Cullen 2011). The first direct interaction among cellular miRNA and virus genome was identified between hepatitis C virus (HCV) and liver-specific miR122 (Jopling, Yi et al. 2005). HCV replication was shown to be dependent of miR122 expression and upon inhibition miR122 by 2'-O-methylated RNA oligonucleotide with exact complementarity to miR122 HCV replication was reduced drastically (Jopling, Yi et al. 2005). Several viruses, including KSHV and mCMV, as well as HIV-1, have been shown to up-regulate host cellular miR132 upon infection (Lagos, Pollara et al. 2010; Chiang, Liu et al. 2013). MiR132 targets transcriptional coactivator p300 and also a transcriptional regulatory protein methyl-CpG binding protein 2 (MeCP2) that in turn inhibits the expression of a number of interferon-stimulated genes, thereby resulting in enhanced replication of these viruses (Lagos, Pollara et al. 2010; Chiang, Liu et al. 2013). Adenoviruses encode a

noncoding RNA called VA1, a 160 nucleotide long structured RNA that is expressed at extremely high levels (approximately $10^8$ molecules/cell) during adenoviral replication (Mathews and Shenk 1991). The high level expression of VA1 in infected cells negatively affects the nuclear export of host pri-miRNAs by Exp5. VA1 can bind to Exp5 and inhibit pre-miRNA export possibly by outcompeting the host pre-miRNAs for Exp5 binding, thus downregulating the production of mature miRNAs (Lu and Cullen 2004). Human papillomavirus (HPV) can modulate expression of host miR203 and thus allowing viral replication to occur in differentiated epithelial cells (Melar-New and Laimins 2010). The HPV life cycle is closely associated with the differentiation program of skin epithelial cells; HPV establishes a latent infection in skin epithelial cells, but when these infected cells begin to differentiate into suprabasal cells, the late promoter of HPV is activated resulting in DNA amplification, late gene expression and start of the productive phase of the life viral cycle. Normally these differentiated suprabasal cells undergo cell cycle arrest mediated in part via the repression of p63 family of transcription factors by the host miR203, but when infected with HPV, viral E6 and E7 oncoproteins block the cell cycle arrest by downregulating miR203 expression, thus allowing differentiated cells to remain in the active state and enabling productive replication to occur (McKenna, McDade et al. 2010; Melar-New and Laimins 2010; Mighty and Laimins 2011).

## 1.13 Rational exploitation of miRNA machinery

### 1.13.1 MiRNA-regulated lentiviral vectors for gene therapy

The first report of exploiting miRNA-machinery for the regulation of a transgene came from the laboratory of Luigi Naldini. They showed that endogenous miRNAs could be used to specifically suppress transgene expression from lentiviral vectors in haematopoietic lineages and maintain expression in non-haematopoietic cells (Brown, Venneri et al. 2006). A major barrier to successful gene therapy has been the development of transgene-specific immunity where transgene expressing professional antigen-presenting cells (APCs) induce immune responses against the transgene. The hypothesis behind the first targeting experiment done in Naldini's laboratory was that by targeting the transgene to haematopoietic-specific miRNA could they prevent the transgene expression in haematopoietic lineages (including APCs) and thus, the induction of the immune responses against the transgene could be avoided enabling stable gene transfer. To test the hypothesis, they constructed a miRNA-regulated lentiviral vector by inserting four tandem copies of a sequence perfectly complementary to a haematopoietic-specific miRNA miR-142 in the 3' untranslated region (3'UTR) of green fluorescent protein (GFP). By developing transgenic mice using this novel virus they showed that the transgene expression was virtually undetectable in all haematopoietic cell lineages but detectable in non-haematopoietic cells. This virus was then systemically administered to immunocompetent mice and was shown to

enable stable gene transfer indicating that by using this novel miRNA-regulated lentiviral vector, high-level and stable expression of a transgene can be successfully established in an immunocompetent mice (Brown, Venneri et al. 2006). To further examine the efficacy of miRNA-regulated lentiviral vectors in a more relevant preclinical model of gene therapy, the same group showed that by using a haematopoietic-specific miR-142 targeted lentiviral vector expressing clotting factor IX (FIX) for the treatment of haemophilia B, it was possible obtain stable correction of the disease (Brown, Cantore et al. 2007). More recently, Matsui et al. showed that miR-142 targeted lentiviral vector pseudotyped with baculovirus GP64 envelope glycoprotein expressing clotting factor VIII (FVIII) was able to mediate stable transgene expression in haemophilia A mice systemically treated with the virus (Matsui, Hegadorn et al. 2011). Gentner et al. developed a miR126-regulated lentiviral vector where the expression of the transgene galactocerebrosidase was restricted in haematopoietic stem cells but robustly expressed in mature haematopoietic cells, thus allowing successful treatment of globoid cell leukodystrophy (Gentner, Visigalli et al. 2010). Apart from de-targeting the transgene expression from the cells of haematopoietic origin, neuronally expressed miRNA has also been exploited for selective lentiviral transgene expression. Colin et al. used neuron-specific miR124 to de-target transgene expression away from neuronal cells towards more restricted expression in astroglial cell populations of the mouse brain (Colin, Faideau et al. 2009).

### 1.13.2 MiRNA-targeted non-replicating virus vectors, virus-like replicon particles and viral amplicons

The first report using miRNA-targeted non-replicating virus vector originated from the laboratory of Hiroyuki Mizuguchi where they used non-replicating adenovirus (Ad) vector regulated by the liver-specific miR122 to de-target suicide gene expression from the liver (Suzuki, Sakurai et al. 2008). They showed that by combining intratumourally administered Ad vector expressing HSV thymidine kinase (Ad-HSVtk) and concomitant ganciclovir administration as a therapeutic strategy for cancer gene therapy, the growth of established tumours in mice could be dramatically suppressed. However, intratumourally injected Ad-HSVtk disseminated into the systemic circulation and efficiently transduced liver giving rise to HSVtk expression in the liver leading to severe hepatotoxicity following ganciclovir administration. By introducing four tandem copies for the liver-specific miR122 into the 3'UTR of the HSVtk expression cassette, the residual HSVtk expression in the liver was dramatically reduced following intratumourally administered miR122-targeted Ad-HSVtk without altering the antitumour effects (Suzuki, Sakurai et al. 2008). By using similar miR122-based targeting strategy, Geisler et al. developed miR122-regulated adeno-associated virus (AAV9) to decrease unwanted liver transgene expression upon cardiac gene transfer (Geisler, Jungmann et al. 2011). Xie et al. further improved the tissue tropism of AAV9 by inserting targets for another highly expressed and tissue-specific miRNA miR1 together with miR122. This miR1 and miR122-regulated virus was shown to have

restricted transgene expression in liver as well as in heart and skeletal muscles allowing more specific expression in the central nervous system (CNS) (Xie, Xie et al. 2011).

MiRNA-targeting has recently been exploited also in the production scheme of virus-like particles (VRP) for Venezuelan equine encephalitis virus (VEEV) vaccine development. Kamrud et al. introduced miRNA target elements for ubiquitously expressed miRNAs into the helper RNAs that provide the alphavirus structural proteins needed in the production of VRPs (Kamrud, Coffield et al. 2010). Since the expression of structural proteins from the helper RNAs is not intented to occur in any cells other than those used to produce VRPs, the targeting of helper RNAs with miRNAs that are evolutionary conserved and ubiquitous leads to the degradation of the targeted helper RNAs in a wide range of cell types. This miRNA-targeting of helper RNAs builds an additional barrier to reconstruction of a functional genome by making any recombinant RNA (as result of a potential recombination event between helper RNAs and replicon RNA) susceptible to miRNA-mediated RNA degradation present in host cells. Lee et al. developed miRNA-regulated helper virus-depended HSV-1-based treatment strategy for prostate cancer (Lee, Rennie et al. 2009). They created ICP4-expression amplicons that contained target elements for miR143 and miR145 in the 3'UTR of ICP4. The miRNAs miR143 and miR145 are expressed in normal tissues but downregulated in many types of cancer cells including prostate cancer. These amplicons can complement the replication of $ICP4^-$ helper virus when present in the same cell. By using this miRNA-targeted amplicon/helper-virus system in human prostate cancer xenograft mouse model, they showed increased tumour specificity and potent suppression of tumour growth as compared to non-targeted amplicon/helper-virus system.

### 1.13.3 MiRNA-targeted replication competent viruses

#### *1.13.3.1 MiRNA-targeted oncolytic viruses*

Oncolytic viruses have been designed to replicate preferentially in tumour cells leaving normal cells unharmed. However, significant toxicity and residual replication in normal tissues can still occur, often limiting their application. Since the discovery that miRNAs can potently suppress foreign genes, attempts have been made to exploit miRNA machinery in order to modify viral tropism of replication. The first reported oncolytic virus that was engineered to exploit miRNA machinery was vesicular stomatitis virus (VSV), a negative-sense RNA Rhabdovirus. Edge et al. incorporated three perfectly complementary target elements for miRNA Let7a into the 3'UTR of VSV matrix (M) gene. Let7a is ubiquitously expressed in normal tissues but has reduced expression in a wide variety of tumours. M protein has an essential role in VSV growth and replication, and it also serves to counteract antiviral responses (Edge, Falls et al. 2008). Edge et al. showed that this Let7a-targeted VSV, $VSV^{let\text{-}7wt}$, had reduced pathogenicity but retained potent antitumour activity in both immune competent and immunodeficient mice models.

Hikichi et al. also exploited Let7a to control the replication specificity of vaccinia virus, a double-stranded DNA virus of the Poxvirus family (Hikichi, Kidokoro et al. 2011). By inserting four target elements for Let7a into the 3'UTR of *B5R* gene, the product of which is essential for robust viral replication in tumours, they were able to develop a highly attenuated oncolytic vaccinia virus that selectively replicated in tumour cells without affecting normal cells. Let7a-targeting has also been used to control the replication specificity of oncolytic adenoviruses. Jin et al. inserted eight target elements for Let7a into the 3'UTR of E1A, a key gene associated with adenoviral replication (Jin, Lv et al. 2011). In that study, it was found that Let7a expression was significantly downregulated in a proportion of primary hepatocellular carcinoma (HCC) tissues as compared to the Let7a expression in normal liver. Based on those findings, SG7011$^{let7T}$, an oncolytic recombinant Ad5 adenovirus containing target elements for Let7a was developed for the treatment of HCC. *In vitro* characterization of SG7011$^{let7T}$ showed that in cell lines resembling normal liver cells (expressing high levels of Let7a) the replication of SG7011$^{let7T}$ was more than 300-fold reduced as compared to that of the wild-type adenovirus 5, whereas in HCC cell lines the replication was similar with both viruses. *In vivo* characterization of SG7011$^{let7T}$ revealed that the oncolytic potency was comparable to the wild-type adenovirus 5 in mice with xenograft tumours expressing reduced levels of Let7a.

In another study aiming at developing an oncolytic virus for the treatment of HCC, Fu et al. constructed an oncolytic Herpes Simplex virus (HSV), a DNA virus of the family *Herpesviridae*, that precisely targets HCC cells via the combined use of a strong liver-specific promoter and miRNA-mediated de-targeting (Fu, Rivera et al. 2012). This HCC targeted oncolytic HSV was constructed by linking the essential viral glycoprotein H gene with the liver-specific apolipoprotein E (apoE)-AAT promoter and by adding target elements for the liver-specific miRNA miR122 together with the neuron-specific miRNA miR124 and Let7a into the 3'UTR of the H gene. The resulting virus, called liver-cancer specific oncolytic virus (LCSOV), was shown to have selective replication tropism towards HCC cells that do not express miR122 and was also shown to leave normal hepatocytes unharmed. Indeed, liver-specific miRNA miR122 has been widely exploited to de-target the viral replication from the liver. This approach has been used especially for developing safer oncolytic adenoviruses, which is a central topic of this study (see later). Other authors who have been active in this field include Cawood et al., who could show that systemically administered miR122-targeted adenovirus 5 was dramatically less hepatotoxic as compared to wild-type adenovirus 5 when injected into mice. They further showed that the miR122-targeted virus had a potent anti-cancer activity in a mouse HepG2 xenograft model (Cawood, Chen et al. 2009; Cawood, Wong et al. 2011). Leja et al. combined the use of miR122 with the neuroendocrine-selective CgA promoter to drive the expression of E1A in order to further improve the selective replication of the miR122-targeted virus for the treatment of liver metastases (Leja, Nilsson et al. 2010). They showed that the replication specificity was enhanced

towards carcinoid cells and that the replication in liver was further reduced by combining miR122-targeting with the CgA promoter driving E1A expression. Sugio et al. combined human telomerase reverse transcriptase promoter driving E1-expression cassette and the incorporation of different combination of target elements for miR122 and for miRNAs that are ubiquitously expressed in normal cells (Sugio, Sakurai et al. 2011). The *in vitro* characterization of these different miRNA-targeted viruses showed significantly improved safety profiles as compared to the telomerase reverse transcriptase promoter-modified virus. Importantly, tumour cell lysis activity was shown to be unaltered.

The tropism of otherwise neurovirulent or neurotoxic viruses has also been successfully modified by the use of neuronally expressed miRNAs. Kelly et al. exploited the neuron-specific miRNA miR125b for the attenuation of VSV neuropathogenicity by incorporating four target elements for miR125b into the 3'UTR of the viral polymerase (L) gene (Kelly, Nace et al. 2010). They showed that intracranially administered miR125-targeted VSV had reduced neurotoxicity as compared to a control virus but retained its antitumour activity in a mouse CT-26 colorectal cancer model. Leber et al. used neuron-specific miR7, which is downregulated in gliomas but highly expressed in normal brain tissue, for the miRNA-targeting of an oncolytic measles virus (MV), a negative-stranded RNA virus of the family *Paramyxoviridae* (Leber, Bossow et al. 2011). By using a mouse strain highly susceptible for MV infection (CD46Ge Ifnar$^{ko}$) they showed that the neuropathogenicity of MV could be completely abrogated by the insertion of 3 target elements for miR7 into the 3'UTR of the viral fusion (*F*) gene. In addition, by using a mouse U87 glioblastoma xenograft model, they showed that the oncolytic potency of the miR7-targeted MV was fully retained.

Finally, muscle-specific miRNAs have also been used to decrease virus pathogenicity. Kelly et al. showed that the lethal myotropism of Coxsackievirus A21 (CVA21, a positive-strand RNA virus of the family *Picornaviridae*) could be prevented by the addition of miRNA target elements for two muscle-specific miRNAs, miR206 and miR133, into the 3'UTR of the viral genome (Kelly, Hadac et al. 2008). They showed in mice that CVA21 is a potent oncolytic virus mediating rapid regression of large tumours over a short time span, which, unfortunately, is followed by severe and progressive muscle paralysis within 12 days of virus infection. However, this myositis could be prevented by the use of muscle-specific miRNA-targeted CVA21 (miRT CVA21). Tumour bearing mice treated with miRT CVA21 showed significantly increased survival as compared to the group of mice treated with CVA21 while retaining its potent antitumour activity.

#### *1.13.3.2 MiRNA-targeted viruses for vaccine development*

Live attenuated virus vaccines are an effective method of immunisation since viral replication elicits strong immune response activating all the components of the immune system. MiRNAs can be exploited in the rational engineering of attenuated

virus vaccines. Indeed, the very first example of miRNA-targeted replication competent virus came from the vaccine studies by the laboratory of Raul Andino, as they showed that poliovirus (a positive-strand RNA virus of the family *Picornaviridae*) replication can be attenuated by the use of Let7a target element inserted into the viral genome (Gitlin, Stone et al. 2005). In a subsequent study they showed that poliovirus replication in the mouse brain can be specifically attenuated by incorporating miRNA target elements for miR124 into the poliovirus genome (Barnes, Kunitomi et al. 2008). This miR124-targeted poliovirus (PV-124) replicated robustly in non-neuronal tissues, but was suppressed in the CNS leading to attenuated neuropathogenicity in the infected mice. Infection with PV-124 induced comparable neutralizing antibody titres to those observed for the Sabin-vaccine strain and immunized mice were protected against lethal challenges of pathogenic wild-type virus. Perez et al. developed a miRNA-mediated complementary attenuation strategy for influenza A virus (segmented negative-strand RNA virus of the family *Orthomyxoviridae*) to increase attenuation and improve vaccine safety (Perez, Pham et al. 2009). By incorporating miRNA target elements for miR93, which is expressed ubiquitously in both human and mouse lung tissues but not in chicken, into the open-reading frame of the viral nucleoprotein, they generated life attenuated influenza vaccines that were attenuated in mice but not in the chicken eggs they were produced in.

## 1.14 Adenovirus 5 biology

Adenovirus virion is a nonenveloped icosahedral particle of 70-90 nm in size with an outer capsid surrounding an inner nucleoprotein core. The capsid is consisting of three major proteins; hexon, penton base and a knobbed fibre and four associating minor proteins; VI, VIII, IX, and IIIa. Six other structural components are situated in the virus nucleoprotein core V, VII, Mu, Iva2, Terminal protein TP and 23K virion protease. From these proteins, V, VII, Mu, Iva2 and TP are associated with the double-stranded DNA genome. The linear DNA is approximately 36 kilobases long and contains inverted terminal repeats (IVR) at both ends to which the terminal protein TP is covalently linked. Genes are encoded on both strands of the DNA with overlapping transcription units (McConnell and Imperiale 2004; Russell 2009).

Adenovirus 5 initial attachment to the cell surface occurs through binding of the fiber knob to the cocsackievirus B and adenovirus receptor (CAR) that is expressed ubiquitously in various human tissues including liver, heart, lung and brain (Meier and Greber 2004). CAR, a type 1 transmembrane protein of the immunoglobulin superfamily, functions normally as a cell-to-cell adhesion molecule on tight junctions and basolateral surface of epithelial cells. After binding to the CAR receptor, an exposed RGD motif on the penton base interacts with αvβ3 or αvβ5 integrin receptors triggering the virus internalization by chlatrin-dependent, receptor mediated

endocytosis (McConnell and Imperiale 2004). The acidic pH of the endosomes triggers virus to penetrate into the cytosol and allows the virus to engage microtubule-associated motors for movements towards the nuclear envelope for docking at the cytosolic side of the nuclear pore complex (NPC) (Greber, Willetts et al. 1993; Suomalainen and Greber 2013). Disassembly of the capsid at the NPC allows for nuclear entry of the viral genome and subsequent start of the viral transcriptional program (McConnell and Imperiale 2004). The first viral transcription unit that is expressed upon infection is E1A. Transcription of E1A leads to multiple mRNA and protein isoforms through differential mRNA processing. Two E1A protein species are produced during early infection: 289R and 243R proteins. These proteins transactivate the other early transcription units E1B, E2, E3 and E4 and induce the host cell to enter the S-phase for optimal virus replication (McConnell and Imperiale 2004). The main mechanism by which E1A forces host cell to enter S-phase is the ability of 289R and 243R to sequester hypophosphorylated pRb that is in complex with transcription factors of the E2F family. The binding of E1A to the pRb releases these transcription factors (E2F and DP) resulting in a stimulation of E2F-dependent transcription and entry into S-phase (Frisch and Mymryk 2002). The E1B transcription unit produces two proteins: E1B-55K and E1B-19K. E1B-55K acts to block p53-dependent apoptosis by directly binding to p53 and abrogating its ability to induce expression of proapoptotic genes. E1B-19K is involved in prolonging cell survival by interacting and ablating the members of the proapoptotic Bax family proteins. The E2 transcription unit produces three proteins: 72 kDa single stranded DNA-binding protein, preterminal protein and DNA polymerase. These proteins are necessary for the replication of the viral genome. The E3 transcription unit produces at least seven different proteins: Adenovirus death protein (ADP), E3-6.7K, E3-12.5K, E3-gp19K, E3-10.4K, E3-14.5K and E3-14.7K. The E3-gp19K is localised in the endoplasmic reticulum (ER) membrane and prevents the translocation of MHC class I heavy chain to the cell surface. The E3-10.4K, E2-14.5K and E3-14.7K have been shown to inhibit the induction of apoptosis by various chemokines. ADP is required for efficient virus release (Russell 2000; Lichtenstein, Toth et al. 2004; McConnell and Imperiale 2004). The E4 transcription unit produces 7 proteins: E4orf1, E4orf2, E4orf3, E4orf3/4, E4orf4, E4orf6 and E4orf6/7. These proteins mainly facilitate viral mRNA metabolism, promote DNA synthesis and shut-off of host protein synthesis (Weitzman 2005). The expression of early genes modulates the host cell to facilitate the genome replication and resultant transcription and translation of the late genes. The late genes are transcribed from the major late promoter (MLP) that produces a 28 kb-long nuclear RNA precursor which is processed into at least 16 different mRNAs that can be arranged into five 3'-coterminal families, L1-L5 (Le Moullec, Akusjarvi et al. 1983). These mRNAs encode the structural components and other proteins involved in virion assembly (Russell 2000).

### 1.14.1 Adenoviral vectors in cancer virotherapy

Adenoviruses have gained a great deal of interest as cancer therapeutics because they can be easily manipulated and exhibit oncolytic properties that can be expanded throughout the tumour mass by viral replication and following spread of the progeny viruses (Choi, Lee et al. 2012). Multiple different strategies have been used to develop tissue restricted and more potent oncolytic adenoviruses. ONYX-015, the first oncolytic adenovirus used to treat human cancers, was generated by deleting E1B-55K gene that rendered the replication slightly more towards p53-deficient cancer cells (Heise, Sampson-Johannes et al. 1997; Kirn, Hermiston et al. 1998). Control of viral gene expression by tissue-specific or tumour-specific promoters (TSPs) has also been exploited. Since E1A is the first gene to be transcribed after infection and essential for viral replication, control of E1A expression by TSPs has been widely used (Choi, Lee et al. 2012). Mutation of E1A by ablating its affinity towards pRb (E1A-Δ24) renders viral replication towards cells with transformed phenotype (e.g tumour cells) (Stolarek, Gomez-Manzano et al. 2004). Transductional targeting has also been exploited to increase infection towards tumour cells or decrease infectivity towards normal cells. Ad5 fibre (which binds to the CAR receptor often modestly expressed in the surface of tumour cells) has been changed to fibres of various other serotypes in order to enhance transduction of tumour cells and to ablate the dependency of the CAR receptor for virus entry (Ranki and Hemminki 2010). Selected modifications used in clinical trials are shown in table 3.

**Table 3. Modifications of adenoviruses used in the clinics.**

| Virus | Modification | Effect | Reference |
|---|---|---|---|
| Adenovirus | E1A-Δ24 | Replicates in cells with downregulated pRb function or abnormal cell cycle control mechanisms | (Stolarek, Gomez-Manzano et al. 2004) |
| | Δ E1B-55k | Resticted replication towards tumour cells | (Heise, Sampson-Johannes et al. 1997) |
| | hTERT promoter driving E1A and E1B expression | Resticted replication towards telomerase expressing cells → tumour cells | (Kawashima, Kagawa et al. 2004) |
| | PPT artificial promoter driving E1A expression | Prostate-specific E1A expression and viral replication | (Cheng, Dzojic et al. 2006) |
| | Ad5 with serotype 3 knob | Targeting adenovirus 5 to the serotype 3 receptor → increased tumour targeting | (Kanerva, Mikheeva et al. 2002) |
| | GM-CSF gene | Enhances immune responses towards cancer cells | (Koski, Kangasniemi et al. 2010) |
| | bispecific adapter sCARhMFE | Targeting transduction towards CEA-expressing tumours | (Li, Everts et al. 2009) |

## 1.15 Semliki Forest virus biology

Semliki Forest virus (SFV) virion is an enveloped icosahedral particle of 65-70nm in size. The genome is composed of a single-strand positive-sense RNA genome approximately 11.5 kb in length. The viral RNA encodes four non-structural proteins (nsp1-4) involved in virus replication and five structural proteins (E1, E2, E3, 6K and capsid) that compose the virion. The viral RNA is encapsidated by the capsid proteins forming a nucleocapsid (NC) that in turn is enveloped in host-derived lipid bilayer membrane on which the viral glycoproteins are situated (Jose, Snyder et al. 2009).

SFV enters the host cell via clathrin-mediated endocytosis (Helenius, Kartenbeck et al. 1980; Helenius, Kielian et al. 1985). Both E2 and E1 spike glycoproteins are involved in the entry: binding to the host receptor induces conformational changes in E2 and E1 proteins and the virion is subsequently endocytosed into the cell (Kielian and Helenius 1985). Inside the cell the virion is in a vesicle called endosome. As the endosome matures to late endosome, it becomes more acidic leading to the destabilization of E2-E1 complex and subsequent exposure of a fusion loop at the tip of the E1 protein. This fusion peptide is inserted into the late endosomal membrane leading to the disassembly of viral spikes and the formation of E1 homotrimers (Li, Jose et al. 2010). This causes the fusion of viral envelope with the late endosomal membrane allowing the virus capsid to be released into the cytoplasm. In the cytoplasm the unenveloped capsid is disassembled and viral RNA is released (Wengler, Koschinski et al. 2003). Three different RNA species is produced from the released viral RNA: positive-strand viral genomic RNA, negative-strand RNA and subgenomic RNA that is used for translation of the structural proteins (Salonen, Ahola et al. 2005). The non-structural proteins are initially translated from the viral RNA as a single polypeptide P1234. This polypeptide is then processed by a protease domain in the nsp2 into mature nsp-proteins as well as cleavage intermediates that have specific functions in virus replication. Nsp4 and the cleavage intermediate P123 produces negative-strand RNA as a first RNA species produced during the infection (Jose, Snyder et al. 2009). As the infection proceeds, the negative-strand RNA production decreases as the continuous nsp2 processing cleaves the intermediates to mature nsp1-3 proteins. The fully matured nsp1-4 proteins form the late replicase that efficiently produces both full length genomic RNA and the subgenomic RNA for the production of structural proteins: capsid (C), E3, E2, 6K and E1 (Leung, Ng et al. 2011). The cleavage of the structural polypeptide occurs cotranslationally and begins with the autoproteolytic cleavage of the capsid protein from the remaining polypeptide. As capsid proteins begin to associate with the newly synthesised genomic RNA, the N-terminus of p62, the precursor of the E3 and E2 proteins, acts as a signal sequence for insertion of the polypeptide into the endoplasmic reticulum (ER) where it hetero-oligomerizes with E1. p62 is processed into E3 and E2 by a host furin-like protease late during the transport in the secretory pathway. Finally the matured E1 and E2 glycoproteins are transported to the cell surface via the secretory route (Melancon and Garoff 1987; Leung, Ng et al.

2011). The genomic RNA containing nucleocapsids assembled in the cytoplasm move to the plasma membrane where they interact with membrane bound viral glycoproteins and acquire the lipid bilayer during budding of the virions (Strauss and Strauss 1994).

### 1.15.1 Semliki Forest virus vectors in cancer virotherapy

Non-replicating SFV vectors have been widely used in cancer virotherapy as these vectors can induce antitumoural activities through apoptosis or induction of immune responses against tumour cells. SFV vectors have been engineered to express various cytokines for the enhancement of antitumour immune responses (Quetglas, Ruiz-Guillen et al. 2010). Various oncolytic SFVs have also been tested in preclinical models of cancer virotherapy. One of the most studied oncolytic SFV is the VA7-EGFP vector, a derivative of the naturally attenuated A7(74) strain (Vaha-Koskela, Tuittila et al. 2003). Preclinical studies using this virus have shown promising efficacy against various human tumours in xenograft mice models (Vaha-Koskela, Kallio et al. 2006; Ketola, Hinkkanen et al. 2008; Maatta, Makinen et al. 2008; Heikkila, Vaha-Koskela et al. 2010). However, recently it has been shown that the potent antitumour efficacy obtained in immunocompromised mice models do not translate into efficacy in immunocompetent mice models harbouring syngeneic tumours (Ruotsalainen, Martikainen et al. 2012; Vaha-Koskela, Le Boeuf et al. 2013). Unexpectedly, in both of these studies it was also reported that the attenuated SFV strains used to treat syngeneic experimental glioma, the viruses were shown to infect normal brain tissue as opposed to tumour tissue, necessitating further increase of the safety of these vectors via e.g. miRNA-mediated neuronal attenuation.

# 2 AIMS OF THE STUDY

The aim of this doctoral thesis was to investigate whether tissue-specific miRNA expression patterns could be exploited to modify the tissue tropism of oncolytic viruses for increased safety. The specific aims were:

1. To study whether liver-specific miR122 expression could be exploited to de-target adenovirus replication from the liver
2. To study whether neuron-specific miR124 expression could be exploited to de-target Semliki Forest virus replication from neurons

# 3 MATERIALS AND METHODS

## 3.1 Cell lines and human liver and tumour tissues

All cell lines used in this study were cultured in the recommended growth media and maintained in a humidified atmosphere in the presence of 5% $CO_2$ at 37 ºC. Table 3 summarizes the cell lines used.

**Table 3. Cell lines used in this doctoral thesis.**

| Cell line | Description | Source | Study |
|---|---|---|---|
| Huh7 | Human hepatocellular carcinoma | Mark Harris | I-III |
| HeLa | Human cervical cancer | ATCC | I,III |
| PC3 | Human prostate cancer | ATCC | I |
| A549 | Human lung cancer | ATCC | I,II |
| 293 | Human embryonic kidney | Microbix | II |
| 293FT | Human embryonic kidney | Life technologies | I |
| Tera-2 | Human embryonal carcinoma | ATCC | III |
| U87 | Human primary glioblastoma | ATCC | III |
| BHK-21 | Syrian hamster kidney fibroblast | ATCC | III |
| Vero | African green monkey fibroblast | University of Turku | III |
| HCT116 | Human colorectal cancer | ATCC | II |
| HEp-2 | HeLa derivative cell line | ATCC | II |

Healthy human liver and colorectal carcinoma samples (**II**) were received from patients undergoing a surgical removal of liver metastasised colorectal carcinoma. Healthy liver was obtained from the resection marginal of the removed tumours. The tissue samples were sliced at 300-500 µm with a Krumdieck precision-cut tissue slicer (Alabama Research and Development Corporation) and cultured on a plate rocker in a humidified atmosphere in the presence of 5% $CO_2$ at 37 ºC. Culture media used was William's medium E (Life Technologies) supplemented with 10% foetal calf serum and 1% Glutamax-I (Life Technologies) and 1% penicillin/streptomycin.

## 3.2 Construction of miRNA-targeted viruses (I-III)

A shuttle vector pSEMII was created from pSE1 (Bauerschmitz, Guse et al. 2006) by engineering a wild-type E1A promoter/enhancer region to drive wild-type E1A expression and a novel MluI site into the 3'UTR of the E1A transcript. A miRNA-targeted shuttle vector pShuttle122 was derived from pSEMII by inserting three target elements for miR122 into the novel MluI site. The target elements were cloned using annealed oligonucleotides (5'- CGCGTGGAGTGTGACAATGGTGTTTGTACCGGT -3' and 5'-CGCGACCGGTACAAACACCATTGTCACACTCCA -3') including the miR122 target element. pShuttleK and pShuttleK-122 was derived from pSEMII and pShuttle122, respectively, by inserting annealed oligonucleotides (5'-CGCGCACCATGGTGTCGACG-3' and 5'-CGCGCGTCGACACCATGGTG-3') into the 5'UTR of the endogenous starts codon (ATG) to create an additional out-of-frame start codon. To create Ad5/3-122 (**I**), a homologous recombination between pShuttle122 and Ad5/3-Δ24 (Kanerva, Mikheeva et al. 2002)

was carried out. To create Ad5/3K (**I**) and Ad5/3K-122 (**I**), a homologous recombination between pShuttleK or pShuttleK-122 and Ad5/3-Δ was carried out respectively. pShuttle 6x122 (**II**) was made similarly to pShuttle122 except instead of three miR122 target elements, six target elements were inserted into the MluI site in the 3'UTR of the E1A transcript. To create Ad5T122 (**II**), a homologous recombination between pShuttle 6x122 and Ad5-Δ24 (Fueyo, Gomez-Manzano et al. 2000) was carried out. The amplification of infectious adenoviral particles were carried out using A549 cells and purified by ultracentrifugation using double cesium chloride gradient or by column filtration (Takara Bio Incorporation). Titres of the purified virus stocks were determined by a 50% tissue culture infectious dose ($TCID_{50}$) method using 293 cells.

To create SFV4-miRT122 and SFV4-miRT124 viruses (**III**), a plasmid containing the genomic sequence of SFV4 (Liljestrom, Lusa et al. 1991) was modified by engineering a novel NdeI site upstream of the sequence coding for protease cleavage site locating between nsp3 and nsp4 coding regions. A synthetic DNA sequence including duplicated sequence coding for protease cleavage site and sequences for six target elements for miR122 or miR124 was inserted between upstream naturally existing XhoI site and the novel NdeI site resulting in a plasmid carrying six miRNA-target elements flanked by sequences coding for the protease cleavage sites. All SFV4 viruses were produced from *in vitro* transcribed RNA by transfection into BHK-21 cells and subsequently titrated by plaque titration method using Vero cells. The correct sequences of miRNA target elements of all viruses created were confirmed by sequencing. All viruses used in this doctoral thesis are summarized in table 4.

**Table 4. Viruses used in this doctoral thesis.**

| Virus | Description | Source | Study |
|---|---|---|---|
| Ad5/3-122 | Ad5 virus with 3xmiR122 target elements in the 3'UTR of E1A + chimeric receptor-binding knob domain of Ad3 | This study | I |
| Ad5/3-Δ24 | Ad5 virus with 24-bp deletion in CR2 of E1A + chimeric receptor-binding knob domain of Ad3 | (Kanerva, Zinn et al. 2003) | I |
| Ad5/3K | Ad5 virus with low E1A expression phenotype + chimeric receptor-binding knob domain of Ad3 | This study | I |
| Ad5/3K122 | Ad5 virus with 3xmiR122 target elements in the 3'UTR of E1A + low E1A expression phenotype + chimeric receptor-binding knob domain of Ad3 | This study | I |
| Ad5T122 | Ad5 virus with 6xmiR122 target elements in the 3'UTR of E1A | This study | II |
| Ad5Luc1 | Non-replicating Ad5 virus with luciferase gene under CMV promoter replacing E1 region | (Krasnykh, Belousova et al. 2001) | II |
| Ad5 | wild type Adenovirus 5 | | II |
| SFV4-miRT122 | SFV4 virus with 6xmiR122 target elements between nsp3 and nsp4 | This study | III |
| SFV4-miRT124 | SFV4 virus with 6xmiR124 target elements between nsp3 and nsp4 | This study | III |
| SFV4 | wild type SFV4 virus | (Liljestrom, Lusa et al. 1991) | III |

## 3.3 Reporter plasmids (I)

Variable copy number of miR122 target elements were introduced by annealing and ligating oligonucleotides 5'- CGCGTGGAGTGTGACAATGGTGTTTGTACCGGT -3' and 5'- CGCGACCGGTACAAACACCATTGTCACACTCCA -3' into the 5'UTR or 3'UTR of firefly luciferase cDNA in the pcDNA3-derived plasmid pSIRNALUC.

## 3.4 Animals (II, III)

Animal experiments were approved by the Finnish Committee for Care and Use of Animals in Experiments (**III**) and the experimental animal committee of the University of Helsinki and the provincial government of Southern Finland (**II**). 3-5 week-old BALB/c mice were purchased from Taconic. 3-5 week-old NMRI nude mice were purchased from Harlan laboratories. 3-5 week-old C57bl/6 mice were purchased from the Laboratory Animal Center of the University of Helsinki. All animals were quarantined for two weeks preceding the experiments.

## 3.5 *In vitro* experiments

### 3.5.1 Dual-luciferase assays (I, II)

Empty or miR122 target elements containing reporter plasmids were transfected together with pCDNA-Renilla and 24 to 48 h posttransfection luciferase activities where measured using a dual-luciferase assay system (Promega). Reporter plasmid transfections were also done in the presence of miRIDIAN miR122 inhibitor (Dharmacon) at final concentration of 100 nM (**I**).

### 3.5.2 Cell viability assays (I-III)

Huh7, HeLa, PC3 and A549 cell lines (**I**) or Huh7, HTC116, Hep-2 and A549 cell lines (**II**) were infected with different adenoviruses at MOI of 0.05 or Huh7, BHK-21, HeLa, Tera-2 and U87 cell lines (**III**) were infected with different SFVs at MOI of 0.1 and cell viability was measured 24 h to 9 days postinfection using CellTiter 96 $AQ_{ueous}$ One solution cell proliferation assay (Promega). A multiwell plate reader (Multiscan EX, Thermo Fisher Scientific) was used to determine the optical density of the reactions at 492 nm.

### 3.5.3 Quantification of viral replication (I-III)

Infected A549 and Huh7 cells (**I**) were harvested at three (A549) and five (Huh7) days postinfection and the amount of cell-associated infectious virus was quantified. Supernatant of the infected cultured human liver and liver metastatic colorectal cancer tissue slices (**II**) were harvested at 1h, 1d, 3d and 5d and the amount of infectious virus was quantified. All adenovirus titres were determined by $TCID_{50}$. Briefly, 293 or 293FT cells were seeded in 96-well plates ($10^4$ cells/well) and the next day eight serial 10-fold dilutions of viral samples were added to the 96-well plates. After 10 days of incubation the wells with CPE were counted and viral titres were calculated by the Spearman-Kärber method. For Semliki Forest virus titrations of mouse tissues (**III**), snap-frozen brain and liver samples were homogenised in 1% bovine serum albumin (BSA)-phosphate buffered saline (PBS) and centrifuged at 4500 g for 5 minutes. The collected supernatant was used for virus titration. All Semliki Forest virus titres were determined by plaque titration.

### 3.5.4 Western blot analysis (I, III)

Infected cells were lysed with 1% NP-40 lysis buffer (150 mM NaCl; 50 mM Tris-HCl, pH 7.9; 1% Nonidet P-40) in the presence of protease inhibitors. Protein concentrations were determined by the Bio-Rad Protein Assay (Bio-Rad), and 50 µg (**I**) or 10 µg (**II**) of each sample was separated by SDS-PAGE and then blotted onto a nitrocellulose membrane (Bio-Rad). After blocking overnight at 4 ºC with PBS + 0.05% Tween 20 containing 5% non-fat dry milk, the membranes were treated with mouse monoclonal antibody specific to adenoviral E1A proteins (Santa Cruz Biotechnology) or adenovirus infected patient serum (a kind gift from Jukka Suni, HUSLAB, Helsinki) as primary antibodies (**I**) or treated with rabbit polyclonal antibodies against nsp3 and nsp4 (kindly donated by Dr. Tero Ahola, University of Helsinki) or rabbit anti-SFV against envelope and capsid proteins as primary antibodies (**III**) and then with horseradish peroxidase-conjugated secondary antibodies against mouse or human immunoglobulins (DakoCytomation) (**I**) or with IRdye 800 secondary antibody against rabbit immunoglobulins (LI-COR) (**III**). Enhanced chemiluminence detection system (ECL; Millipore) (**I**) or Odyssey infrared imaging system (LI-COR) (**III**) was used for detection and quantification of viral proteins.

### 3.5.5 Quantitative RT-PCR (I, III)

Total RNA was extracted from infected cells using GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich). A RevertAid M-MuLV reverse transcriptase (Thermo Fisher Scientific) was used for cDNA synthesis (**I, III**). In the study (**I**): Oligonucleotide sequences for amplification of E1A S13 gene were: forward primer 5'-TCCGGAGCCGCCTCACCTTTC-3' and reverse primer 5'-GGCTCAGGTTCAGACACAGGACTGTAG-3'. Oligonucleotide sequences for amplification of the human house-keeping gene GAPDH were: forward 5'-GAGTCAACGGATTTGGTCGT-3' and reverse 5'-TTGATTTTGGAGGGATCTCG-3'. PCR was run using a LightCycler Instrument (Roche) with DyNAmo™ Capillary SYBR® Green qPCR Kit (Finnzymes). After an initial incubation at 95°C for 10 min, 35 amplification cycles were conducted as follows: denaturation at 95°C for 10 s, annealing at 55°C for 10 s, and extension at 72°C for 12 s. To quantify the differences of E1A mRNA levels in Huh7 cells the LightCycler data point curves were compared to similar analyses obtained using a dilution standard, and graphed as percentage relative to the specimen with highest expression. In the study (**III**): Oligonucleotide sequences for amplification of SFV positive strand genome were: forward primer 5'-TGGAGCTGACCACAGACTTG-3' and reverse primer 5'- GGCCACAACGTCAGTATCTC-3'. PCR was run using an Mx3005p QPCR System (Stratagene) with Power SYBR green PCR master mix (Applied Biosystems). After an initial incubation at 95°C for 10 min, 40 amplification cycles were conducted as follows: denaturation at 95°C for 15 s, annealing and extension at 60°C for 1min.

### 3.5.6 Immunohistochemistry (II, III)

Brain tissue samples from mice displaying neurological symptoms (**III**) or human liver and liver metastatic colorectal cancer tissue slices (**II**) were fixed and embedded into paraffin blocks. The samples were cut into 7-µm sections using microtome and IHC analyses were performed using a Vectastain ABC kit (Vector laboratories) with a polyclonal anti-SFV antibody (II) or using Dako envision system (Dako) with an antibody against adenovirus 5 E1A protein (Thermo Fisher Scientific). Detailed descriptions of sample preparations can be found in the original publications.

## 3.6 *In vivo* experiments

### 3.6.1 Animal experiments in study II

For the experiments assessing liver toxicity, adult 5-7 week old female C57bl/6 mice were systemically infected with $1x10^9$ PFU of Ad5 or Ad5T122 by tail vein injection. After 72h post-infection blood was taken from mice by cardiac puncture and serum samples were added to ALAT reagent (Thermo Fisher Scientific) and the change in absorbance (340nm) per minute was monitored. Units of ALAT activity were calculated according to manufacturer's instructions. For the experiments assessing oncolytic potency of the viruses, adult 5-7 week old female NMRI nude mice were subcutaneously injected with $3x10^6$ A549 cells into both flanks and animals were

monitored for tumour appearance. Mice were treated on days 0, 2 and 4 by intratumoural injection at $10^7$ PFU/tumour with either Ad5 or Ad5T122. Tumour volumes were measured using hand-held calliper and MedCalc software was used to calculate serial measurements for statistical analysis. More detailed description of the experiments can be found in the original publication (**II**).

### 3.6.2 Animal experiments in study III

Adult 5-7 week old female Balb/c-mice (Taconic) were infected with SFV4, SFV4-miRT122 or SFV4-miRT124 by injecting $1x10^6$ PFU intraperitoneally or $100\text{-}1x10^5$ PFU intracranially. For intracranial injections mice were sedated and kept under isoflurane gas anesthesia. Viruses were injected into the caudate putamen of mice. After virus injection mice were monitored for neurological symptoms and distress. Symptoms were graded as follows: 0: no symptoms; 1: mild symptoms (ruffled fur, hunched back, weakness of limbs, optic neuritis); 2: partial paralysis of hind limbs; 3: paralysis of hind or front limbs, 4: severe paralysis, tetraplegia, 5: moribund/dead. In the case of severe neurological symptoms, paralysis (grade 3 or higher) or distress mice were sacrificed and tissue samples (brain, liver and blood) were collected. More detailed description of the experiments can be found in the original publication (**III**).

# 4 RESULTS

## 4.1 Development of liver de-targeted adenoviruses (I, II)

### 4.1.1 Targeted destruction of adenovirus E1A mRNA by liver-specific miR122 (I)

To test the potential of miR122 to suppress foreign genes containing miR122 target elements perfectly complementary to miR122 sequence, we constructed reporter plasmids that contained variable copy number of miR122 target elements in the 5' or 3'UTR of a firefly luciferase (FFluc) gene (see fig. 1A in **I**). Dual-luciferase assays with plasmids containing miR122 target elements showed that increasing copy number of target elements had cumulative effect on the suppression of the FFluc expression in liver-derived Huh7 cells expressing high amounts of miR122 but not in non-hepatic cells devoid of miR122 expression. Co-transfection with synthetic miR122 inhibitor (antagomiR-122) released the suppression of FFluc expression in Huh7 cells confirming that the inhibitory effect of miR122 target elements was specifically mediated by miR122 (see figs. 1B and C in **I**).

To test whether miR122 target elements could suppress the expression of E1A of a replication-competent adenovirus 5, we constructed an adenovirus containing three target elements for miR122 in the 3' UTR of E1A gene (Ad5/3-122). E1A protein levels in Ad5/3-122-infected Huh7 cells where drastically lower than in Ad5/3-Δ24-infected Huh7 cells whereas in A549 cells the E1A levels were similar with both viruses indicating that miRNA-mediated suppression of E1A in the context of a replication-competent adenovirus can occur (see fig. 2A in **I**). To further test if the miRNA-mediated suppression of E1A was due to reduced levels of E1A mRNA rather than reduced E1A translation, RNA was extracted from Huh7 and A549 cell cultures infected for 1-3 days with Ad5/3-Δ24 or Ad5/3-122 viruses and E1A mRNA levels were measured by quantitative RT-PCR. The levels of E1A mRNA produced in A549 cells were similar for both viruses whereas E1A mRNA levels of Ad5/3-122 produced in Huh7 cells were significantly reduced compared to that of Ad5/3-Δ24 (see fig. 2B in **I**), indicating that the miRNA-mediated suppression occurs via mRNA destruction (endonucleolytic cleavage-pathway) rather than via translational repression.

Despite the very potent and miR122-specific suppression of E1A mRNA levels, the viral replication of Ad5/3-122 was only moderately attenuated as compared to Ad5/3-Δ24 in Huh7 cells based on cell viability assays and the notion that the levels and expression of total adenoviral proteins revealed similar patterns for both viruses (see fig. 2A in **I**). Thus, although E1A levels could be drastically reduced by miRNA-mediated suppression in Huh7 cells, the level of reduction was not sufficient to have a significant impact on viral replication. In order to overcome this problem, we generated an adenovirus that has a uniformly reduced E1A expression in all cell types by inserting an ectopic translation initiation site into the 5'UTR of E1A mRNA in the wrong reading frame relative to E1A reading frame. This adenovirus, named Ad5/3K, replicated similarly to

Ad5/3-Δ24 in both Huh7 and A549 cells despite the reduced E1A expression (see fig. 3A in **I**). Based on this finding, we generated a miRNA-targeted derivative of Ad5/3K containing three target elements for miR122 in the 3' UTR of E1A gene (Ad5/3K-122). The infection of Huh7 cells with Ad5/3K or Ad5/3K-122 displayed dramatic differences in the E1A protein levels. The E1A levels in the Ad5/3K-infected Huh7 cells increased gradually over the 5-day incubation period, whereas the E1A levels in the Ad5/3K-122-infected Huh7 cells remained undetectable in all time points examined (see fig. 3B in **I**). Also, in contrast to the increasing levels of total adenoviral proteins in Ad5/3K-infected Huh7 cells, the levels of total adenoviral proteins in Ad5/3K-122-infected Huh7 cells remained undetectable in all time points examined (see fig. 3B in **I**). Importantly, no difference in viral protein levels between Ad5/3K-infected A549 cells and Ad5/3K-122-infected A549 cells was observed (see fig. 3B in **I**). In agreement with the undetectable levels of adenoviral proteins in Ad5/3K-122-infected Huh7 cells, infection with Ad5/3K-122 did not induce detectable cytopathic effect (CPE) in Huh7 cells even upon 2-week observation period. In other cell lines tested the CPE induced with Ad5/3K-122 infection was similar to CPE induced with Ad5/3K infection (see fig.4A in **I**).

To quantify the suppression of replication by miR122, we measured the cell-associated infectious virus produced by Huh7 and A549 cells infected with Ad5/3K-122 or Ad5/3K. Five days postinfection with the viruses at a MOI of 0.05, only 79 PFU/ml of Ad5/3K-122 compared to $1.0x10^6$ PFU/ml of Ad5/3K could be recovered from Huh7 cells. In A549 cells, both viruses had similar rate of replication confirming miR122-specific suppression of Ad5/3K-122 replication (see fig. 5 in **I**).

### 4.1.2 Construction and *in vitro* characterization of miR122-targeted adenovirus based on serotype 5 (II)

To generate a miRNA-targeted derivative of wild-type adenovirus 5 (Ad5T122), we inserted six target elements for miR122 in the 3' UTR of E1A gene (see fig. 1 in **II**). To compare the oncolytic potency of Ad5T122 and wild-type adenovirus 5 (Ad5) *in vitro*, a panel of cancer cell lines where infected with these viruses and a non-replicating control virus (Ad5Luc1) and the amount of lytic cell killing was measured using a cell viability assay. As expected, the non-replicating A5Luc1 did not have a significant effect on the cell viability of any of the cell types tested. Infection with Ad5 lead to potent lysis in all the cell lines tested. Infection of the non-hepatic cell lines with Ad5T122 lead to lysis similar to that of Ad5. In contrast, lysis caused by Ad5T122 was strongly suppressed in the liver-derived cell line Huh7, indicating a miR122-specific attenuation of Ad5T122 (see fig. 2 in **II**). To further confirm that the attenuation of Ad5T122 in Huh7 cells was due to silencing by miR122, we generated stable cell lines in which the key component of the miRNA machinery, Argonaute 2 (Ago2) had been targeted for silencing with anti-Ago2 short hairpin RNAs (shRNAs). Infection of those cell lines with Ad5 or Ad5T122 resulted in very similar production of CPE. The effect of miR122 inhibition on virus replication was also examined by transfecting Huh7 cells with

MIRIDIAN miR122 inhibitor prior to infection with Ad5 or Ad5T122. Also in this case, the suppressive effect of miR122 target elements on Ad5T122 replication was abolished (see supplementary fig. S2 in **II**)

### 4.1.3 Characterization of Ad5T122 replication in human liver and in colorectal carcinoma (II)

To further examine the miR122-specific suppression of Ad5T12, we used an experimental system based on *ex vivo* culturing of precision-cut human liver tissue slices. This *ex vivo* human liver infection model is one of the best models for preclinical evaluation of adenoviral induced liver toxicity, and allows the examination of productive adenoviral infection in the presence of all the cell types within the context of the normal three-dimensional architecture of the human liver. This same experimental system was also used to examine the replication of Ad5 and Ad5T122 in human tumour tissue, more precisely, in human colorectal carcinoma liver metastases. To examine the suppression of Ad5T122 replication in human liver, we infected liver tissue slices with Ad5 or Ad5T122 and followed the infections for up to 5 days. Similarly, human tumour tissue slices were also infected with these viruses and were followed for up to 5 days. The immunohistochemical examination of the Ad5-infected tissue slices with an antibody against E1A protein showed considerable increase in signal intensity in liver as well as in tumour as the infection proceeded indicating productive replication in both tissues. The immunohistochemical examination of the Ad5T122-infected tumour tissue slices showed similar increase in signal intensity as observed with Ad5. In contrast, the immunohistochemical examination of the Ad5T122-infected liver tissue slices did not show any specific signal and appeared identical to the uninfected liver tissue controls (see fig. 3 in **II**).

To directly examine the rate of productive replication of Ad5 and Ad5T122 in the human liver and tumour tissues, the amount of infectious virus was quantitated at different time points after initial infection of liver and tumour tissues with Ad5 or Ad5T122. The infectious titre of Ad5 in the liver tissue supernatant increased gradually during the five-day follow up period, exceeding the input dose by 68-fold for liver #1 and by 27-fold for liver #2 at 5d postinfection (p.i). By contrast, very little Ad5T122 was found in the liver tissue supernatant, and even at 5d p.i. the titre for liver #1 only slightly (1.3-fold) exceeded the input level (1h p.i.) and for the liver #2 the titre decreased to less than half (0.47-fold) of the input level (see fig. 4A in **II**). In the tumour tissue samples, both viruses replicated to the same extent (see fig. 4B in **II**).

### 4.1.4 Evaluation of liver toxicity of systemically administered Ad5T122 (II)

Although human adenovirus 5 does not productively replicate in mouse tissues, systemic viral injection is associated with hepatotoxicity manifesting in elevated serum levels of liver enzyme alanine aminotransferase (ALAT). To investigate the toxicity of systemically administered Ad5T122, we infected $1x10^9$ PFU of Ad5, Ad5T122 or PBS into the tail vein of C57bl/6 mice. 72h post-injection the animals were sacrificed and

serum ALAT levels were measured. Mice injected with Ad5 showed markedly increased ALAT levels compared to PBS injected mice, indicating hepatotoxicity of Ad5 following systemic administration. By contrast, mice injected with Ad5T122 showed ALAT levels indistinguishable from those of PBS injected mice (see fig. 5 in **II**).

### 4.1.5 Oncolytic potency of Ad5T122 in a lung cancer xenograft model (II)

To confirm that the severe attenuation of Ad5T122 replication in liver was not associated with reduced replication in tumour tissue *in vivo*, we compared the oncolytic potency of Ad5T122 and Ad5 in a mouse lung cancer xenograft model. Nude mice bearing an established subcutaneous A549 lung cancer xenografts were treated intratumourally on days 0, 2, and 4 with $1x10^7$ PFU of Ad5, Ad5T122 or a corresponding volume of PBS, and tumour growth was monitored for 12 days following the start of the treatment. Tumours in the PBS treated group of mice showed rapidly increasing growth and by the end of the 12-day follow up, tumours had reached more than 5x the initial volume. Tumours in the Ad5 and Ad5T122 treated groups of mice showed similarly reduced growth and starting from the day 8, a statistically significant reduction in tumour growth was observed with both viruses compared to the PBS treated group (see fig 6 in **III**).

## 4.2 Development of miRNA-targeted Semliki forest viruses for the attenuation of lethal neurovirulence (III)

### 4.2.1 Construction and *in vitro* characterization of miRNA-targeted Semliki forest viruses (III)

For the development of neuronally attenuated Semliki Forest virus (SFV), we inserted six target elements for a neuron-specifc miRNA miR124 into the genome of SFV4 (SFV4-miRT124). As a control virus for efficient evaluation of the miRNA-targeting in the context of replication competent SFVs *in vitro*, we inserted six target elements for liver-specific miR122 into the genome of SFV4 (SFV4-miRT122). Initially, three different strategies to generate miRNA-regulated SFV4 were tested. Target elements for miR122 were inserted in 5'UTR, 3'UTR or in the middle of the genome between nsp3 and nsp4. When inserted in the middle of the genome, an additional protease cleavage site was introduced between nsp3 and nsp4 and the target elements were inserted between the additional and original protease cleavage sites. Characterization of these modified viruses revealed that the most effective place for the target insertion was between nsp3 and nsp4, and this strategy was then chosen for all subsequent experiments (see fig 1 in **III** for schematic presentation of the miRNA-targeted SFV4 viruses).

To see whether the insertion of additional protease cleavage site and miRNA-target elements between nsp3 and nsp4 affected the correct proteolytic processing of nsp3 and nsp4, BHK-21 cells were infected with SFV4-miRT122, SFV4-miRT124 or wild-type

SFV4 at MOI of 1. Twelve hours p.i. cells were collected and the proteolytic processing of nsp3 and nsp4 was analysed by Western blotting. All viruses showed bands of similar size for both nsp3 and nsp4 indicating correct processing of these proteins from the polypeptide (see fig. 2A in I**II**).

To evaluate the potency of miRNA-targeting to restrict SFV4 replication, we infected miR122-expressing Huh7 cells and miR122-negative A549 cells with SFV4-miRT122, SFV4-miRT124 or wild-type SFV4. Six hours, 12h or 24h postinfection of Huh7 cells and 24h postinfection of A549 cells, the capsid and envelope protein production was monitored by Western blotting. In BHK-21 cells, SFV4-miRT122 and SFV4-miRT124 produced similar amounts of capsid and envelope proteins as compared to SFV4. In Huh7 cells, SFV4-miRT124 and SFV4 produced similar amounts of capsid and envelope proteins at all time points analysed whereas viral protein production of SFV4-miRT122 was strongly reduced, most strikingly at the early time points see figure 2B in **III**). To test if the miRNA-mediated suppression of viral proteins in SFV4-miRT122-infected Huh7 cells was due to endonucleolytic cleavage of viral genomic RNA rather than translational suppression, we infected Huh7 and A549 cells with SFV4-miRT122, SFV4-miRT124 or wild-type SFV4 at an MOI of 0.1 and measured the levels of genomic RNA by quantitative RT-PCR at 24h p.i. (see fig. 2C in **III**). In BHK-21 cells, no differences in the levels of viral genomic RNA were seen between the viruses. In Huh7 cells infected with SFV4-miRT122, the levels of viral genomic RNA were strongly (over 83%) reduced as compared to the cells infected with SFV4 or SFV4-miRT124. These results confirmed that the miRNA-specific suppression occurred via endonucleolytic cleavage of the viral genomic RNA.

For the *in vitro* evaluation of the oncolytic potency of miRNA-targeted SFV4 viruses, we infected a panel of cell lines with SFV4-miRT122, SFV4-miRT124 or wild-type SFV4 at an MOI of 0.1 and the amount of cell destruction was measured using a cell viability assay. Infection with SFV4 or SFV4-miRT124 lead to a potent destruction of all cell lines tested. By contrast, infection with SFV4-miRT122 lead to a potent destruction of all cell lines except miR122-expressing Huh7 cell line, thus indicating that the replication of SFV4-miRT122 was specifically suppressed by miR122. Also, the insertion of miRNA target elements into the SFV4 genome did not affect the oncolytic potency of these viruses *in vitro*.

### 4.2.2 *In vivo* characterization of SFV4-miRT124 (III)

To evaluate the replication and viral spread of the miRNA-targeted SFV4s *in vivo*, adult 5-7-week old BALB/c mice were infected intraperitoneally with $1x10^6$ PFU of SFV4-miRT122, SFV4-miRT124 or wild-type SFV4. Since miR122 is not expressed in the CNS, we used SFV4-miRT122 as a control virus to rule out any nonspecific effects on viral replication due to the insertion of the target elements into the viral genome. Mice infected with SFV4 started to display neurological symptoms at day 4 p.i. leading to severe symptoms or death in 4 out of 8 mice within the 14 day follow-up period. Mice

infected with SFV4-miRT122 also started to display neurological symptoms at day 4 p.i. eventually leading to severe symptoms or death in 6 out of 8 mice. No statistical difference in survival between SFV4- and SFV4-miRT122-infected groups of mice could be observed. By contrast, in the group of SFV4-miRT124-infected mice, only 1 mouse out of 8 mice developed severe neurological symptoms and had to be sacrificed within the 14 day follow-up period. Importantly, a significantly increased survival benefit was obtained over the control SFV4-miRT122 group (P=0.0106, log-rank test), indicating an attenuated replication of SFV4-miRT124 in the CNS.

To examine whether the severe neurological outcome of the single mouse from the group of SFV4-miRT124 infected mice was due to mutational loss of the target elements, we isolated RNA from the brain of this animal. The region containing the target elements was amplified by RT-PCR and 18 individual clones containing the amplified PCR fragments were sequenced. 16 out of 18 clones showed no mutations in the target element region and 1 clone had lost 2 target elements but the remaining 4 were intact. Only 1 clone out of 18 had lost all the target elements and had reverted to wild-type like virus. Although these result showed that mutations in the target region can occur, the late emergence and low frequency of the escape mutants indicates that this might be relatively rare event and unlikely to explain the severe outcome of infection in this animal.

Viral replication in the periphery was analysed by titration of the sera of the infected mice at days 1, 2 and 3 p.i. High level of viremia was seen with all viruses at 1d p.i. rapidly dropping to near undetectable levels at the following days. All viruses showed comparable serum titres indicating a wild-type-like replication of SFV4-miRT124 and SFV4-miRT122 in the periphery (see fig. 4B in **III**). Viral load from the brains of the infected mice was also analysed. Titres of the SFV4- and SFV4-miRT122 infected mice brain homogenates showed comparable, continuously increasing amount of virus between days 2 and 4 p.i. Titres of the SFV4-miRT124-infected mice brain homogenates showed an increase in viral load until day 3 p.i., and remained at the same level at day 4 p.i. However, the average brain titre for SFV4-miRT124 remained at $10^3$ PFU/g while the average titres for SFV4 and SFV4-miRT122 increased to $10^5$ to $10^6$ PFU/g (see fig. 4C in **III**). To further study the attenuation of SFV4-miRT124 in the infected mice brain, we evaluated the antiviral interferon responses elicited by the viruses by measuring the levels of interferon-β (IFN-β) from the brain homogenates of infected animals. High levels of IFN-β could be measured from the brains of SFV4-miRT122- and SFV4-infected animals, indicating a clear host response against these viruses. By contrast, no IFN-β could be measured from the brains of SFV4-miRT124-infected animals correlating well with the attenuated replication of SFV4-miRT124 in the CNS (see fig. 5 in **III**).

To study the distribution and amount of viruses in brain, immunohistochemical analysis of brain samples collected from the infected mice was carried out. Analysis of a brain sample from the SFV4-miRT124-infected mouse suffering from severe neurological symptoms revealed only a limited focal infection of the brain whereas the brains of SFV4- and SFV4-miRT122-infected mice suffering from neurological symptoms showed a wide-spread neuronal infection of the midbrain, thalamus and cerebellum (see fig. 6 in **III**).

### 4.2.3 Pre-infection with SFV4-miRT124 induces protective immunity against lethal SFV challenge (III)

To test whether the SFV4-miRT124-infected mice that survived the intraperitoneal virus administration had develop immunity against SFV, an indicator of robust peripheral replication, 5 surviving mice were intraperitoneally injected with $1x10^6$ PFU of the lethal L10 strain (see fig.7 in **III**). All mice previously infected with SFV4-miRT124 survived the lethal L10 challenge and remained asymptomatic for the 20-day follow-up period. By contrast, all mice without preceding SFV4-miRT124 infection died within 4 days.

### 4.2.4 Intracranially administered SFV4-miRT124 leads to restricted infection of the corpus callosum (III)

To examine the neuronal attenuation of SFV4-miRT124 when administered directly into the brains of mice, 100, 10 000 and 100 000 PFU of SFV4-miRT122, SFV4-miRT124 or SFV4 was intracranially injected into groups of BALB/c mice. While a significantly increased survival benefit ($P \leq 0.0009$, log-rank test) of the SFV4-miRT124-infected mice was obtained over the SFV4-miRT122 and SFV4-infected groups of mice, all mice eventually developed lethal neurological symptoms (see fig. 8 in **III**). Immunohistochemical analysis of the brains of these mice revealed that while SFV4-miRT122 caused a widespread infection of the brain involving also the Purkinje neurons of the cerebellar cortex, infection with SFV4-miRT124 was dramatically restricted in the neurons. Only a few infected neurons were seen, and the virus was predominantly infecting the oligodendrocytes of the corpus callosum (see fig. 9 in **III**).

# 5 DISCUSSION

## 5.1 Developing liver-detargeted adenoviruses for increased safety (I,II)

Oncolytic adenoviruses are considered to be a promising tool for the treatment of cancer. However, liver tropism restricts their systemic use and adenoviruses can cause liver toxicity. Various approaches have been developed to circumvent the liver toxicity and to increase tumour selectivity, including the use of tumour specific promoters to drive the expression of E1A, deletion of CR2 in the E1A, deletion of E1B55k reading frame, chemical or genetic modifications of capsid proteins (Kanerva and Hemminki 2004). We wanted to test whether miRNAs can be exploited to de-target adenovirus replication from the liver.

In the study **I**, we showed that the expression of a liver-specific microRNA miR122 can be exploited to create an adenovirus that fails to replicate in the liver-derived cells. We first tested the potency of miR122 to regulate foreign genes when artificial target elements for miR122 were introduced into the 5'UTR or 3'UTR or into both the 5' and 3'UTR of the firefly luciferase (FFluc) mRNA. Dual-luciferase assays showed profound and miR122-specific suppression of FFluc activities when tested with various different cell lines. These results encouraged us to test the ability of miR122 to suppress adenovirus E1A expression following infection of liver-derived Huh7 cells.

We constructed a chimeric adenovirus (Ad5/3-122) by inserting three perfectly complementary target elements for miR122 in the 3'UTR of the E1A mRNA. In vitro characterization of this virus showed that the E1A expression was specifically suppressed in Huh7 cells and the mode of suppression was degradation of the E1A mRNA. Although E1A mRNA expression from Ad5/3-122 in Huh7 cells was less than 10 % of the E1A mRNA levels of the control virus (Ad5/3-Δ24), no significant differences in the rate of replication between the viruses were observed. This finding is in agreement with previous studies where it has been shown that adenovirus can replicate normally in cultured HeLa cells despite greatly reduced levels of E1A protein (Hitt and Graham 1990). To overcome this problem, we created a virus that had non-specifically reduced E1A expression in all cells (Ad5/3K), but replicated similarly to the original virus. A miRNA-targeted derivative of Ad5/3K, named Ad5/3K-122, showed liver cell-specific suppression of E1A and in contrast to Ad5/3-122, the replication of this virus was also dramatically reduced in Huh7 cells (4 orders of magnitude difference in replication compared to Ad5/3K). The uniformly reduced E1A expression of Ad5/3K-122 combined with the miRNA-mediated suppression of E1A was sufficient to decrease the E1A mRNA levels below a threshold at which no viral replication was supported in Huh7 cells. However, the requirements for the amount of E1A might be different in transformed cell lines than in primary cells. In primary cells, in addition to its transactivation properties, E1A is required to inactivate Retinoblastoma (RB) proteins in order to drive the infected cells into the S-phase of cell cycle that is necessary for robust viral

replication (Berk 2005). In transformed cell lines such as HeLa and Huh7, the cell cycle regulation is compromised by default and these cells are in a continuously active state permitting viral replication to proceed normally despite reduced levels of E1A protein. In addition, although Huh7 cells express miR122 to a very high amount, these levels are only 8% of the miR122 levels observed in human primary hepatocytes (Chang, Nicolas et al. 2004), suggesting that the miRNA-mediated suppression of E1A might be even more potent in these cells. In a subsequent study using similar approach, Leja et al. 2010 also combined miRNA-targeting with additional measures in order to decrease E1A expression and replication of a miRNA-targeted adenovirus in cultured primary hepatocytes and in liver-derived cell line Huh7.5 (Leja, Nilsson et al. 2010). Cawood et al. 2009 and 2011 used the miRNA-targeting approach as well to show that luciferase expression from a miRNA-targeted E1A-firefly luciferase fusion gene in Ad5 was potently suppressed by miR122 in cultured primary hepatocytes and Huh7 cells (Cawood, Chen et al. 2009; Cawood, Wong et al. 2011).

While these studies clearly demonstrated the potency of miRNA-targeting in combination with other modifications to de-target the adenovirus from the liver-derived cells, the replication kinetics of adenoviruses exploiting miRNA-targeting as the only modification to the adenoviral genome had not been evaluated in primary hepatocytes or in human liver tissue. To this end, we constructed a miRNA-targeted derivative of wild-type adenovirus 5 carrying six target elements for miR122 in the 3'UTR of the E1A mRNA (Ad5T122 in the study **II**). No other modifications were included in the genome of this virus. With the use of this virus, we wanted to investigate whether miRNA-targeting alone was sufficient to suppress adenovirus replication in human liver. For this purpose, we used an experimental system based on *ex vivo* culturing of precision-cut human liver tissue slices. Considering that human adenovirus 5 is normally only capable of an abortive replication in mouse tissues, this *ex vivo* human liver infection model is a superior study system for preclinical evaluation of adenoviral induced liver toxicity, and allows the examination of productive adenoviral infection in the presence of all the cell types within the context of the normal three-dimensional architecture of the human liver. This ex vivo experimental system was also used to study the oncolytic potency of Ad5T122 in human tumour tissue. Immunohistochemical analyses of Ad5- and Ad5T122-infected liver and tumour slices showed clear difference in the intensity and extend of E1A signal between the viruses. Ad5-infected liver as well as tumour tissues showed considerable increase in E1A signal intensity as the infection proceeded, thus indicating robust replication and spread in both tissues. Ad5T122-infected tumour slices showed similar E1A signal intensities as Ad5-infected tumour slices, thus indicating that the insertion of miR122 target elements does not affect the replication of Ad5T122. By contrast, Ad5T122-infected liver slices did not show any specific signal for E1A indicating a potent and miR122-specific suppression of E1A expression. While these experiments demonstrated that in normal human liver miR122 can be exploited to suppress E1A

expression, the replication of these viruses could not be measured with this approach since adenoviral replication can proceed normally even with reduced amounts of the E1A protein (as was shown in the study **I**). To directly examine the productive replication of these viruses, the amount of virus in the culture media of Ad5- and Ad5T122-infected liver and tumour slices was quantitated at different time points after the initial virus administration. The infectious titre of Ad5 in the liver and tissue supernatants increased robustly during the 5-day follow-up period confirming that Ad5 can replicate well in human liver. In contrast, very little Ad5T122 was found in the liver tissue supernatants, a direct evidence of the suppressive potential of miR122 target elements on adenoviral replication in human liver. In the samples of colorectal cancer liver metastasis tissue both viruses replicated to the same extend, thereby confirming that the insertion of miR122 target elements had not compromised the replicative potential of Ad5T122. Taken together, these data demonstrated that six target elements for miR122 in the 3'UTR of E1A mRNA is sufficient without any other modifications to prevent productive replication of adenovirus 5 in normal human liver.

Although human adenovirus 5 does not productively replicate in mouse tissues (Jogler, Hoffmann et al. 2006), systemic viral administration is associated with hepatotoxicity such as elevated serum levels of the liver enzyme alanine aminotransferase (ALAT). Ad5T122 did not induce hepatotoxicity as measured by serum ALAT levels, confirming results obtained by Cawood et al. 2009 using very similar virus (Cawood, Chen et al. 2009). Cawood at al. 2011 showed that miRNA-targeted Ad5-mir122 (four target elements for miR122 inserted into the 3'UTR of E1A mRNA) had potent anti-cancer efficacy in a Hep2G xenograft mouse model and that the virus was less hepatotoxic than wild-type Ad5 (Cawood, Wong et al. 2011). However, their treatment regimen included different dosages for Ad5-mir122 and wild-type Ad5 (mice received 10-times more Ad5-mir122 than Ad5) complicating the comparison of the oncolytic potency of these viruses. We used a lung cancer xenograft model for direct comparison of the oncolytic potency of Ad5T122 and Ad5. Ad5T122- and Ad5-infected groups of tumour bearing mice received the same amount of virus intratumourally. Both Ad5T122- and Ad5-infected groups of mice had significantly reduced tumour growth as compared to PBS-treated group of mice. These data conclude that the oncolytic potency of Ad5T122 was not reduced compared to wild-type Ad5.

Our data provides a definitive proof of concept and preclinical validation for the use of miR122 target elements for reducing the risk of liver toxicity in adenovirus-based cancer virotherapy applications. We showed that the insertion of target elements for the liver-specific miR122 into the adenovirus 5 genome prevents productive replication in liver-derived cells (**I**) or normal human liver (**II**). This modification did not compromise the replicative capacity of the modified viruses in cells (**I, II**) or tissues of non-hepatic origin (**II**). Also, the oncolytic potency of Ad5T122 was similar to wild-type Ad5 (**II**). This miR122-based targeting adds a valuable new safety measure addressing

the liver toxicity of therapeutic adenoviruses. Ad5T122 replicated efficiently in liver metastatic colorectal cancer emphasizing its potential in the development of better systemic treatments for patients with liver metastasized cancer or primary hepatocellular carcinoma. In the future clinics, adenoviruses might be "armed" with one or more additional gene products that enhance the direct cell killing or immune-mediated destruction of infected cells via promoting the development of systemic anti-tumour immunity (Cody and Douglas 2009). To prevent the expression of these potentially dangerous genes in hepatocytes could be even more important than suppressing the viral replication itself (as has been shown with Ad-HSVtk vectors see (Suzuki, Sakurai et al. 2008)). However, in combination with the targeting of E1A gene, these transgenes could also be placed under the control of miR122 for more efficient restriction of unwanted expression in hepatocytes.

## 5.2 MiRNA-mediated attenuation of Semliki Forest virus neurovirulence (III)

In study **III** we showed that the neuropathogenicity of Semliki Forest virus can be selectively attenuated by inserting six perfectly complementary target elements for the neuron-specific miRNA miR124 between the non-structural proteins nsp3 and nsp4 in the viral genome. *In vitro* characterization of the miRNA-targeted SFV4 viruses showed that the insertion of the target elements did not affect the correct processing of the nsp polyprotein as both nsp3 and nsp4 were produced identically to wild-type SFV4. In line with our previous results using perfectly complementary target elements in adenoviruses (study **I** and **II**), the miRNA-mediated suppression of viral proteins was shown to occur via endonucleolytic cleavage of viral genomic RNA. *In vivo* characterization of these viruses in BALB/c mice showed that intraperitoneally injected SFV4-miRT124 had strongly attenuated neurovirulence but showed similar peripheral replication as compared to SFV4-miRT122 control virus. This strongly indicated that the reduced neuropathology was not due to attenuated peripheral replication of the SFV4-miRT124 virus. Interferon-β measurement from the brain tissue following intraperitoneal infection correlated well with the viral loads, and consequently, showed that whereas both control viruses SFV4 and SFV4-miRT122 induced high levels of interferon-β, SFV4-miRT124 did not induce detectable levels of INF-β. These results are in line with previous studies where the expression of proinflammatory cytokines in the CNS was shown to correlate with the rate of SFV (Tuittila, Nygardas et al. 2004).

Although SFV4-miRT124 had significantly attenuated neurovirulence compared to the SFV4-miRT122 control virus, 1 mouse out of 8 developed severe neurological symptoms following intraperitoneal infection. Sequence analysis from the brain tissue of this animal revealed that majority of the viruses (89%) contained the original sequence introduced between the nsp3 and nsp4 and only a single wild type-like

revertant was found. Although these result showed that mutations in the target region can occur, the late emergence and low frequency of the escape mutants indicates that this might be relatively rare event and unlikely explains the severe outcome of infection of this animal.

Pre-infection of mice with SFV4-miRT124 elicited strong antiviral immunity and complete protection against an otherwise lethal challence with a highly neurovirulent wild type SFV strain L10. In addition to clearly indicating a robust peripheral replication of SFV4-miRT124, this result also suggests that miRNA-targeting could be exploited in designing more potent yet safer alphaviral vaccine vectors. In a recent study, Kamrud et al. incorporated target elements for ubiquitously expressed miRNAs into Venezuelan equine encephalitis virus (VEEV) replicon helper RNAs that were used for virus-like replicon (VRP) production. The rationale behind this approach was to prevent expression of helper RNAs and functional recombinants between the replicon and helper RNAs in VRP-vaccinated individuals in order to create an additional safety measure for VEEV vaccine development (Kamrud, Coffield et al. 2010).

To further evaluate the neuronal attenuation of SFV4-miRT124, we tested intracranial administration of the viruses. Intracranial injection of SFV4 and SFV4-miRT122 viruses resulted in widespread infection throughout the brain of the infected mice, whereas SFV4-miRT124 infection resulted in a restricted infection pattern and a predominant infection of the oligodendrocytes of corpus callosum correlating well with known expression patterns of miR124 in the mouse brain (Deo, Yu et al. 2006; Pena, Sohn-Lee et al. 2009). The observed infection of the corpus callosum might explain the attenuated but still lethal phenotype of SFV4-miRT124 in intracranially infected animals. However, similar infection pattern was previously observed with intracranially administered SFV strain A7(74) resulting necrotic cell death of the oligodendrocytes of corpus callosum but this infection did not lead to death of the infected mice (Fazakerley, Cotterill et al. 2006). In light of this report, there is a possibility that a hitherto unidentified population of infected non-neuronal cells in the CNS might be responsible for the lethal phenotype witnessed in mice intracranially administered with SFV4-miRT124. Taken together, intracranial infection with SFV4-miRT124 (even with enormous amount of 100 000 PFU of virus) showed efficient neuronal de-targeting, confirming the results obtained from the experiments using intraperitoneally administered virus.

Our results show that the tissue tropism of a wild type Semliki Forest virus can be selectively modified by the insertion of tissue-specific miRNA target elements into the viral genome. By the use of target elements for the neuron-specific miR124, we were able to strongly attenuate the neurovirulence of SFV4, thus providing new opportunities for studies on SFV4 in preclinical mice models. Indeed, the severe neurovirulence of SFV4 has prevented its use as an oncolytic virotherapy agent. The

neuronally attenuated SFV4-miRT124 allows overcoming this problem and enables the use of SFV4 in preclinical mice cancer models. Furthermore, our findings together with the data by Kamrud et al. clearly show that miRNA-targeting can also be exploited in alphavirus vaccine development.

# 6 CONCLUSIONS

In this thesis we showed that miRNAs can be efficiently exploited to de-target viral replication specifically from tissues accounting for viral pathogenicity. Remarkably, miRNA-targeting was successfully used in two very different viruses, one being a double-stranded DNA virus and the other being a positive-sense single-stranded RNA virus. These results together with a large number of recent publications using different RNA- and DNA-viruses are supporting the idea that miRNA-targeting can be used across the entire spectrum of viruses. That is particularly important since the most suitable and efficient viruses for different applications might yet to be found. Also, given the fact that there are a wide variety of differentially expressed, tissue-specific miRNAs that could be exploited to de-target viral replication from virtually any given tissue, miRNA-targeting approach has a great potential to significantly improve the safety of a wide variety of virotherapeutic applications including gene therapy, cancer gene therapy and vaccine development.

# 7 ACKNOWLEDGEMENTS

This study was carried out at the Department of Virology, Haartman Institute, University of Helsinki. I thank the head of the department, Professor Kalle Saksela for the very warm and welcoming working environment at the department.

I would like to express my sincere gratitude to my supervisor, Professor Kalle Saksela who took me under his wing and gave me the opportunity to do my thesis research in his laboratory. I truly thank you for your outstanding guidance and for always patiently hearing me out when I had something on my mind. Thank you Kalle!

I thank Docent Maarit Suomalainen and Professor Veijo Hukkanen for reviewing this thesis and for providing valuable and constructive comments that improved the thesis significantly. I also thank Professor Magnus Essand for accepting the role of the opponent in my thesis dissertation.

I thank all the collaborators and co-authors without whom this thesis could not have been possible to obtain. Ari Hinkkanen, Miika Martikainen, Sergio Lavilla-Alonso, Akseli Hemminki, Sari Jäämaa, Markus Vähä-koskela, Johanna Arola, Heikki Mäkisalo, Taija af Hällström, Tanja Hakkarainen, Tapio Visakorpi and Raul Andino are all thanked for excellent collaboration and their critically important contributions to this work.

The members of my thesis committee, Docents Maarit Suomalainen and Tero Ahola, I am most thankful for your invaluable comments and critical, yet constructive evaluation of the progress of the thesis work over all these years.

This thesis has been partly financed by Helsinki Biomedical Graduate School and Jenny and Antti Wihuri –foundation, both of which are deeply acknowledged.

My warmest gratitude is expressed to all the current and former members of the Saksela lab. Kalle, Tapio, Virpi, Hannamari, Constanze, Leena, Jarno, Kristina, Annika, Silja, Tina, Matjaz, Yoke, Hyunseok, Jubauer, Arunas, Jacob, Iivari, Sergio, Johanna, Anette and Virpi K are all thanked for, in addition to scientific help, creating a wonderfully friendly working atmosphere at the lab. Especially I would like to thank Tapio for all the guidance and discussions (work related as well as totally off topic), my Danish friend Jacob for all the fun stuff at the lab and outside, Arunas for all those boy's night out -moments, Hannamari for all the support (scientific and non-scientific), Kristina for various interesting discussions and Virpi for keeping all things (including me) in order at the lab. Thank you Sergio for the team effort we made to get the research done.

I would also want to thank my friends who have supported me throughout these years as a grad student. Teka, Tuomas, Jassu, Tero and Mikko from the bottom of my heart I thank you for your friendship and support.

I would like to express my deepest gratitude to my parents who have always encouraged me to do things I want to do and have always supported my decisions made in life in general. Words cannot describe how grateful I am to my wife Leena who has stood by me all these years and has always supported me. Thank you Leena! I also want to thank the two most important people in my life, Aura and Emil for keeping daddy busy with also other things than science.

This thesis is dedicated to the loving memory of my little brother Tuukka. You only got to see the start of my thesis journey before you were taken away from us. I promised myself I would complete my thesis in honour of you. I miss you so much…

Espoo, April 2013

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