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Molecular interactions between adenoviral RNAs
and the cellular RNA interference machinery

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1 Abstract

Adenovirus infections represent a considerable cause of life-threatening complications in immunocompromised patients. Among hematopoietic stem cell transplant recipients, mortality rates are particularly high, reaching values up to 80% (1-5). Available antiviral drugs such as nucleoside or nucleotide analogues suffer from efficacy against adenovirus infections or show severe side effects (6). Thus, the identification of alternative targets for therapeutic intervention is essential for improving the treatment of such infections. Unraveling the molecular interplay between the virus and its host cell may aid in the development of novel anti-adenoviral treatment strategies.

It has been shown that adenovirus interacts with the cellular RNA interference (RNAi) machinery by expressing small virus-associated (VA) RNAs. These RNAs are processed into micro(mi)RNA-like subfragments (mivaRNAs) which saturate the key enzymes of RNAi, Exportin-5, Dicer, and the Argonaute proteins that make up the RNA-induced silencing complex (RISC) (7, 8). mivaRNAs are suggested to target cellular mRNAs and thus alter host cell gene expression (9). In this study I investigated several aspects of these molecular interactions.

To screen for mivaRNA target mRNAs, I used an immunoprecipitation assay to biochemically isolate RISC and its associated miRNAs and their target mRNAs from mivaRNA-transfected cells. Inspection of these putative targets by microarray analysis and bioinformatics target prediction software revealed a set of genes implicated in various biological functions such as signaling, metabolism and immune response. Targeting of these genes by the virus may be of importance for the successful establishment of a lytic viral infection or may aid in hiding from the host during a latent infection.

Another proposed function of mivaRNAs is the protection of viral mRNA from cellular miRNA-mediated targeting (7). I employed RISC-immunoprecipitation to screen for the enrichment of viral mRNA in the presence or absence of mivaRNAs. When a mutant virus lacking the VA-RNAs was used to infect A549 cells, a set of adenoviral mRNAs was found enriched in RISC. This enrichment pattern differed from that obtained with the wild-type virus, possibly substantiating the proposed function of mivaRNAs to act as defense molecules against cellular RNA interference.

2 Zusammenfassung

Adenovirale Infektionen in immunsupprimierten Patienten stehen im Zusammenhang mit einer hohen Anzahl an lebensbedrohlichen Komplikationen. Insbesondere nach hämatopoetischen Stammzelltransplantation können disseminierte Infektionen fatale Folgen haben und sind mit einer Mortalitätsrate von bis zu 80% assoziiert (1-5). Derzeit verfügbare Medikamente wie etwa Nukleosid- oder Nukleotidanaloga zeigen nur beschränkte Wirksamkeit gegen Adenovirusinfektionen oder führen zu schwerwiegenden Nebeneffekten (6). Deshalb ist es notwendig, alternative Angriffspunkte für eine therapeutische Intervention zu identifizieren, um die Behandlungsmöglichkeiten solcher Infektionen zu verbessern. Dabei könnte ein grundlegendes Verständnis des Wechselspiels zwischen einem Virus und seiner Wirtszelle zur Entwicklung neuer anti-adenoviraler Behandlungsstrategien beitragen.

Es wurde gezeigt, dass Adenovirus mit der zellulären RNA-Interferenz (RNAi)-Maschinerie interagiert. Dies erfolgt über die Expression von kleinen virus-assoziierten (VA) RNAs. Diese RNAs werden zu miRNA-ähnlichen Subfragmenten (mivaRNAs) prozessiert, welche die Schlüsselenzyme der RNAi – Exportin 5, Dicer und den RNA-induced silencing complex (RISC) – sättigen (7, 8). Es wird angenommen, dass mivaRNAs die Genexpression ihrer Wirtszelle beeinflussen können, indem sie an zelluläre mRNAs binden (9). In dieser Studie wurden verschiedene Aspekte dieser molekularen Interaktionen untersucht.

Um möglichen Ziel-mRNAs von mivaRNAs zu identifizieren wurde eine Immunpräzipitationsmethode angewandt. Dabei wurden Zellen mit mivaRNAs transfiziert und anschließend RISC Komplexe, sowie darin vorhandene miRNAs und deren Ziel-mRNAs biochemisch isoliert. Anschließend erfolgte eine kombinierte Microarray- und Bioinformatik-Analyse der potenziellen Ziel-mRNAs. Diese ergab ein Set an Genen, welche in verschiedenen biologischen Funktionen wie Signaltransduktion, Metabolismus und Immunantwort eine Rolle spielen. Man kann vermuten, dass ein Targeting dieser Gene durch mivaRNAs von besonderer Wichtigkeit für die erfolgreiche Etablierung einer lytischen Virusinfektion ist, oder dazu dienen kann sich während einer latenten Infektion vor dem Wirt zu verbergen.

Des Weiteren wird vermutet, dass mivaRNAs als Schutzmechanismus für virale mRNAs fungieren (7). Die RISC-Immunpräzipitationsmethode wurde dazu verwendet, die Anreicherung viraler mRNAs in der An- oder Abwesenheit der

mivaRNAs zu untersuchen. Dabei wurde eine Virus-Mutante verwendet, welche die VA-RNAs nicht exprimieren kann. Wenn die Mutante anstelle des Wildtyp Virus für die Infektion von A549 Zellen verwendet wurde konnte gezeigt werden, dass bestimmte adenovirale Transkripte in RISC angereichert sind. Diese Beobachtung untermauert die für mivaRNAs vorgeschlagene Funktion als Mediatoren eines molekularen Schutzmechanismus gegen zelluläre RNAi.

3 Introduction

3.1 Human adenoviruses

3.1.1 Classification

Human Adenoviruses (HAdVs) are members of the viral family *Adenoviridae* and belong to the genus *Mastadenovirus*. They have a linear, double-stranded DNA (dsDNA) genome and an icosahedral, non-enveloped capsid of approximately 90 nm in diameter (10). First isolated in the early 1950's from adenoid tissue (11, 12) the family comprises nowadays more than 50 serotypes, which can be further subgrouped into six species (A to F), based on their DNA sequence or oncogenic and hemagglutinating properties (4, 13, 14)

3.1.2 Clinical features

Adenovirus infections in immunocompetent individuals are often subclinical and self-limited. Diseases affecting the upper respiratory tract, the eyes as well as the gastrointestinal tract are the most frequent manifestations (14). Furthermore, chronic persistent infections in mucosal lymphocytes have been described, bearing the possibility for a reactivation of viral replication (15). Adenoviruses are highly prevalent in the human population since 80% of individuals are estimated to become infected early in life. Respiratory illness caused by adenovirus is common among children and people living in close proximity to each other such as military recruits (16, 17). The main routes of transmission are aerosolized droplets or by conjunctival inoculation. Furthermore, spread of the virus can occur by infected blood or tissue (14).

The lack of defence mechanisms against pathogens renders immunocompromised individuals susceptible to more severe disease upon adenovirus infection. Manifestations can include serious pneumonia, hemorrhagic cystitis and meningoencephalitis. Finally, dissemination of the infection can lead to fatal outcome through multi-organ failure (3, 18).

Allogeneic stem cell transplantation (HSCT) is a current treatment option for patients suffering from haematological malignancies. However, the post-transplant period is characterized by an overall immunosuppression which is accompanied by a high risk for viral infections (19). The incidence rates for adenovirus infections following HSCT are alarmingly high. In a prospective multicenter trial including 130 HSCT-recipients, 35 positive cases were diagnosed and among 36 patients who died during the study, nineteen were infected with adenovirus (20). A different study with similar results was conducted at the St. Anna Children's Hospital, Vienna Austria, including 132 paediatric patients who underwent allogeneic stem cell transplantation. Overall, 27% of these patients were tested positive for adenovirus infection. In most cases, adenovirus belonged to group C. Particularly in the cohort of patients who were diagnosed positive for Adenovirus in the peripheral blood, which is an indication for disseminated disease, mortality rates were significantly higher compared to Adenovirus-negative patients, reaching a value of 82% (4).

3.1.3 Therapy of adenovirus infections

To counteract the severe clinical manifestation of adenovirus infections in immunocompromised patients, efforts were made to use existing antiviral drugs such as cidofovir or ribavirin to cure infected patients.

The drug cidofovir, which is successfully used to clear infections with other viruses such as the cytomegalovirus (CMV), has been shown to be effective against adenoviruses as well (21). Cidofovir belongs to a class of antiviral drugs termed acyclic nucleoside phosphonates (ANP). These compounds are 2',3'-dideoxynucleotide analogues and are acting as polymerase inhibitors. ANPs show a higher affinity for the viral DNA polymerase than for the cellular isoform, which would restrict their action to virus-infected cells (6, 22, 23). However, due to accumulation in the renal proximal tubules, administration of ANPs is linked to severe nephrotoxic side effects (22).

Ribavirin, which is another agent used in the treatment of viral infections has been evaluated for anti-adenoviral effects as well. Cell culture studies on the sensitivity of adenoviruses to Ribavirin revealed a species-dependent susceptibility. Among different serotypes from species A to F, an inhibitory effect could only be

observed for species C (24). Further studies have only revealed an overall low efficacy against adenoviruses (25).

Adoptive immunotherapies using T-cells secreting Interferon- γ were shown to result in protection from complications related to adenovirus infections. However, T-cell stimulation is laborious and the major disadvantage is the restricted availability of antigens required for their stimulation (reviewed in reference 26).

Recently, alternative approaches for the inhibition of adenovirus multiplication *in vitro* were presented. An adenoviral vector-based system was used to express the Herpes simplex virus thymidine kinase (HSV-TK) gene in virus-infected cells. This would render adenovirus susceptible to the highly efficient anti-herpetic prodrug ganciclovir (27). Furthermore, successful efforts were made to knock down adenoviral gene expression by small interfering RNAs (siRNAs) (28).

Despite these promising prospects, the fundamental understanding of the complex interaction between the virus and its host is still incomplete. Therefore, a complete comprehension of host-pathogen interplay will be crucial to provide novel opportunities for therapeutic interventions.

3.1.4 Molecular organization of adenoviruses

3.1.4.1 Structural characteristics

A high-resolution view of the adenoviral capsid was revealed by X-ray crystallographic studies (29-31) (Fig. 1). The most abundant proteins of the capsid shell are the homotrimeric hexon (protein II), the penton base (protein III) and the protruding fibre proteins (protein IV). They are therefore termed the major components. Other structural elements of the capsid are the so-called minor proteins IIIa, VI, VIII and IX (32, 33). Inside the virus core, the viral genome is associated with five other structural components (V, VII, μ , IVa2, and the terminal protein TP) (a detailed description can be found in reference 33).

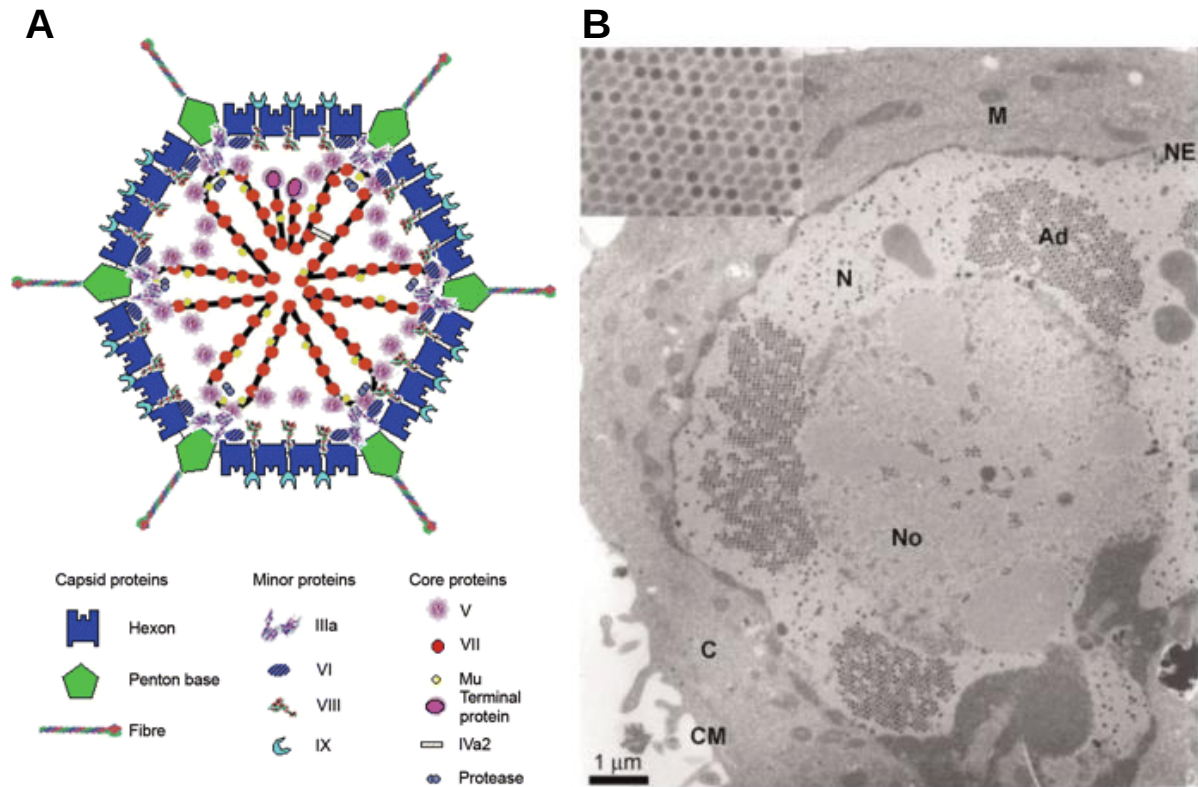


Figure 1: Structural properties of adenovirus. (A) (Taken from reference 33). The organization of viral shell proteins is depicted. These consist of capsid and minor components defined by structural studies using cryo-electron microscopic and crystallographic techniques. The arrangement of the core proteins and the viral genome is rather speculative. (B) (Taken from reference 10). Transmission electron microscope image of a HeLa cell infected with adenovirus taken 48 hours post-infection. No, nucleolus; N, nucleoplasm; NE, nuclear envelope; C, Cytoplasm; M, Mitochondria; CM, cell membrane.

3.1.4.2 The adenoviral genome organization

The dsDNA genome consists of approximately 36 kb and is flanked by inverted terminal repeats which function as an origin of replication. A *cis*-acting packaging sequence is located next to the left terminal repeat. The genome of HAdVs contains five early transcription units (E1A, E1B, E2, E3 and E4), named after the time points when they are produced during the viral replication cycle (E = early, L = late). Furthermore three delayed early regions (IX, IVa2 and E2 late) as well as one late transcription unit are described. The latter is further processed into five late mRNAs (L1, L2, L3, L4 and L5). All of the transcription units are transcribed by RNA

polymerase II and are subject to alternative splicing, which results in a complex and fine-tuned gene expression program.

Additionally, depending on the serotype, adenovirus encodes one or two virus-associated RNAs (VA RNAs), which are transcribed by RNA polymerase III. In general, the transcribed gene loci are asymmetrically distributed between the two DNA strands of the genome (Fig. 2). Thus, both strands encode different transcription units. A detailed description of the genome organisation of adenovirus is reviewed in reference 34.

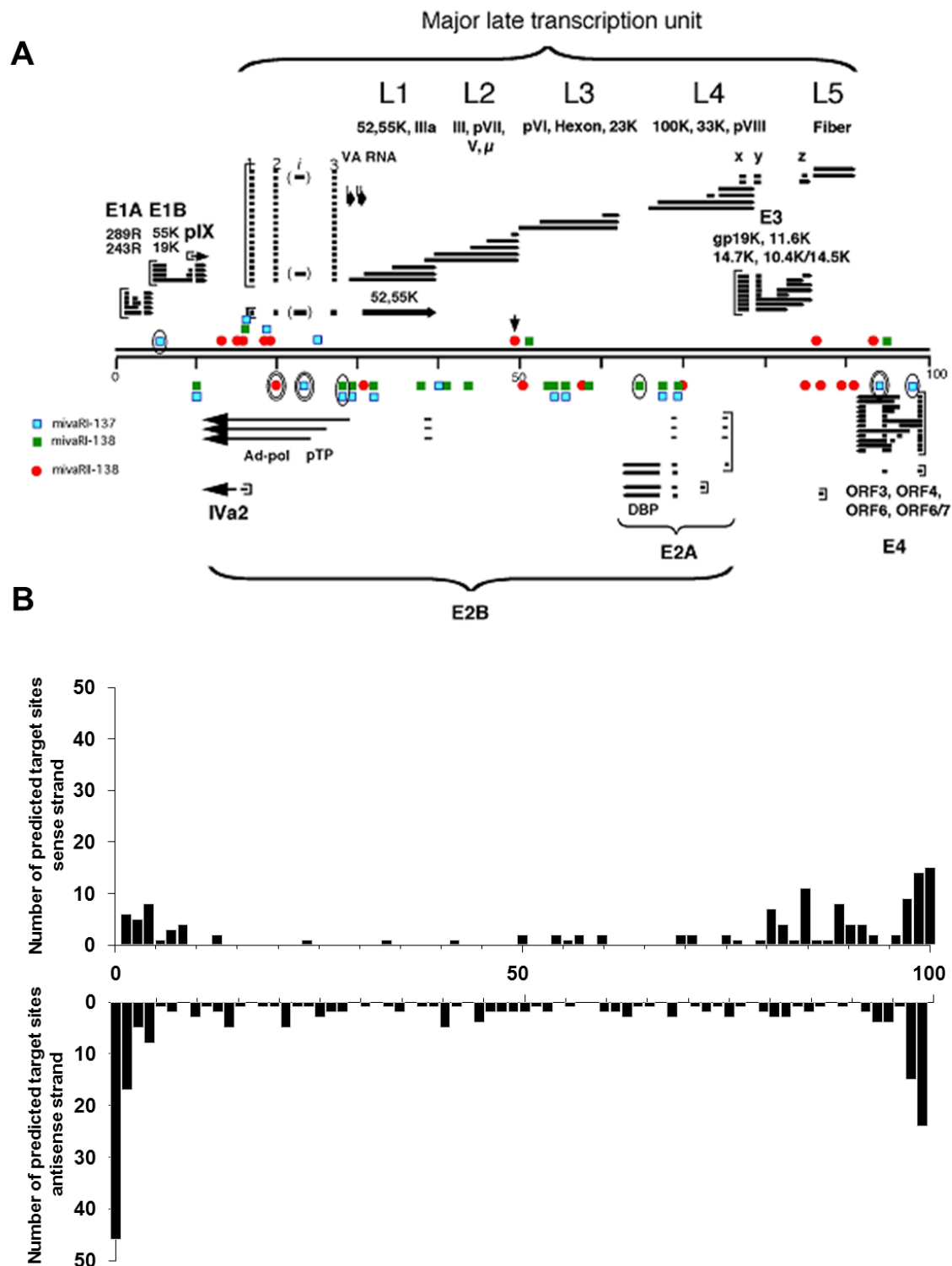


Figure 2: The adenovirus transcription map. (A) (Taken from reference 35). Adenoviral mRNAs are transcribed from either the sense (upper) or the antisense (lower) strand of the double stranded DNA genome. The transcription map shows splice variants of the distinct transcripts. Additionally, the supposed miVaRNA binding sites are depicted. (B) The panel shows the distribution of predicted target sites of cellular miRNAs over the adenovirus genome. The predictions were calculated by TargetScan.

3.1.5 The adenovirus life cycle

In most cases, adenovirus enters the host through the mouth, the nasopharynx or the ocular conjunctiva (23). As a first event in the replicative cycle, the virus attaches to the surface of cells located in its primary infection site by binding to primary high-affinity receptors via the carboxyterminal knob domain of its trimeric fiber protein (13, 34). For all adenovirus species except species B, the coxsackievirus and adenovirus (CAR) receptor has been shown to be an efficient docking site for the fiber protein (36). The main physiological function of this receptor is to mediate cell-to-cell adhesion through the formation of tight-junctions (37). Additionally, interactions with other receptors such as CD46, sialic acid, or lactoferrin have been shown to mediate attachment of different adenovirus species (reviewed in reference 38).

Following interaction with the receptor, the virus is internalized by clathrin-mediated endocytosis. This process is triggered by an additional interaction between the viral penton base and α v-integrins located on the cell surface (33). This results in a release of the fiber proteins and the uptake of the virus by clathrin-mediated endocytosis (39). After the maturation of clathrin-coated vesicles into endosomes, the virus particles eventually evade into the cytosol (40). Subsequently, the virion is disassembled by an ordered removal of structural proteins to allow the viral genome to enter the nucleus via the nuclear pore complex (34, 41, 42).

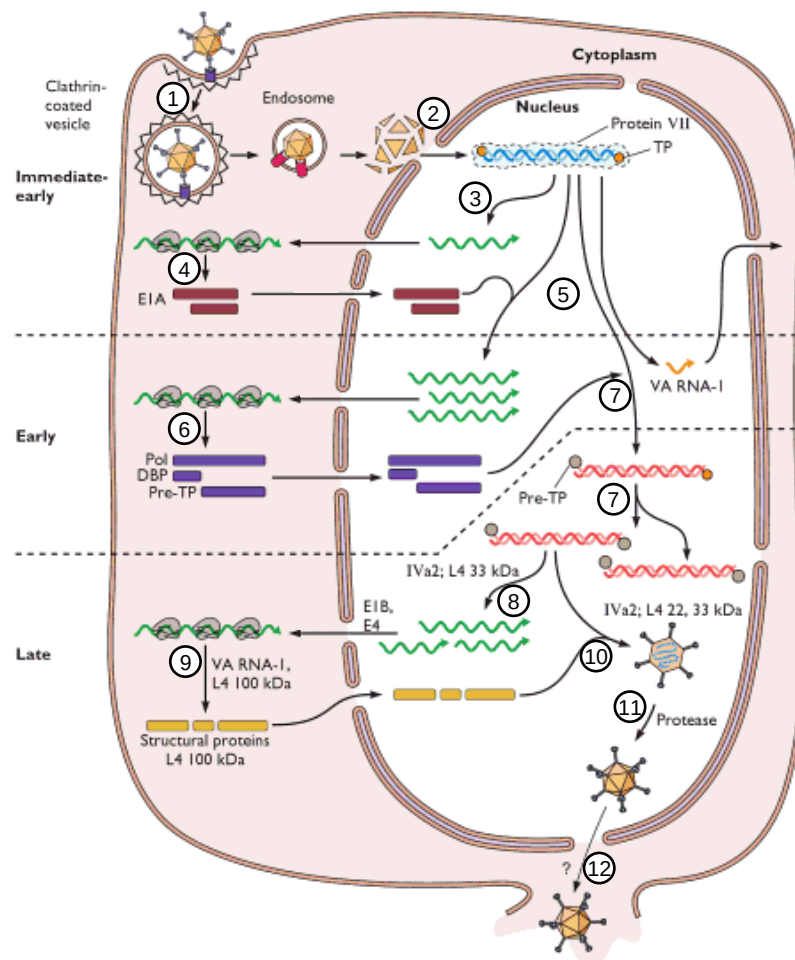


Figure 3: The adenovirus replicative cycle. (Adapted from reference 43). After attachment to a proper receptor on the host cell surface and internalization of the virus by endocytosis (1), the capsid is disassembled and the viral genome is translocated into the nucleus (2). The E1A mRNA is transcribed and exported into the cytoplasm where the E1A proteins are synthesized (4). These are shuttled back to the nucleus again, where they activate transcription of cellular as well as other early viral genes (5). In parallel, expression of the VA-RNAs is set on. The early viral genes are translated in the cytoplasm (6), and back in the nucleus, initiate replication of the viral genome (7). Late proteins are transcribed from *de novo* synthesized viral genomes (8) and exported from the nucleus. In the cytoplasm, structural proteins are synthesized (9) and imported into the nucleus. New immature capsids are assembled, and the viral DNA is packaged into them (10). As immature particles contain precursors of some structural components, they have to undergo cleavage reaction by the viral protease to allow the formation of fully infectious particles (11). The newly synthesized virions are released, usually accompanied by lysis of the host cell (12).

Shortly after the viral DNA has reached the nucleus, the E1A transcription unit is being transcribed. The primary transcript is alternatively spliced and gives rise to

two major proteins with a respective length of 289 (termed E1A-13S) and 243 (termed E1A-12S) amino acid residues (44). Sequence analysis of E1A identified four regions which have high sequence homologies among primate adenovirus subgroups and are therefore designated conserved regions (CR 1 to 4) (45). CR2 has been shown to play a critical role in driving the host cell into S phase by sequestering the retinoblastoma protein (pRb) and its family members p107 and p130. This leads to the displacement of the E2F transcription factors, which can activate transcription of several genes necessary for S-phase entry (46). The displacement of pRb from E2F by E1A circumvents the mechanisms of normal cell cycling. A regular S-phase entry would implicate the phosphorylation of pRb by G₁ cyclin-dependent kinases (CDK) upon mitogenic stimuli. Furthermore, E1A has been shown to interfere with CDK-inhibitors (CKI) such as p16^{INK4a} and p27^{KIP1}, which would otherwise hinder cell cycle progression (47). This virus-induced cell cycling is essential for the progression of the virus lifecycle and provides an optimal environment for its replication.

However, irregular S-phase entry would result in an induction of the gatekeeper p53, which would lead to cell cycle arrest and apoptosis (48). Therefore E1B-55k, a protein which is translated from the E1B mRNA, acts in concert with E4orf6 (encoded in the E4 transcription unit) and several cellular proteins to form an ubiquitin ligase complex. The latter can mediate the polyubiquitination of p53 which leads to its proteasomal degradation (34).

Another mechanism to inhibit apoptosis is mediated through E1B-19k, which is the second protein originating from the E1B transcription unit. It is homologous to the apoptosis-regulating protein Bcl-2 and can counteract TRAIL- and Fas-induced programmed cell death (49).

The accumulation of E2 transcripts as a consequence of stimulation by E1A-13S and the E2F transcription factor is a prerequisite for viral DNA replication. The E2-derived DNA-binding protein (DBP), the precursor terminal protein (pTP) and the DNA polymerase (Pol) cooperate with cellular factors to form the replication machinery (34, 50).

The main function of the E3 genes is to suppress host defence mechanisms. A prominent mechanism involves E3-19K, which sequesters MHC-I in the endoplasmic reticulum to evade cytotoxic T-Lymphocyte (CTL) – mediated cell lysis (51). Additionally, the E3 region encodes the adenovirus death protein (ADP), which

facilitates the release of newly synthesized virions by catalysing virus-mediated cell lysis (52). Other proteins are involved in the inhibition of pro-apoptotic pathways (50).

Moreover, adenovirus interferes with the cellular DNA damage response via proteins synthesized from the E4 region. This leads to genomic instability and is considered a 'hit and run' strategy, necessary to prevent nucleolytic cleavage of the viral genome (53-55). Besides that, the E4 unit encodes multiple functions, represented by a minimum of 18 distinct mRNAs derived from this region. These are involved in mRNA metabolism, facilitate replication of viral DNA and regulate apoptosis (50, 56).

The late transcripts are produced at only low level during the early phase of infection. Simultaneously with viral DNA replication, an efficient expression of these genes is set on. The IX and IVa2 gene products, together with cellular transcription factors orchestrate the activation of the major late promoter. A large primary transcript is synthesized and processed into five groups of late transcripts (L1 to L5) coding mainly for structural components of the virion. Finally, viral genomes are packed into newly synthesized particles and the virus progeny is released into the cytoplasm. The host cell membrane is disintegrated and the viruses are released from the host cells (33, 34).

3.1.6 The virus-associated RNAs

Human adenoviruses encode a class of small non-coding RNAs transcribed by RNA polymerase III. These were in particular investigated in the group C adenoviruses, which possess two species of them, VA RNAI and VA RNAII. VA RNAs are about 160 nucleotides in size and have a highly conserved secondary structure. Three structural characteristics are the terminal stem, the central domain and the apical stem-loop (57, 58). The transcription of the VA RNA genes is already initiated during the early phase of infection. During the late phase of infection, the transcription is enhanced, leading to the accumulation of about 1×10^8 molecules of VA RNAI and approximately 1×10^7 molecules of VA RNAII per infected HeLa cell (34).

To investigate the impact of the VA RNAs on adenovirus replication, mutant adenoviruses type 5 (Ad5), lacking either the VA RNAI or the VA RNAII gene were used. An impaired growth compared to wild-type was observed for the VA RNAI mutant, but not for the VA RNAII mutant. However, when a double-mutant lacking

both, VA RNAI and VA-RNAII, was used, virus replication declined even further. This effect was explained by an inefficient translation of viral mRNAs during the late stages of infection (59, 60).

Protein kinase R (PKR) is known to phosphorylate the translation initiation factor eIF2 in an inhibitory manner. This results in a general inhibition of protein synthesis. PKR is mostly activated by interferon- α and interferon- β as well as double-stranded RNA (dsRNA) and thus represents a cellular anti-viral defence mechanism (reviewed in reference 61). However, VA RNAI prevents the activation of this response by binding to PKR in an inhibitory way, thus allowing the efficient translation of viral mRNAs (57, 62, 63)

Finally, both VA RNAs were shown to influence the cellular RNAi pathway. The RNase III enzyme Dicer processed dsRNA into short RNAs of ~22 nucleotides (nt) in length. These are incorporated into the RNA-induced silencing complex (RISC) and guide the degradation of mRNAs containing complementary sequences (a more detailed description of the RNAi pathway can be found in the section “RNA interference”). It has been shown, that VA RNAs are processed into miRNA-like small RNAs (referred to as svaRNAs or mivaRNAs), which are incorporated into RISC and may target cellular mRNAs (9, 35, 64). Moreover, it has been reported that adenovirus is able to block the activity of Exportin-5, Dicer as well as RISC and probably Drosha at late stages of infection. Due to the high abundance of VA RNAs, this might be explained by oversaturation effects (7, 8).

However, the extent to which mivaRNAs may contribute to a successful viral replication is still the object of intensive research. In particular the reliable identification of target genes remains the major challenge and is one major object of this thesis.

3.2 RNA interference

In 1998, Mello and Fire could demonstrate for the first time, that dsRNA, when injected into the nematode *Caenorhabditis elegans* can interfere with endogenous gene activities in a highly efficient manner and were thus awarded the Nobel prize for Physiology or Medicine in 2006 (65, 66). Subsequently, it has been shown that the introduction of artificially synthesized short interfering RNAs (siRNAs) can trigger gene silencing also in mammalian cells (67). This discovery has shaped the future for the development of RNAi-based therapeutics (68).

3.2.1 The canonical RNA interference pathway

Sequence-specific mRNA degradation is mainly mediated by ~22-nt small interfering RNAs (67). These are incorporated into the RISC, where they can mediate silencing of their target genes either by repressive epigenetic modifications or post-transcriptionally. Post-transcriptional silencing can occur either by direct cleavage of the target mRNA or by translational repression and subsequent mRNA decay. The mechanism induced depends on the degree of complementarity between the small RNA guide sequence and the target RNA. Small interfering RNAs showing perfect complementarity encourage nucleolytic cleavage by a core protein of RISC termed Argonaute 2 (Ago2). In contrast, particularly when nucleotides 2 to 8 of the guide sequence (seed sequence) show reduced complementarity to their target, translational repression and RNA decay is the preferred mechanism (Fig. 4). This mechanism is utilized by miRNAs (68).

In the first step of miRNA biogenesis, primary (pri)-miRNAs are transcribed from miRNA genes by RNA polymerase II or III. pri-miRNAs form pronounced stem-loop secondary structures. These stem-loops are cleaved off by the RNase III Drosha, which is part of the nuclear microprocessor complex. Next, a different RNase III, termed Dicer, processes pri-miRNAs to ~22 base-pair duplex RNA. Finally, one strand (the guide strand) is loaded into the RISC, whereas the unincorporated strand (passenger strand) is degraded. The incorporated strand acts as a mature miRNA by guiding the recognition of its target sequence (69). This usually results in silencing of the target gene, although it has been proposed that miRNAs may also enhance the translation of several proteins (70, 71).

3.2.2 The physiological functions of miRNAs

Up to now, more than 1500 miRNAs encoded in the human genome have been proposed and the number of their putative target mRNA is consistently increasing (72-74). It is generally accepted that a controlled expression of miRNAs is indispensable for a variety of biological and cellular functions such as development, differentiation, cell cycle control, or apoptosis (reviewed in reference 75). It is therefore obvious, that a deregulated expression of one or more miRNAs might have a pathogenic outcome. In Alzheimer's disease, decrease of the miR-29a/b-1 cluster is associated with an increase of the A β protein, the major component of amyloid plaques (76). Furthermore, in patients suffering from Parkinson's disease, deficiency for miR-133b in midbrain tissue was reported, which may be conducive to the loss of midbrain dopaminergic neurons (77). Additionally, a role for miRNAs in the development of a wide range of cancers has been proposed. A prominent example is the downregulation of the *let-7* family, which correlates with poor prognosis in lung cancer. Moreover, an implication of miRNA profiling for cancer diagnostics has been reported. Expression analysis allowed determining the developmental lineage and differentiation state of a tumor, as well as predicting the primary origin of metastasis (75). The essentiality of the miRNA pathway is further substantiated by the fact, that loss of Dicer or Argonaute proteins is lethal for most animals. For example in mice lacking Dicer, stem cell development is highly impaired, which results in embryonic lethality (78).

Despite rapid progress in the field of miRNA research, many biological functions of miRNAs are poorly understood and object of intensive research. Due to the facts that one miRNA may have multiple target mRNAs and one single mRNA might be targeted by several miRNAs creates an unmanageable complexity and the future challenge will be to unravel these labyrinthine interactions.

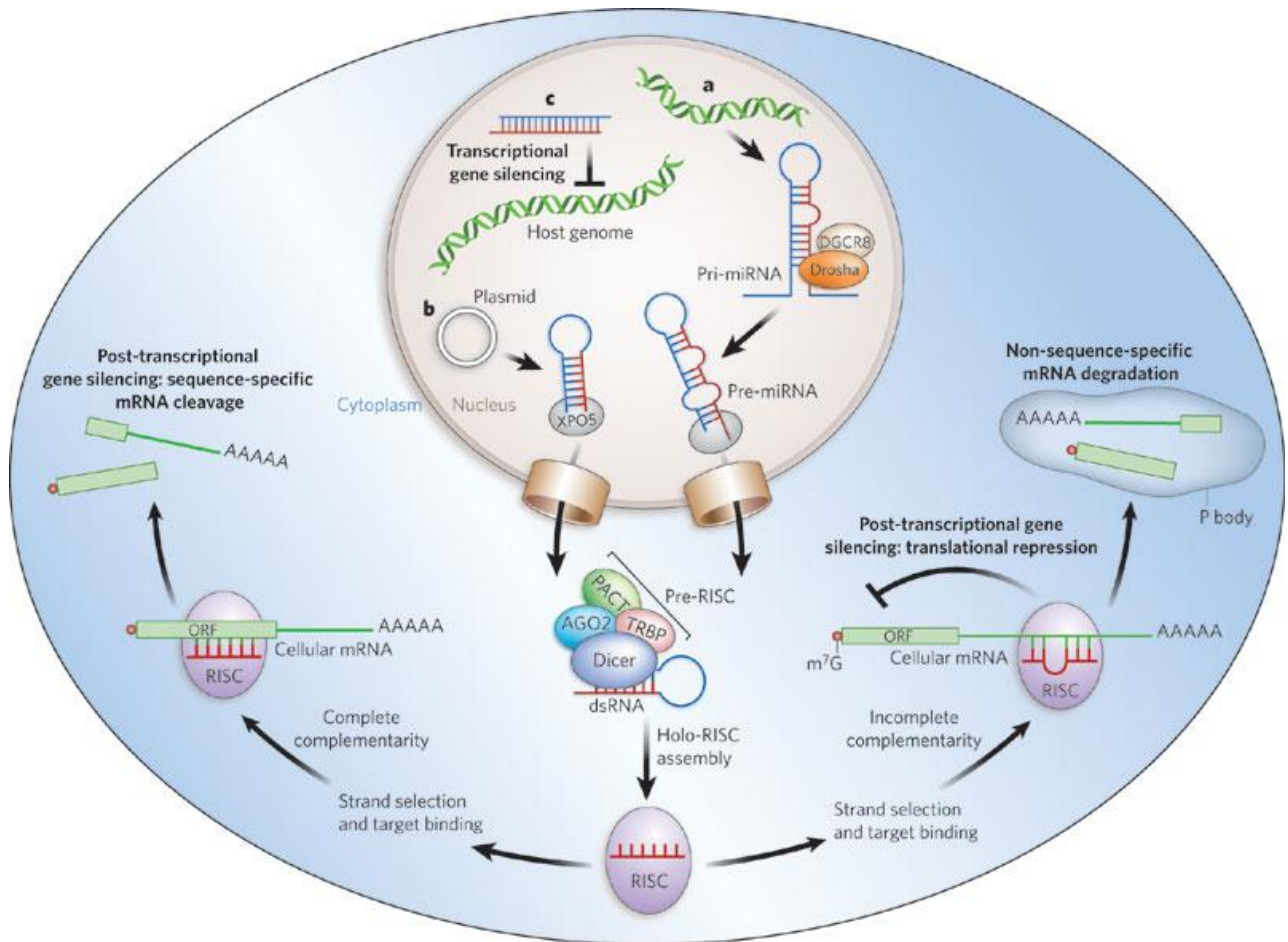


Figure 4: Mechanisms of cellular gene silencing. (Taken from reference 68). (A) In the first step the enzymes Drosha and DGCR8 catalyze the processing of primary microRNAs (pri-miRNAs) into precursor RNAs (pre-miRNAs). The latter are exported out of the nucleus by Exportin-5 (XPO5). In the cytoplasm they are recognized and processed into short double stranded RNA fragments by Dicer, which is part of the pre-RISC protein complex. One strand which is also referred to as the guide strand is selectively incorporated into the holo-RISC by Argonaute 2 (Ago2). The guide sequence can base-pair with corresponding target mRNAs. If a full complementarity is achieved (Fig. 4, left side), Ago2 catalyzes the cleavage and degradation of the target mRNA. If the guide strand is not fully complementary to its mRNA target, but base-pairing of the nucleotides 2-8 of the miRNA (defined as the seed region) occurs, mechanisms of translational inhibition take place. These can include the accumulation and non-sequence-specific degradation of silenced mRNAs in p bodies. (B) The introduction of artificial short hairpin(sh)RNAs (e.g., by the transfection of plasmid vectors), leads to the activation of the RNAi pathway. shRNAs are exported from the nucleus by XPO5. In the cytoplasm they are processed by Dicer and loaded into the RISC machinery. Those siRNAs can lead to the degradation of their target mRNAs by specific cleavage through Ago2.

(C) siRNAs in the nucleus which are base-pairing to promoter sequences can cause epigenetic modifications of chromatin which may lead to transcriptional gene silencing.

3.2.3 Viruses and RNAi

Besides being involved in gene regulatory functions, RNAi has been proposed to be a natural antiviral defence mechanism in invertebrates and plants. Virus-derived long dsRNAs are processed into siRNAs, which, after amplification, prime RNAi. However, this mechanism has not been shown for vertebrates yet. In vertebrates, the interferon (IFN) response is the primary antiviral defence mechanism of the innate immunity, leading to an overall halt of protein synthesis, cellular RNA degradation and in many cases death of the host cell (79). Nevertheless, RNAi may contribute to mammalian antiviral responses as well and this hypothesis is supported by a series of evidence. Matskevich *et al.* (80) could demonstrate that Dicer-knockdown in Influenza A-infected cells led to an increase of virus production and enhanced apoptosis rates. Furthermore, the adenovirus VA RNAI and VA RNAII are processed by Dicer, incorporated into RISC and thus suppress RNAi (a more detailed description can be found in the section “The virus-associated RNAs”) (7). Finally, the HIV-1 trans-activation-responsive RNA sequence (TAR) was shown to interfere with RNAi by sequestering the TAR RNA-binding protein (TARBP), which is essential for correct function of Dicer (81).

General mechanisms to suppress RNAi may have evolved to escape inhibitory effects of host miRNAs on virus replication. Evidence for such mechanism comes from experiments which have demonstrated that interferon beta (IFN β) rapidly changes the miRNA expression pattern in a cell by upregulating anti-viral miRNAs. Some of them were shown to have complementary sequences to hepatitis C virus (HCV) transcripts or the HCV genome. The induction of such miRNAs creates an antiviral environment within the cell, mostly by directly targeting HCV, leading to an inhibition of hepatitis C virus (HCV) replication and infection (Fig. 5) (82).

A different study demonstrated that a panel of human miRNAs is enriched in primary resting CD4⁺ cells. Some of them were shown to target the 3' ends of HIV-1 mRNAs. In CD4⁺ cells, HIV-1 replication is inhibited and the virus favors to establish latent infections. To test the hypothesis whether cellular miRNAs may contribute to virus persistence, a combination of five antisense-inhibitors against putative antiviral-

miRNAs were used. The transfection led to a substantial increase of HIV-1 protein and viral particles, assigning cellular miRNAs an important role in the suppression of virus replication (83).

Recently, the existence of antiviral-miRNAs was further highlighted by similar experiments conducted for human papillomavirus and hepatitis B virus (84 and references therein).

Besides the proposed function of host-miRNAs to provide a defence against viruses by targeting their transcripts, a contrary role has been suggested as well. A prominent example affects the life cycle of HCV. miR-122 is specifically expressed in the liver where it represents about 70% of the whole miRNA population (85, 86). It has been proven, that the expression of miR-122 affects HCV replication in a positive manner (Fig. 5). This may provide a possible explanation for the tissue tropism of the virus (87, 88).

In summary, different host miRNA expression patterns may either promote or delay the infection cycle of a virus and additionally may be involved in the establishment of latent infections.

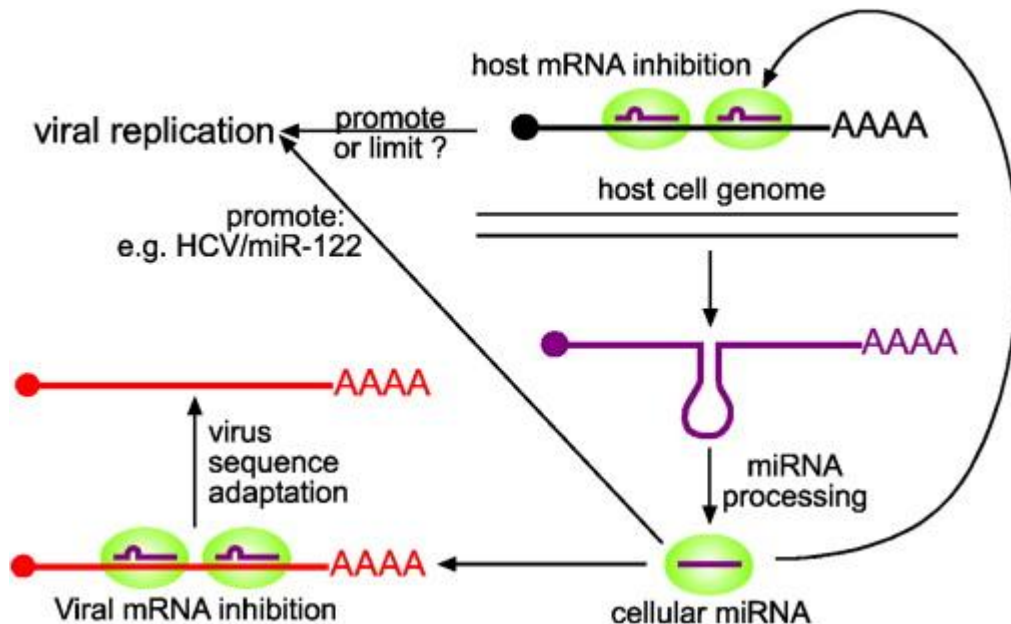


Figure 5: Potential Effect of Cellular miRNAs on Virus Replication. (Taken from reference 88). Viral replication may be affected in either a promoting or an inhibitory manner due to cellular miRNAs (purple) binding to viral RNAs (red). One example would be miR-122 which was shown to promote the replication of Hepatitis C Virus (HCV). Furthermore, an

indirect promotion or limitation of virus replication by cellular miRNAs regulating their cellular target RNAs would be conceivable.

Another aspect of the interplay between viruses and RNAi comprises the field of virally encoded miRNAs. These small RNAs can directly modulate viral and/or cellular gene expression (Fig. 6) (88). The existence of viral miRNAs has been demonstrated for a wide range of nuclear DNA viruses comprising adenoviruses, *Heliothis virescens* ascoviruses, polyomaviruses and the whole herpesvirus family (89). One possible scenario involving virally encoded miRNAs is the interference with the host RNAi machinery by either competing for binding sites or by directly targeting host transcripts and thus creating an environment favourable for virus replication. Such an example would be the human cytomegalovirus, which encodes a miRNA targeting a cellular gene related to the major histocompatibility complex I. This leads to its downregulation and subsequent escape of the infected cell from natural killer cell-mediated destruction (90). The adenovirus-encoded small RNAs counteract cellular RNAi by saturating Exportin-5, Dicer, RISC and possibly Drosha (7, 8). A description of proposed biological functions of the VA RNAs can be found in the chapter “The virus-associated RNAs”.

Besides the interference with host-processes, viral miRNAs can also act as regulators of viral gene expression. Such mechanisms have been shown for herpesvirus, where the virally encoded miR-UL112-1 downregulates immediate-early genes. Suggested biological consequences of such regulators are optimisation of virus replication in different cellular environments by fine-tuning viral gene expression. Furthermore, downregulation of viral genes during the infection cycle may be crucial for the establishment and maintenance of persistent infections (91, 92). Similar mechanisms would be conceivable for adenovirus as well, as it was shown that sequences complementary to the virus-derived miRNAs appear in the viral genome (Fig. 2A) (35). However, this assumption is rather speculative and intensively investigated.

Several ways by which viral miRNAs may strive to mediate the regulation of their own gene expression or to interfere with cellular processes are summarized in Fig. 6 and extensively reviewed in (88) and (93).

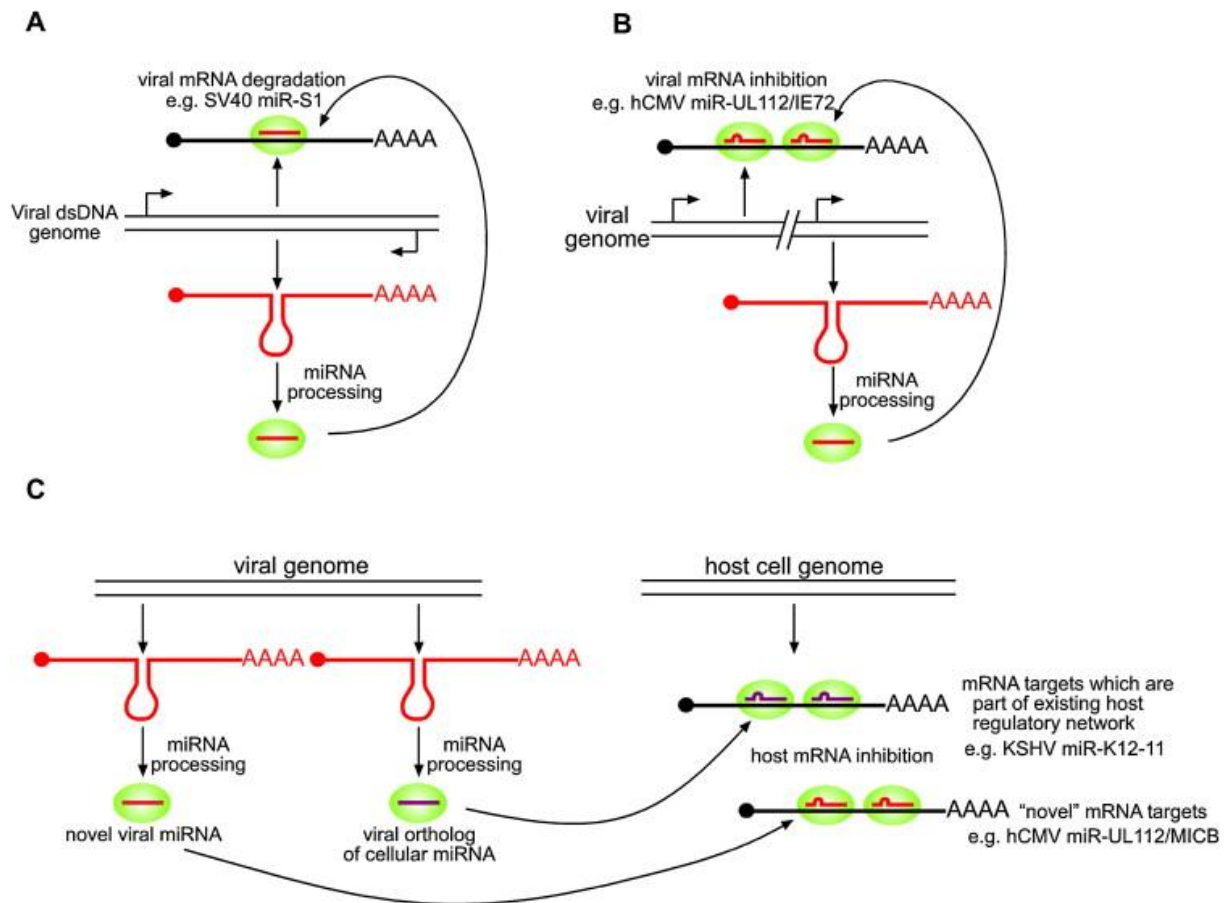


Figure 6: Suggested functions of viral miRNAs. (Taken from reference 88). (A) Virus-encoded miRNAs are loaded into the RISC (green circle) and bind to viral mRNAs with perfect complementarity, which causes site-specific cleavage by Ago2. (B) Translational inhibition of viral transcripts by virus-encoded miRNAs. Viral miRNAs may have non perfect sequence complementarity to their targets mRNAs, which leads to repression of protein synthesis. (C) Virus-encoded miRNAs may disturb endogenous miRNA-regulated networks by directly targeting cellular mRNAs (red) or by mimicking the functions of cellular miRNAs (purple).

3.2.4 Experimental approaches to study miRNA-mRNA regulatory networks

The mechanisms by which miRNAs recognize their target sites were intensively studied during the last years, leading to the definition of rules for target site prediction. It is generally accepted, that the highly conserved Watson-Crick pairing between the seed region of the miRNA and the sequence of its target mRNA, usually located within the 3' untranslated region (UTR), is sufficient to prime RNAi (94 and references therein). Based on this fact, attempts were made to design algorithms for

the prediction of miRNA binding sites. However, miRNA-mRNA interactions may be more sophisticated. It was for example shown that strong complementarity of the miRNA 3' end can compensate for reduced binding of the seed region (95). Furthermore centered binding sites, base-pairing to 11-12 nucleotides in the central region of a miRNA, have been suggested (96). These dynamics in the definition of a target site is usually not considered in target prediction programs. To account for such alternative binding mechanisms will be a challenge for the future and will help to predict a more accurate spectrum of target sites.

The predominant experimental techniques to screen for miRNA targets are to overexpress the miRNA of interest or to inhibit its expression, followed by analysis of differentially expressed genes. However both approaches are reported to yield high false positive rates, as indirect changes in gene expression cannot be distinguished from miRNA-mediated ones. Finally, mRNAs which are translationally repressed rather than cleaved upon miRNA targeting will not necessarily show altered gene expression upon overexpression or inhibition of a miRNA (97, 98).

Given the limitations in computational miRNA target identification, efforts were made to develop novel approaches. Co-Immunoprecipitation of RISC along with its incorporated miRNAs and target mRNAs has been proven to be a powerful method. (99-104). The RNA immunoprecipitation (RIP)-Chip approach consists of an immunoprecipitation of RISC followed by microarray analysis of the target mRNAs. Modified protocols make use of deep sequencing instead of microarrays and are referred to as RIP-Seq (105). This method has been already successfully employed to identify the targetome of several viruses such as the Kaposi's sarcoma-associated herpesvirus and Epstein-Barr virus (106). Here, a similar approach was used to study the overall impact of adenovirus on the targetome of an infected cell and to further elucidate the RNAi-coupled functions of miRNAs by identifying their targets genes.

Alternative approaches to RIP-Chip/RIP-Seq were introduced recently. The major difference is the use of ultraviolet light prior to immunoprecipitation to induce crosslinking of RNA to RNA-binding proteins. High-throughput sequencing of RNA isolated by crosslinking immunoprecipitation (HITS-CLIP), has been shown to be efficient, however the method has been mainly criticized for its low efficiency of crosslinking (98). A novel approach termed photoactive-ribonucleoside-enhanced crosslinking (PAR-CLIP) introduced by Hafner *et al.*, uses photoactivatable nucleosides in cell culture medium to enhance the cross linking efficiency and may

hold promise for the future (107). Detailed information about the advantages and disadvantages of the distinct techniques used to assay miRNA-mRNA interaction can be found in reference 98.

4 Aims of this thesis

Given the fact, that the adenovirus reproductive cycle is closely linked to cellular RNAi mechanisms, the goal of the present work was to gain a deeper insight into this complex interplay. Therefore emphasis was laid on the following points:

1. Establishment of a RIP-assay as a reliable tool for studying miRNA-mRNA interactions
2. Investigation if VA RNA-derived miRNAs target cellular mRNAs
 - Design and functional validation of artificially generated mivaRNAs (mivaRNA mimics)
 - Screening for cellular mRNA targeted by mivaRNA mimics using a RIP-Chip approach and bioinformatic analysis
 - Validation of the RIP-Chip results with further molecular biological methods
3. Characterization of the protective function of mivaRNAs on adenoviral mRNAs
 - Comparison of steady-state levels of viral transcripts of adenovirus type 5 wild-type and a mutant lacking the VA RNA genes
 - Identification of viral transcripts targeted by miRNAs using the RIP-assay and computational prediction

5 Materials and Methods

5.1 Cell culture

Cells were usually maintained in 75 cm² cell culture flasks at 37°C in a 5% CO₂ humidified atmosphere. A549 (human lung carcinoma cell line; ATCC CCL-185) and HEK293 (human embryonic kidney; ATCC CRL-1573) were maintained in DMEM high glucose medium with stable glutamine (PAA) supplemented with 10% fetal bovine serum (FBS; PAA). When reaching confluency, cell cells were split (1:10) using 0.25% trypsin/EDTA at 37°C for enzymatic cell removal. BJAB (EBV-negative B lymphoma cell line; (108)), THP-1 (Human acute monocytic leukemia cell line; ATCC TIB-202) and U937 (Human monocytic cell line; ATCC CRL-1593.2) were grown in RPMI 1640 (PAA) including 10% FBS (PAA). Cells were cryopreserved in the presence of DMEM media, supplemented with 10% dimethylsulfoxide (DMSO) and 20% DMSO and stored in liquid nitrogen.

5.2 Amplification and purification of virus

For propagation of adenovirus and adenoviral vectors, HEK293 cells were seeded into cell culture flasks and grown to approximately 70% confluence. Subsequently, virus was added and cells were incubated until showing cytopathic effects. After harvesting, adenovirus-containing cells were subjected to three freeze-thawing cycles to lyse the cells and to liberate virus particles. This was followed by centrifugation for 15 min at 3000 rpm to pellet the cell debris. The supernatant was filtered through a 0.45 µm filter. Adenovirus crude suspensions were purified by two rounds of standard CsCl gradient centrifugation. Adenoviral bands were isolated from the gradient and dialyzed against a buffer containing 10% glycerol (87%), 20 mM HEPES pH 7.3 and 50 mM NaCl. Purified adenovirus was stored at -80°C after mixing in a ratio of 1:1 with storage buffer (70% glycerol, 20 mM HEPES pH 7.3, 50 mM NaCl and 0.1% BSA). Infectious virus particle titers were determined on HEK293 cells by 50% tissue culture infective dose (TCID₅₀) assays.

5.3 FACS

In a first attempt to determine the infection efficiency of Ad5, a cytomegalovirus (CMV) promoter-controlled, enhanced green fluorescent protein (EGFP)-expressing recombinant adenoviral vector (pAD-CMV-EGFP, R. Klein, unpublished) was used. A549 cells were seeded at a density of 1×10^5 cells per well of a 24-well plate the day before infection. Cells were infected with the adenoviral vector at a multiplicity of infection (MOI) of 1, 10, 30, and 50 in duplicate. The virus-containing medium was removed 4 hours post-infection; cells were washed with PBS and supplied with fresh medium. BJAB, THP-1, and U937 cells were infected at an MOI of 50, 100, 200 and 500. Therefore, 1×10^5 cells were collected and resuspended in 100 μ l RPMI 1640 medium w/o FCS. Infections were carried out in 96-well tissue culture plates. 4 hours post-infection the cells were transferred into microcentrifuge tubes. Cells attached to the surface of the plate were removed by treatment with 0.25% trypsin/EDTA at 37°C. Cells were washed with PBS and supplied with fresh medium containing 10% FBS. 24 hours after infection, cells were harvested and analyzed with a FACS analyzer (FACS Calibur, Becton Dickinson) to determine the percentage of infected, EGFP-expressing cells.

5.4 Vector construction

Target vectors for miRNAs were generated by gene synthesis (Eurofins MWG, Ebersberg, Germany). Therefore, oligonucleotides corresponding to the miRNA target sequences were synthesized and cloned into psiCHECK-2 (Promega, Mannheim, Germany). Vector maps can be found in the section „Appendix“.

5.5 Synthesis and transfection of miRNA mimics

Single-stranded RNA oligonucleotides (Complementary sense and antisense strand of the respective mimic; Table 1) were purchased from RiboTask (Odense, Denmark). Oligonucleotides were annealed to produce dsRNA. Therefore, equimolar amounts (20 μ M) of the two oligonucleotides were combined in a 75 μ l reaction

containing 50 mM Tris (pH 7.5) and 100 nM NaCl in nuclease-free H₂O. The solution was incubated at for 2 minutes at 95°C and allowed to slowly cool down to room temperature for one hour. Finally the solutions were cooled down to 4°C for another hour. The double-stranded mivaRNA mimics were analyzed by gel electrophoresis (Appendix) and stored at -20°C.

Table 1: Sequences of the RNA oligonucleotides used for producing mivaRNA mimics

RNA oligo name	Sequence (5' to 3')
mivaRI(A)-137-5'	AGCGGGCACUCUCCGUGGUCUGG
mivaRI(A)-137-3'	AGACAACGGGGGAGUGCUCCUUUU
mivaRI(G)-137-5'	GGGCACUCUCCGUGGUCUGG
mivaRI(G)-137-3'	AGACAACGGGGGAGUGCUCCU
mivaRII-138-5'	GGCUCGCUCCCUGUAGCCGGA
mivaRII-138-3'	CGGAAACAGGGACGAGCCCC

For RIP-assays, 3e+06 A549 cells were reverse transfected with 30 nM of the individual mivaRNA mimic or a non-targeting control siRNA using Lipofectamine 2000 (Invitrogen/LifeTechnologies Austria, Vienna, Austria). For each transfection, 30 µL Lipofectamine 2000 was diluted with 1470 µL OptiMEM medium (Invitrogen/LifeTechnologies Austria, Vienna, Austria). After 5 minutes of incubation, 1500 µL diluted Lipofectamine 2000 was mixed with 1500 µL of a specific siRNA mix. After 20 minutes of incubation, the mixes were combined with 12 ml of cell suspension and seeded into 10-cm cell culture dishes.

5.6 Purification of nucleic acids

Circular plasmid DNA was extracted with EasyPrep Pro Plasmid Mini-prep Kits (Biozym, Oldendorf, Germany) or EndoFree Plasmid Maxi Kit (Qiagen, Hilden, Germany). Total RNA was isolated using an RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Genomic DNA was digested using the RQ1 RNase-Free DNase (Promega, Mannheim, Germany) according to the standard protocol. Different RNA isolation methods were used in RNA immunoprecipitation experiments and are described in the respective section. The

nucleic acid concentration as well as the 260/280 nm and 260/230 nm ratios were assessed using a spectrophotometer (NanoDrop ND-1000, Thermo Fisher Scientific, Wilmington, DE, USA).

5.7 cDNA synthesis and RT-qPCR

Reverse transcription was carried out using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems/Life Technologies Austria, Vienna, Austria). mRNA levels were determined by TaqMan RT-qPCR assays carried out on a LightCycler® 2.0 (Roche Diagnostics, Vienna, Austria) instrument. RT-qPCR was performed with a Light Cycler® TaqMan® Master Mix (Roche Diagnostics, Vienna, Austria), 300 nM of the primers, 100 nM of the hybridization probes and 5µl of the cDNA sample in a total reaction volume of 20µl. Sequences of primer/probe sets specific for individual adenoviral or cellular mRNAs are listed in Table 2. Thermocycling conditions consisted of a pre-denaturation step at 95°C, followed by 50 cycles of amplification: denaturation 10 sec at 95°C, annealing and elongation for 15 sec at 60°C to 68°C.

Table 2: Primer and probe sequences used for RT-qPCR assays

Primer name	Sequence (5' to 3')
E1A 289R-cDNA-f1	GCATGTTTGTCTACAGTCCTGTGTC
E1A 289R-cDNA-r1	GGCGTCTCAGGATAGCAGGC
E1A 289R-cDNA-p1	AGGCTCCGGTTCTGGCTCGGG
E1A-9S-cDNA-f1	AGCTGATCGAAGAGGTCCTGTG
E1A 289R-cDNA-r1	GGCGTCTCAGGATAGCAGGC
E1A 289R-cDNA-p1	AGGCTCCGGTTCTGGCTCGGG
E1B_mRNA4-f1	GCGAGTGTGGCGGTAAACAT
E1B_mRNA4-r1	GGCGGCTGCTCAATCTGTAT
E1B_mRNA4-p1	TGCACCCGCGCTGAGTTTGGC
E1B_mRNA5-f1	AATGTTGTACAGCAGCCGCC
E1B_mRNA5-r1	TGGAGCCCATCACATTCTGAC
E1B_mRNA5-p1	TCATATTTGACAACGCGCATGCCC
Hex-cDNA-f1	CAGTCACAGTCGCAAGAGGAGC
Hex-cDNA-r1	AGGTACTCCGAGGCGTCCTG
Hex-cDNA-p1	ACCACTGCGGCATCATCGAAGGG
IVa2-cDNA-f1	GGAAACCAGAGGGCGAAGA

IVa2-cDNA-r1	AGGGTCCTCGTCAGCGTAGTC
IVa2-cDNA-p1	CTTGAGGCTGGTCCTGCTGGT
ML-cDNA-f1	CAGCTGTTGGGCTCGCG
ML-cDNA-r1	CGGGTTTCCGATCCAAGAGTAC
ML-cDNA-p1	AGGACAAACTCTTCGCGGTCTTTCC
DBP-cDNA-f1	GCGATATCAGGGGAGAAGGAA
DBP-cDNA-r1	ACGACACGTCCTCCATGGTT
DBP-cDNA-p1	TCGCGCTGCTCCTCTTCCCGA
E3-cDNA-f1	CGCGACTCCGTTTCAACC
E3-cDNA-r1	GTTCCCGGCAGGTAAGGAG
E3-cDNA-p1	CCTCTCCGAGCTCAGCTACTCCATCAG
E4-f1	ACAGGAAACCGTGTGGAATATAACA
E4-r1	CCAACACACAGAGTACACAGTCCTTT
E4-p1	TCCCCGGCTGGCCTTAAAAAGCA
Pol-cDNA-f1	ATGGCCTTGGCTCAAGCTC
Pol-cDNA-r1	GCGTAGGTTGCTGGCGAAC
Pol-cDNA-p1	CGCCTCTGCGTGAAGACGACGG
pTP-cDNA-f2	AAACCAACGCTCGGTGCC
pTP-cDNA-r2	GGACGCGGTTCCAGATGTT
pTP-cDNA-p2	CGCGCGCAATCGTTGACGCT
GAPDH-f1	TGCACCACCAACTGCTTAGC
GAPDH-r1	GGCATGGACTGTGGTCATGAG
GAPDH-p1	CCTGGCCAAGGTCATCCATGACAACTT
CCNE1-f1	GCTGCTTCGGCCTTGTATCA
CCNE1-r1	GGAACCATCCACTTGACACAGTTC
CCNE1-p1	CAAAAGGTTTCAGGGTATCAGTGGTGCG
HMGA2-f1	AACCGGTGAGCCCTCTCCTA
HMGA2-r1	CTCCAGTGGCTTCTGCTTTCTT
HMGA2-p1	CCCAAAGGCAGCAAAAACAAGAGTCCC
hRluc-f2	TGAGAGTGTCTGGACGTGATC
hRluc-r2	CACCATTTTCTCGCCCTCTTC
hRluc-p2	CGAGTGGCCTGACATCGAGGAGGAT
FLuc-f3	GGACCGCCTGAAGTCTCTGA
FLuc-r3	CTCCGGCGTCGAAAATGTTA
FLuc-p3	AAGGGCTACCAGGTGGCCCCAGC

For the validation of microarray data, RT-qPCR was performed using the iQ SYBR Green supermix (Bio-Rad, Vienna, Austria). Therefore, 600 nM of each primer was combined with 5 µl cDNA in a 20 µl reaction. Thermocycling conditions consisted of a pre-denaturation at 95 °C, followed by 50 cycles of amplification: denaturation 10 sec

at 95 °C, annealing for 10 sec at 63°C and elongation for 15 sec at 72°C. Primer sequences are listed in Table 3:

Table 3: Primers used for RT-qPCR to validate microarray data

Primer name	Sequence (5' to 3')
CRIPAK-f1	GAGTCAGACGCTGTTACCGT
CRIPAK-r1	ACGTTATCTCGAGGTGCAGAG
IL12A-f1	TGCTCCAGAAGGCCAGACAA
IL12A-r1	AAACAGGCCTCCACTGTGCT
ISOC1-f1	CCGAGGAGTCGAGGTTTAC
ISOC1-r1	TATGATCCCGGTTTCGAGCGA
TMEM222-f1	TGCTCTACGGGAAGTACGTCA
TMEM222-r1	CCGGAGGTTAAAGACCAGGC
GPR63-f1	CCGTAGGAAACCCCGACCTG
GPR63-r1	GCCTGGTAGCCTGGATTGGT
SUPT7L-f1	GGAGGAACCTGTGAGCGACA
SUPT7L-r1	CCCAAGCACTCCCATAGGCA
GUK1-f1	GTATGGCACGAGCAAGGTG
GUK1-r1	GCGGCTGCACAGAGATGTAG
PRR3-f1	CCTCGAAGGCTCAAAAGCTG
PRR3-r1	CGGATTTGTCTTCCATAACCTGG
LY6K-f1	ACCTGACTGCGAGACAACGA
LY6K-r1	TGGGTTCTGGCACTCGAAAGT
LEPROTL1-f1	TTTGATGCTTGGATGTGCCCT
LEPROTL1-r1	GCCCGTTGTAAGAAAGATGGC
SRI-f1	CAGAAGGCCCTGACAACAATG
SRI-r1	GGCGATGTAGTCGTCGAAGG
LGALS3-f1	TGGTGCCTCGCATGCTGATA
LGALS3-r1	GCGTGGGTTAAAGTGGAAGGC
RPL34-f1	TGGTGGTTCCATGTGTGCTAA
RPL34-r1	GCTTGTGCCTTCAACACTTTC
ZNF768-f1	AGGATGTGCAGAGTTCTGACG
ZNF768-r1	TCGACTTCATAGTCCCCACTG
COMMD1-f1	GCTACGGAGCCAGCTATATCC
COMMD1-r1	GGTTTGAGCAGTCAAGAATGCC
COMMD7-f1	TCTCTGAATTTGCCACCACCA
COMMD7-r1	GCACCATTTGGAACCAGAAGG
COMMD9-f1	CGAGGCAATTCTGGCTCTCTT

COMMD9-r1	GGCTTCGGTTCTCCAAGTAGAC
NAT14-f1	GCGGGAGATGAGGGAAGATG
NAT14-r1	GTTTTCCGTGTCCTTCACGC
SMAGP-f1	ACCAGCCTCCTGACTACTCC
SMAGP-r1	CTGCAATGAGTGCTGTGCTG
BOK-f1	CAGTCTGAGCCTGTGGTGAC
BOK-r1	GCATACAGGGACACCACCTT

5.8 Luciferase reporter gene assay

1.5e+05 HEK 293 cells were seeded into the wells of 96-well tissue culture plates. Cells were reverse transfected using Lipofectamine 2000 (Invitrogen/LifeTechnologies Austria, Vienna, Austria). For one single transfection 200 ng of pAdVantage vector (Promega, Mannheim, Germany) and 100 ng of the reporter vectors were used. miRNA mimics were used at a final concentration of 10 nM per transfection. For each well, 0.5 µL Lipofectamine 2000 was diluted with 24.5 µL OptiMEM medium (Invitrogen/LifeTechnologies Austria, Vienna, Austria) and incubated for 5 minutes. Next, 25 µL of a specific siRNA/reporter vector mix (diluted in OptiMEM) was added. After 20 minutes of incubation, the mixes were pipetted directly into the wells of a 96-well plate and freshly harvested cells were added. After 24 hours, medium was exchanged and cells were incubated for another 24 hours. Firefly and *Renilla* luciferase activities were determined at 48 hours post-transfection using the Dual-Glo luciferase assay (Promega, Mannheim, Germany), following the manufacturer's instructions. Briefly, 75 µL of Dual-Glo Reagent was added to cells grown in 75 µL medium, and after 10 min of incubation at room temperature, Firefly luciferase activity was measured. Next, one volume of Dual-Glo Stop & Glo reagent was added to each well, plates were incubated for an additional 10 min at room temperature and *Renilla* luciferase activity was determined. Luminescence was measured on a Wallac Victor 1420 Multilabel Counter (Perkin Elmer Austria, Brunn am Gebirge, Austria). Knockdown rates were calculated by normalizing *Renilla* luciferase activities to Firefly luciferase activities, and comparing dual-luciferase ratios between miRNA mimics and a non-targeting control siRNA and between the respective reporter-vectors and an unmodified psiCHECK™-2 vector (Promega, Mannheim, Germany).

5.9 Western blotting

Cell lysates and beads-ribonucleoprotein (RNP) complexes from immunoprecipitations were mixed with NuPAGE[®] LDS Sample Buffer (NP0007; Novex[®], Life technologies Austria, Vienna, Austria) and NuPAGE[®] Sample Reducing Agent (NP0009; Novex[®] Life technologies Austria, Vienna, Austria) at the recommended concentrations. Samples were heated to 70°C for 10 minutes prior to loading onto the gel. To recover proteins from beads, samples were heated to 100°C for 5 minutes. The mixture was centrifuged at 2,000 x g for one minute at room temperature and the supernatant was used for electrophoresis. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on NuPage[®] 10% Bis-Tris gels (Invitrogen/Life Technologies Austria, Vienna, Austria) and transferred to nitrocellulose membranes (Whatman, Dassel, Germany). Membranes were stained with Ponceau S and subsequently blocked with blocking reagent (Roche Diagnostics, Vienna, Austria). Argonaute proteins were detected using the anti-pan Ago antibody (Clone 2A8, MABE56; Merck Millipore). A control antibody (sc-32233; Santa Cruz Biotechnology Inc., Santa Cruz, USA) was used to detect GAPDH which served as a reference. Bands were visualized using a secondary fluorescent goat anti-mouse antibody (35521; Pierce Biotechnology Inc, Rockford, USA). Fluorescence was measured using a LI-COR Odyssey detection system (Li-Cor Biosciences, Lincoln, NE, USA).

5.10 Preparation of cell lysates for immunoprecipitation

Cells were collected and washed five times with PBS to remove traces of cell culture medium. Cells were lysed using an aqueous buffer containing 25 mM Tris-HCl, pH 8.0 (15567027; Invitrogen/Life Technologies Austria, Vienna, Austria), 150 mM NaCl (AM9760G; Invitrogen/Life Technologies Austria, Vienna, Austria), 2 mM MgCl₂ (AM9530G; Invitrogen/Life Technologies Austria, Vienna, Austria), 0.5% IGEPAL[®] CA-630 (I8896; Sigma Aldrich St. Louis, MO, USA), 5mM DTT (43816; Sigma Aldrich St. Louis, MO, USA), protease inhibitors and 250U/ml RiboLock[™] RNase Inhibitor (10389109; Fermentas, Glen Burnie, MD, USA). Cells were resuspended in lysis

buffer and incubated for 10 minutes on ice to lyse cells. 1 ml of lysis buffer was used to lyse $\sim 6 \times 10^6$ cells. Nuclei and debris were removed by centrifugation and the lysates were subjected to preclearing.

5.11 Immunopurification of RISC and isolation of associated RNAs

RISC and associated RNAs were isolated using an adapted protocol of a previously described RNA-immunopurification assay (available at <https://ruo.mbl.co.jp/gtf/1/1/RN1001.pdf>). For immunoprecipitation, an anti-pan Ago antibody (Clone 2A8, MABE56; Merck Millipore, Darmstadt, Germany) and a mouse IgG1 isotype control antibody (Clone 1E2.2, CBL610; Merck Millipore, Darmstadt, Germany) was used respectively. Prior to immunoprecipitation, antibodies (15 μ g) were coupled to Protein G sepharose beads (Recombinant Protein G – Sepharose® 4B, 10-1242; Invitrogen/Life technologies Austria, Vienna, Austria). For one single Immunoprecipitation (IP), 25 μ l of beads were washed three times with 1 ml of nuclease-free, ice-cold PBS (AM9624; Invitrogen/Life technologies Austria, Vienna, Austria). Antibodies were allowed to attach to the beads in 1 ml of nuclease-free PBS containing the protease inhibitors aprotinin (10 μ g/ml, 10236624001; Roche, Vienna, Austria), leupeptin (5 μ g/ml, 11017101001; Roche, Vienna, Austria), phenylmethylsulfonyl fluoride (100 μ g/ml, P7626; Sigma Aldrich St. Louis, MO, USA) and pepstatin (1 μ g/ml, 10253286001; Roche, Vienna, Austria) at 4°C using an end-over-end rotator. After 6 hours of coupling, to reduce unspecific binding, a blocking solution consisting of 1.2 mg/ml bovine serum albumin (A7906, Sigma Aldrich St. Louis, MO, USA) and 0.6 mg/ml yeast tRNA (10109517001, Roche, Vienna, Austria) was added. Beads were blocked overnight at 4°C under rotation. Just before immunoprecipitation, beads were washed once with PBS and twice with cell lysis buffer. Both buffers were supplemented with blocking solution.

To reduce unspecific binding during immunoprecipitation, lysates were precleared: For one single IP, 60 μ l of beads were washed three times with 1 ml nuclease-free, ice-cold PBS and resuspended in 1 ml of nuclease-free PBS containing protease inhibitors. Beads were blocked under rotation overnight at 4°C in blocking solution. Immediately before preclearing, beads were washed twice with cell lysis buffer.

For preclearing, lysates were combined with beads and incubated under rotation for 1 hour at 4°C. 1/20 of the precleared lysate was used for isolation of total RNA (referred to as input) and another 1/20 was used for western blotting. The remaining precleared lysate was combined with the antibody-immobilized protein G sepharose beads and incubated with rotation for 3 hours at 4°C.

To wash the antibody-immobilized protein G sepharose beads-RNP complex, beads were centrifuged at 2,000 x g for 1 minute at 4°C. The post-IP supernatant was stored in TRIzol reagent (15596-026; Life Technologies Austria, Vienna, Austria) for quality control. The beads were washed twice with lysis buffer containing blocking solution. Then, the beads were washed trice using an aqueous high-salt buffer containing 25 mM Tris-HCl pH 8.0 (15567027; Invitrogen), 900 mM NaCl (AM9760G; Invitrogen), 2 mM MgCl₂ (AM9530G; Invitrogen), 1% IGEPAL® CA-630 (I8896; Sigma Aldrich), 5mM DTT (43816; Sigma Aldrich), blocking solution, protease inhibitors and RNase inhibitor. This was followed by washing twice with cell lysis buffer containing blocking solution. Finally the beads were washed with a low-salt buffer containing 25 mM Tris-HCl pH 8.0 (15567027; Invitrogen), 150 mM NaCl (AM9760G; Invitrogen), 2 mM MgCl₂ (AM9530G; Invitrogen), 0.05% IGEPAL® CA-630 (I8896; Sigma Aldrich), 5 mM DTT (43816; Sigma Aldrich), blocking solution, protease inhibitors and RNase inhibitor. 1/20 of the beads were used for western blotting, the remaining beads were subjected to RNA isolation.

For RNA isolation, input samples and immunoprecipitates were homogenized after the addition of TRIzol. Input samples derived from virus-infected cells were additionally incubated for 30 minutes at 60°C with vortexing every 10 minutes to break open the capsids and allow for efficient digestion of genomic DNA. Chloroform (0.2 ml per 1 ml TRIzol) was added to homogenized lysates; samples were vigorously shaken for 15 seconds and incubated at room temperature for 3 minutes. After centrifugation at 12,000 x g for 15 minutes at 4°C, the aqueous phase was removed. 15 µg of glycogen (10814-010; Invitrogen/Life Technologies Austria, Vienna, Austria) was added to each sample. RNA was precipitated at -20°C for ~12 hours after the addition of 2-propanol. Thereby, an amount of isopropanol corresponding to the volume of the aqueous phase was used. The RNA was collected by centrifugation (10 minutes at 12,000 x g) and the pellet was washed twice with 75% Ethanol. The pellet was resuspended in 20 µl of RNase-free H₂O and incubated at 55°C for 10 minutes.

Contaminating DNA was digested using TURBO™ DNase (AM2238; Invitrogen/Life Technologies Austria, Vienna, Austria), according to the supplier's protocol. DNase was inactivated by an additional TRIzol extraction.

To remove yeast tRNA used in the blocking solution, RNA samples isolated from RISC and supposed to be used for microarray experiments were subjected to cleanup using the RNeasy MinElute Cleanup Kit (74204; Qiagen).

5.12 Quantification of miRNA expression levels

RNA was isolated using TRIzol as described in "Immunopurification of RISC and isolation of associated RNAs". For miRNA quantification, miR-16 levels were determined using a pre-designed miRNA quantification assay. Briefly, 10 ng RNA was reverse transcribed using the TaqMan® MicroRNA Reverse Transcription Kit (4366596; Applied Biosystems/Life Technologies Austria, Vienna, Austria) following the manufacturer's instructions. miR-16 levels were determined in a subsequent RT-qPCR reaction carried out on a LightCycler® 2.0 (Roche Diagnostics) instrument. Therefore, 1.4 µl of the reverse transcription product was combined with 10 µl of LightCycler® TaqMan® Master Mix (Roche Diagnostics) and 1 µl TaqMan® microRNA Assay (4427975; Applied Biosystems/Life Technologies Austria, Vienna, Austria; 20 x; forward primer, reverse primer and probe) in a 20 µl reaction. Thermocycling conditions consisted of a pre-denaturation step at 95 °C, followed by 50 cycles of amplification: denaturation 10 sec at 95 °C, annealing and elongation for 15 sec at 60°C.

5.13 RNA amplification

The RNA obtained from RIP-assays was amplified using the Whole Transcriptome Expression kit (4411973; Ambion, Life Technologies Austria, Vienna, Austria). Briefly, first-strand cDNA synthesis was primed using a mixture of random hexamers and oligo(dT) primers containing the promoter sequence of the T7 RNA polymerase as an extension. Next, single-stranded cDNA was converted to double-stranded cDNA using DNA polymerase contained in the kit. This was followed by *in vitro* transcription catalyzed by the T7 RNA polymerase using the second-strand cDNA as template.

Amplified cRNA was purified by using nucleic acid binding beads according to the manufacturer's instructions. Second-cycle cDNA was synthesized using random primers. A buffer mix containing dUTP at a fixed ratio relative to dTTP was used, allowing the fragmentation of DNA for subsequent labeling. RNA template was hydrolyzed using RNase H and amplified second-cycle cDNA was purified using nucleic acids binding beads.

5.14 Gene Chip microarray assay

Purified second-cycle cDNA, containing dUTP residues, was fragmented using a combination of uracil DNA glycosylase (UDG) and apurinic/apyrimidinic endonuclease 1 (APE 1) followed by terminal labeling with biotin (WT Terminal Labeling Kit, Affymetrix, Inc., Santa Clara, CA, USA). Between 0.6 and 3.0 µg fragmented and labeled DNA was hybridized to an Affymetrix Human Gene 1.1 ST Array Plate. For hybridization, washing, staining and scanning an Affymetrix GeneTitan® system was used. Sample processing was performed at an Affymetrix Service Provider and Core Facility, "KFB - Center of Excellence for Fluorescent Bioanalytics" (Regensburg, Germany; www.kfb-regensburg.de).

5.15 Microarray data analysis

Probe set summarization, normalization and background correction was performed using the RMA (109) algorithm with the Affymetrix GeneChip Expression Console Software. Subsequent analyses were performed in R statistical environment using Bioconductor packages (110). Comparisons between different experiments were performed using a moderated t test from the "limma" package (111).

5.16 Target site prediction

Prediction of miRNA target sites in the human genome was performed using the "Targetscan" program with default parameters (72). 3'UTR sequences and Perl

scripts were downloaded from
http://www.targetscan.org/cgibin/targetscan/data_download.cgi?db=vert_61.
Coding sequences were downloaded from: ftp://ftp.ensembl.org/pub/release-67/fasta/homo_sapiens/cdna/

6 Results

6.1 Establishment of RISC IP

The reliable identification of mRNAs targeted by miRNAs remains a challenging field of research. Although recent advances in bioinformatics have facilitated the discovery of miRNA-mRNA interactions through the implementation of various prediction algorithms, these approaches still suffer from a variety of limitations. Since prediction software is based on probability calculations, actual miRNA-binding sites may be missed and thus the prediction may not comprise the entire spectrum of interaction partners or may lead to false positive results. To fill this technological gap, biochemical RIP-assays were employed, capable to identify RISC-incorporated miRNAs and their target mRNAs and are therefore contributing to a better understanding of miRNA-mediated regulatory processes in cells. Compared to conventional computing-based methods, they can facilitate the reduction of false-positive and negative prediction rates. Nevertheless, bioinformatic analysis is essential to obtain optimal results and the combination between bioinformatics and biochemistry provides the most robust insight into miRNA target networks. However, such RIP-assays have to undergo stringent functional testing, ensuring accurate and error-free performance.

6.1.1 Establishment of a dual-luciferase vector based reporter gene assay

In a first approach I assessed the detection limits of the RIP-method by employing a reporter gene assay-based test system. Briefly, A549 cells were co-infected with adenoviral vectors bearing the *Renilla* luciferase (hRLuc) gene (pAd-Luc-as, R. Klein, unpublished) and an artificial miRNA known to directly target the 3'UTR of the hRLuc mRNA (mi+; pAd-FLuc-mi1, R. Klein, unpublished), respectively. A distinct vector carrying a non-targeting miRNA gene (mi-; pAd-mi-, R. Klein, unpublished) was used as a control. 24 hours and 48 hours post-infection, total RNA was isolated and reverse transcribed as described in "Materials and Methods". Relative mRNA levels were determined by RT-qPCR. hRLuc mRNA levels were normalized to FLuc and GAPDH mRNA levels, respectively. 24 hours after co-infection with the luciferase and

the mi⁺ vector, a significant decrease in the relative hRLuc mRNA levels was observed. This effect was further enhanced at 48 hours post-infection. No significant reduction of hRLuc mRNA was noted when the non-targeting control vector was used, suggesting a miRNA-mediated silencing of the target gene (Fig. 7A). Thus, this system provides an appropriate readout for the establishment of a RIP-assay protocol.

6.1.2 Quantification of RISC-associated luciferase target mRNA levels

Next, the RIP-assay was established by co-infecting A549 cells with pAd-Luc-as and either pAd-FLuc-mi1 or the control vector pAd-mi-. At 24 hours post-infection, cells were lysed and RISC-complexes were isolated by immunoprecipitation using an antibody directed against the argonaute proteins Ago 1-4 (Fig. 7B). A distinct IgG-isotype control antibody was used to determine background noise. A detailed description of the procedure can be found in the “Materials and Methods” section. Total RNA was isolated from 1/20 of the input fraction as well as from the immunoprecipitated fraction. RT-qPCR analysis of the input fraction revealed reduced relative hRLuc mRNA levels when cells were co-infected with the pAd-Luc-as and the pAd-FLuc-mi1 vector compared to the co-infection with the negative control vector (Fig. 7C, left panel). Next, mRNA derived from the immunoprecipitated fraction was quantified. Relative mRNA levels were determined by normalizing these values to the levels of the input fraction. When the cells were co-infected with the pAd-Luc-as and the pAd-FLuc-mi vector, hRLuc mRNA obtained from the immunopurified RISC complex was found enriched over the control IP (Fig. 7C). No significant enrichment was obtained when the non-targeting miRNA vector was co-introduced. Furthermore, no enrichment was detected for the housekeeping gene GAPDH. This gave rise to the conclusion that GAPDH expression is not or at least not extensively regulated by endogenous miRNAs in A549 cells and was thus considered as a constant factor for calculating mRNA enrichments. In this experiment, the relative hRLuc mRNA isolated from the Ago-IP was approximately 9-fold enriched over GAPDH when the cells were co-infected with pAd-Luc-as and the vector producing the targeting miRNA. A significant enrichment was neither detected when the isotype-control antibody was used, nor when the cells were co-infected with the non-targeting miRNA (Fig. 7D). Furthermore, immunoblot analysis of the

immunoprecipitated fraction showed specific enrichment of argonaute proteins. An antibody against GAPDH was used as a control (Fig. 7E).

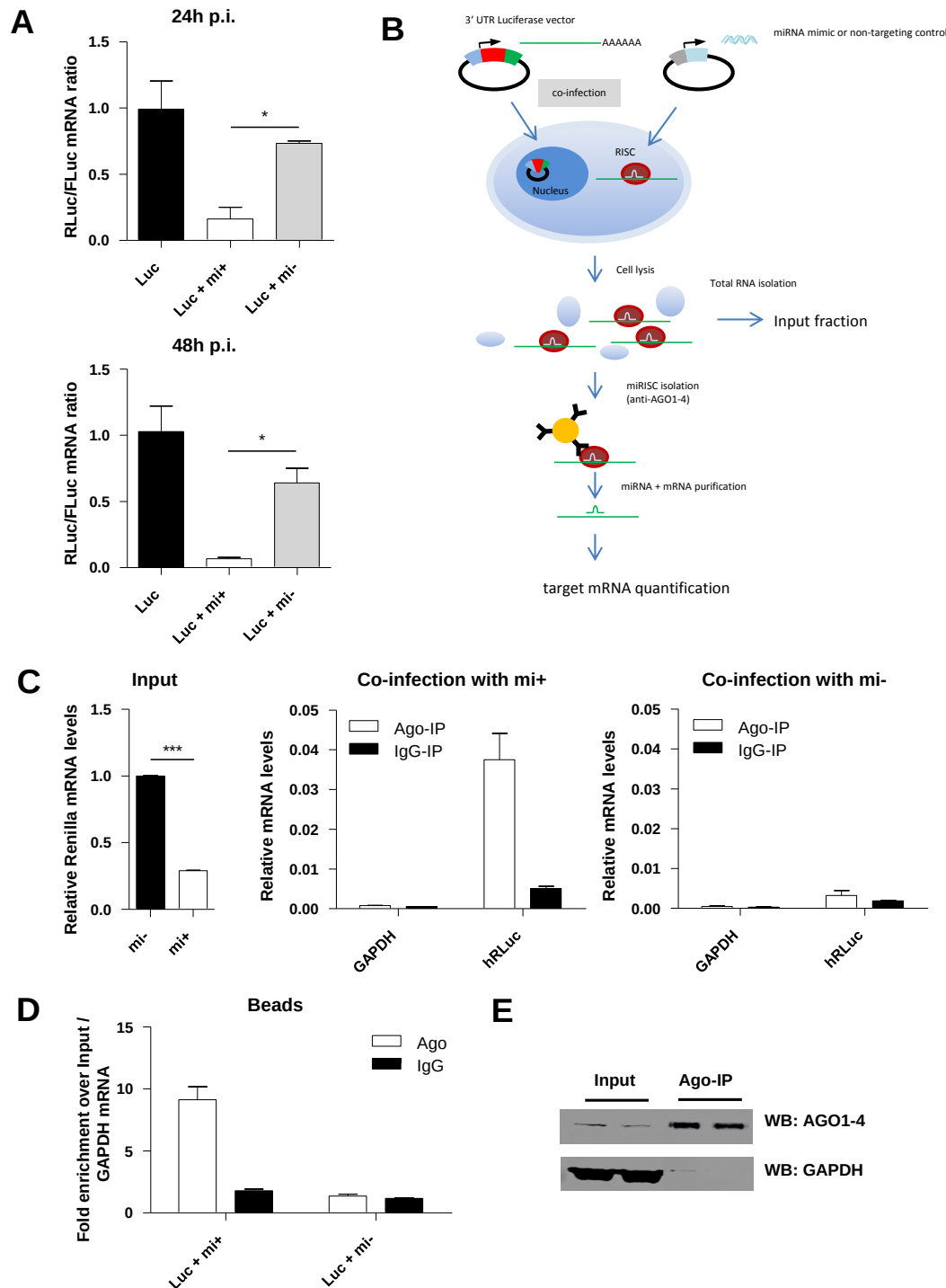


Figure 7: Establishment of a RIP-assay for the identification of miRNA targets. (A) An artificially induced miRNA-mRNA interaction served as an initial readout for setting up the assay. A549 cells were co-infected with distinct adenoviral vectors. A dual-luciferase based reporter vector bearing the *Renilla* (hRLuc) and the Firefly luciferase (FLuc) genes (pAd-Luc-as) together with a vector encoding a miRNA targeting hRLuc (mi+) were used. A distinct

non-targeting miRNA gene (mi-) served as a negative control. At 24 and 48 hours post-infection hRLuc mRNA levels were determined by RT-qPCR, respectively. 24 hours after co-infection with the Luc and the mi+ vector, a significant decrease of relative hRLuc mRNA levels could be observed. This effect was even more elevated at 48 hours post-infection. In both cases, no significant decrease could be detected when Luc was introduced together with mi-. mRNA levels were normalized to GAPDH and FLuc. The mRNA levels are shown relative to the hRLuc mRNA obtained upon infection with Luc alone. (B) Schematic representation of the RIP-assay. Cells are co-infected with the Luc and the mi+ vector. The mi- vector is used as a negative control. Cells are lysed and RISC complexes containing miRNAs and their target mRNAs are immunopurified using antibodies against argonaute 1-4 (anti-Ago1-4) coupled to sepharose beads. Total RNA is isolated from a part of the total cell lysate (referred to as Input fraction) and serves as a reference for subsequent normalization of RNA levels. Furthermore, miRNAs and their target mRNAs are isolated from the immunopurified RISC complexes and target mRNAs are quantified by RT-qPCR. (C) RT-qPCR analysis of the two distinct fractions obtained from an immunoprecipitation experiment. An anti-Ago1-4 antibody (Ago-IP) or an IgG-control antibody (IgG-IP) was used for immunoprecipitation. Cells were either co-infected with pAd-Luc-as and pAd-Luc-mi1 or pAd-Luc-as and pAd-mi- vectors. Left panel: Relative input hRLuc mRNA levels after co-transfection with pAd-Luc-mi1 compared to the co-transfection experiments in which the non-targeting control miRNA was used. Middle panel: mRNA levels obtained from the immunoprecipitates were normalized to the respective input levels. Right panel: Cells co-infected with the non-targeting miRNA. (D) The mRNA levels obtained from the immunoprecipitates were first normalized to their respective input levels and then normalized to GAPDH mRNA levels (E) Quality control for the immunoprecipitation of RISC complexes. 1/20 of the input fraction was used for western blotting using an anti-Ago1-4 antibody. A GAPDH antibody was used as control.

6.1.3 Enrichment of endogenous miRNAs and target mRNA

To further evaluate the assay performance I investigated whether endogenous miRNAs and their target mRNAs can be enriched by IP. It has been proven that the Cyclin E1 (CCNE1) mRNA is post-transcriptionally regulated by miR-16 in A549 cells. This results in mRNA degradation and thus downregulation of the CCNE1 mRNA levels (112). Thus, I investigated if CCNE1 mRNA and its corresponding targeting miRNA miR-16 can be enriched by the established method. RIP-assay of A549 cells revealed highly significant enrichments (~18,600 fold; p-value = 0.0098) of miR-16

levels over the miRNA levels obtained using the IgG control antibody (Fig. 8A). In line with this result, also the CCNE1 mRNA was enriched when the antibody against the argonaute proteins was used for IP (~10.5 fold; p-value = 0.001) (Fig. 8B). Since again no significant enrichment of GAPDH was observed, relative CCNE1 mRNA levels were normalized to the respective mRNA levels of the housekeeping gene. This resulted in a normalized enrichment over input of approximately 10-fold (Fig. 8C).

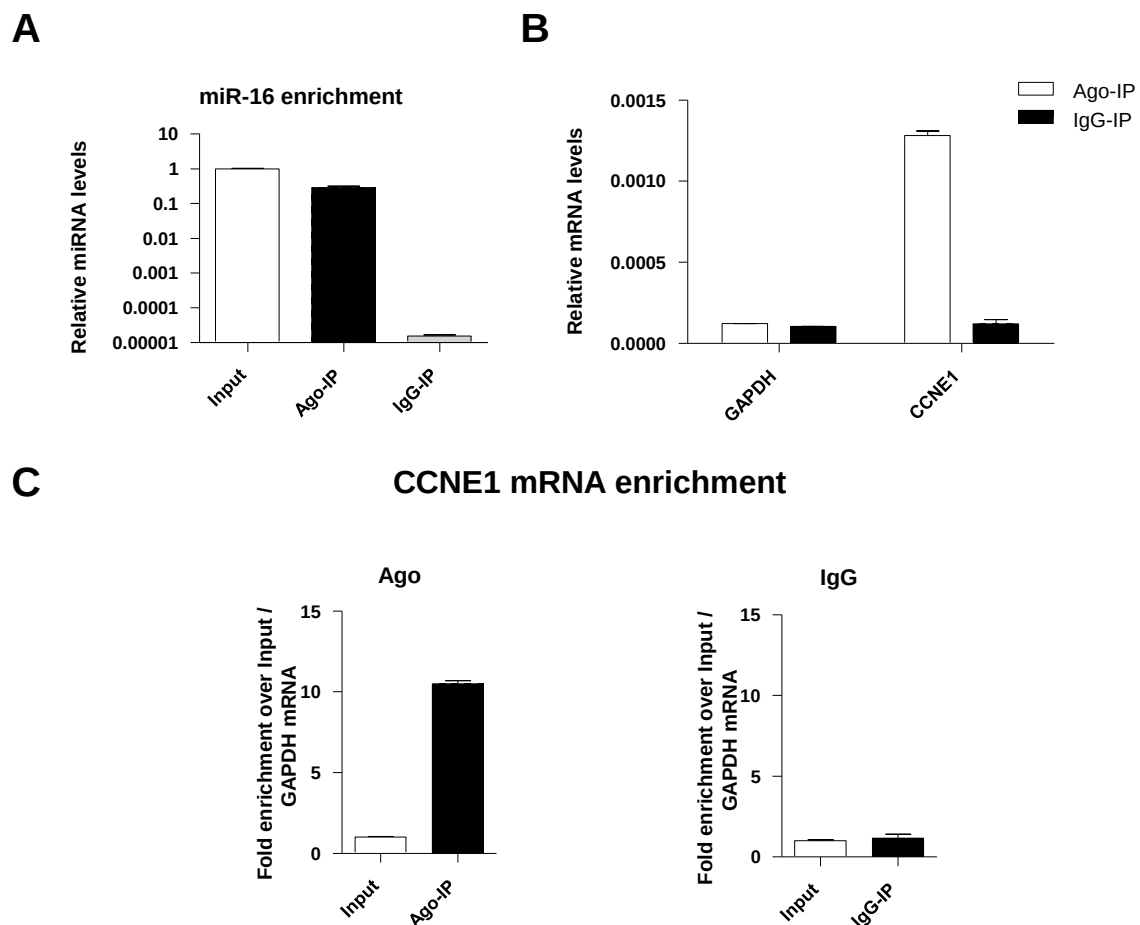


Figure 8: Enrichment of endogenous miRNAs and their target mRNAs. A549 cells were grown to confluence for 30 hours and subsequently subjected to RIP-assay. Ago-IP: Relative mRNA levels obtained in RISC IPs performed with the pan-Ago antibody. IgG-IP: Relative mRNA levels obtained when the IgG-control antibody was used for IP (A) Enrichment of miR-16 by immunoprecipitation of Argonaute 1-4. The amount of miRNA was determined by RT-qPCR and normalized to the level obtained from the input fraction. Input levels were set at 1. (B) Relative mRNA levels of CCNE1, a prominent target mRNA of miR-16. GAPDH mRNA levels served as a control gene determined by RT-qPCR. All mRNA levels were normalized to their respective input values. (C) Right panel: Normalized enrichment of relative CCNE1

mRNA levels obtained through Ago-IP. CCNE1 mRNA levels measured by RT-qPCR were first normalized to the input values and then relativized to GAPDH mRNA levels. Right panel: Normalized enrichment of CCNE1 mRNA obtained when an IgG control antibody was used for IP.

Altogether, these results provide a proof-of-principle that miRNA-targeted mRNAs can be reliably identified by the RISC-IP approach. Thus, this method was subsequently employed to investigate interactions between mivaRNAs and cellular mRNAs.

6.2 The interplay between adenoviral gene products and the cellular RNA interference machinery

6.2.1 Identification of cellular mRNAs targeted by virally encoded miRNAs

The adenovirus encoded VA RNAs have been shown to be processed into functional miRNAs which are incorporated into RISC (9). However, little is known about their cellular target genes. Efforts to identify putative targets were made by Aparicio *et al.* (64). They performed microarray analysis to elaborate a set of genes which were downregulated in the presence of mivaRNAs. Furthermore, they combined this genomic approach with bioinformatics to substantiate their findings. However, the major problem of such conventional approaches is their inability to distinguish between direct miRNA-mediated downregulation of gene expression or secondary effects. RIP-Chip, however, allows to identify true RISC-associated mRNAs.

6.2.2 Functional validation of mivaRNA target vectors

In a first approach, to evaluate the ability of synthetic mivaRNA mimics to mediate regulation of gene expression by Ago2-guided target mRNA cleavage I performed dual-luciferase reporter gene assays. Therefore I used genetically engineered reporter constructs based on the psiCHECKTM-2 vector. The latter contains the sequences of the *Renilla* and the Firefly luciferase gene under control of distinct promoters. A multiple cloning site (MCS), located downstream of the *Renilla*

luciferase open reading frame, allows insertion of target sequences, resulting in a fusion mRNA. I used reporter constructs containing the target sequences for VA RNA-derived miRNAs in their 3' UTR (Fig. 9A). Upon transfection into cells, active luciferase protein is produced, which can be quantified by measuring luminescence (Fig. 9B). However, when active, miRNAs are present, they would specifically recognize their target sequence fused to the *Renilla* luciferase mRNA and thus promote its degradation. This would result in abolished synthesis of *Renilla* luciferase protein and consequently *Renilla* luciferase activity (Fig. 9C).

6.2.3 Validation by using the pAdVantage vector

To assess the reliability of the reporter gene assay, I used the pAdVantage vector (Promega), which harbors the VA RNAI and VA RNAII genes. HEK293 cells were either transfected with the empty psiCHECKTM-2 vector alone, or co-transfected with the pAdVantage vector and the distinct psiCHECKTM-2 vectors containing the miRNA target sites. These vectors are referred to as pmiVAI3, pmiVAI5, pmiVAII3 and pmiVAII5 and a detailed description can be found in the "Appendix" section. A co-transfection experiment using the pAdVantage vector combined with the empty psiCHECKTM-2 vector served as a negative control. I could observe a highly significant decrease in luciferase activity upon co-transfection of pAdVantage and the vectors bearing the target sequences for miRNAs derived from both strands of VA RNA I. I also detected a pronounced decrease, when the vector carrying the target sequence for the miRNA derived from the 3' end of VA RNA II was co-transfected. However, luciferase activity was not abolished when the vector containing the target sequence for the 5' end of VA RNAII-derived miRNAs was used (Fig. 9D). This results is consistent with the literature, as Xu *et al.* could demonstrate that only a small fraction (about 2,5%) of the RNAs derived from the 5' end of the VA RNAII is incorporated into the RISC complex (35).

6.2.4 Confirmation by using adenovirus type 5 wild-type

Next, I confirmed these results by repeating the experiment with adenovirus-infected cells. Therefore, HEK293 cells were again transfected with the reporter gene

constructs bearing the target sites for miRNAs. The next day, cells were infected with Ad5 wild-type and luciferase activity was assayed 24 hours post-infection. The luciferase levels dropped significantly as a consequence of the production of miRNAs (Fig. 9E). The most significant decrease could be observed for pmiVAI3 ($88\% \pm 0.187\%$, $p\text{-value} < 0.0001$), indicating that the miRNA produced from the 3' strand of VA RNAI was the most efficient in terms of silencing its target mRNA. Furthermore I noted a significant down-regulation of luciferase activity also for the pmiVAI5 as well as the pmiVAII3 target vector. These observations are in accordance with the previous results and suggest that the adenovirus-encoded miRNAs recognize their target sequences in a highly efficient manner. Interestingly, in this experimental setup, a significant downregulation of the luciferase gene could also be observed for the target vector of the miRNA derived from the 5' strand of VA RNA II. This effect was not observed in previous experiments, in which the pAdVantage vector was used instead of adenovirus. However, one simple explanation might be that much higher levels of miRNAs were present in the virus-infected cells compared to cells transfected with the plasmid. This would render it likely that inefficient RISC-incorporation may be compensated by a high abundance of the molecule.

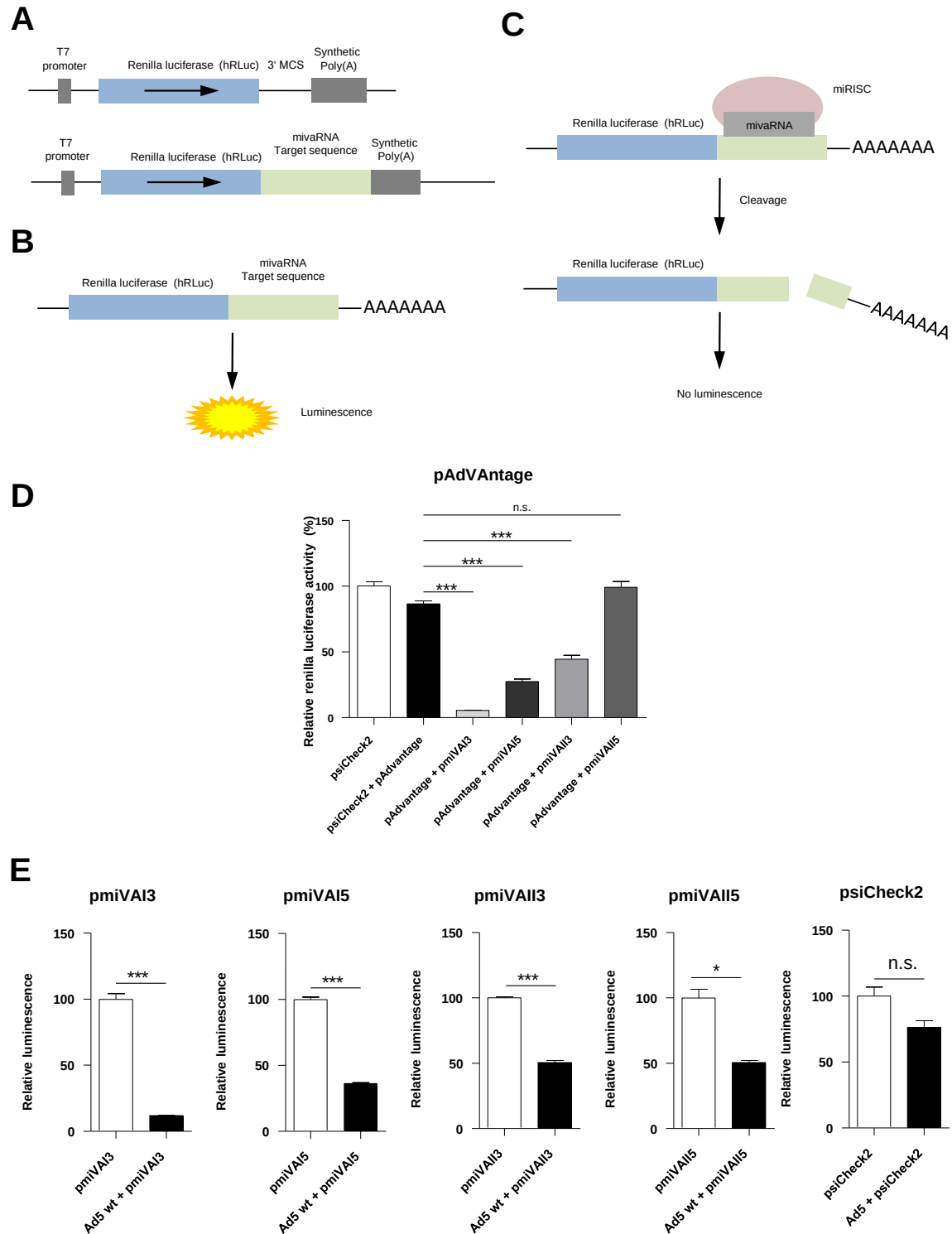


Figure 9: Functional validation of mivaRNA mimics. To functionally validate the ability of mivaRNAs to recognize and degrade their target mRNAs, a dual-luciferase reporter gene assay was performed. The reporter constructs are based on the psiCHECKTM-2 vector. Statistical evaluation of data was performed using the unpaired Student's t-test, considering a p-value less than 0.05 as statistically significant. (A) The binding sites for mivaRNAs were inserted into the 3' MCS of the *Renilla* luciferase gene (hRLuc). (B) Upon transfection into cells, the hRLuc mRNA is produced and translated into the corresponding protein, whose

activity can be quantified by measuring the luminescence. (C) In the presence of RISC-incorporated miRNAs, the hRLuc mRNA is recognized by the active RISC and catalytically cleaved by argonaute 2. This leads to mRNA degradation and to reduced protein levels resulting in diminished luminescence. (D) HEK293 cells were co-transfected with the pAdVantage vector and luciferase reporter constructs containing the target sequences for miRNA I and II either derived from the 3' end or the 5' end of the corresponding VA RNA (pmiVAI3, pmiVAI5, pmiVAII3, pmiVAII5). The unmodified psiCHECKTM-2 vector was used as a control. At 24 hours post-transfection, luminescence of Firefly and *Renilla* luciferase was measured. *Renilla* luciferase luminescence levels were normalized to Firefly luciferase luminescence levels. (F) HEK293 cells were transfected with luciferase reporter constructs. The unmodified psiCHECKTM-2 vector was used as a negative control. The next day, cells were infected with Ad5 wild-type. At 24 hours post-infection, the enzymatic activities of the Firefly and *Renilla* luciferases were determined. All *Renilla* luciferase luminescence measurements were related to the corresponding Firefly luciferase values. These normalized *Renilla* luciferase levels are depicted as relative to the luciferase levels in uninfected cells. No significant decreased in *Renilla* luciferase activity could be observed when the unmodified psiCHECKTM-2 vector was used. (n = 3; mean values $\pm$ SD; * p<0.05, ** p<0.01, ***p<0.0001, n.s. = not significant)

6.2.5 VA RNA mimic-mediated down regulation of target sequence

To investigate the biological functions of the adenovirus-derived miRNAs, miRNA mimics were designed. It has been shown that the VA RNAs can give rise to distinct small RNAs in dependence of the cleavage site of Dicer. Thereby, the small RNAs miRNAI-137 and miRNAI-138 are among the predominantly produced miRNAs, termed after their starting position in the VA RNA molecule (Fig. 10A) (35). Furthermore, transcription of VA RNAI can start from two different sites, hence giving rise to even more miRNA species. Named after their first nucleotide, VA RNAI(A) and VA RNAI(G) have been described (113 and references therein). In this study I used small double stranded RNA oligonucleotides mimicking miRNAI(A)-137 and miRNAI(G)-137 as well as miRNAII-138. These artificially synthesized small RNAs represent a reliable tool to perform gain-of-function studies on miRNAs and hence allow an extensive functional analysis of the different miRNAs.

I first tested the ability of the mimics to direct RISC-catalyzed target mRNA cleavage in luciferase reporter gene assays. Therefore I used again the genetically engineered reporter constructs bearing the mivaRNA target sequences. HEK293 cells were co-transfected with the mimics and their corresponding target vectors. A non-targeting siRNA was used as a negative control in all experiments and a siRNA against the adenovirus pTP mRNA, co-transfected together with its target vector was used as a positive control ("Appendix"). mivaRNA mimics derived from the 3' region of VA RNAI performed best (Fig. 10B). Again, these results are consistent with the literature as it has been shown that the 3' end-derived mivaRNAs are incorporated into RISC at much higher efficiency. Despite its inefficient incorporation, the 5'end-derived mivaRNA has been shown to catalyze the cleavage of its target mRNA in a more efficient way (113). This may compensate for its low incorporation efficiency and may thus provide an explanation for the reduced luciferase levels in both cases.

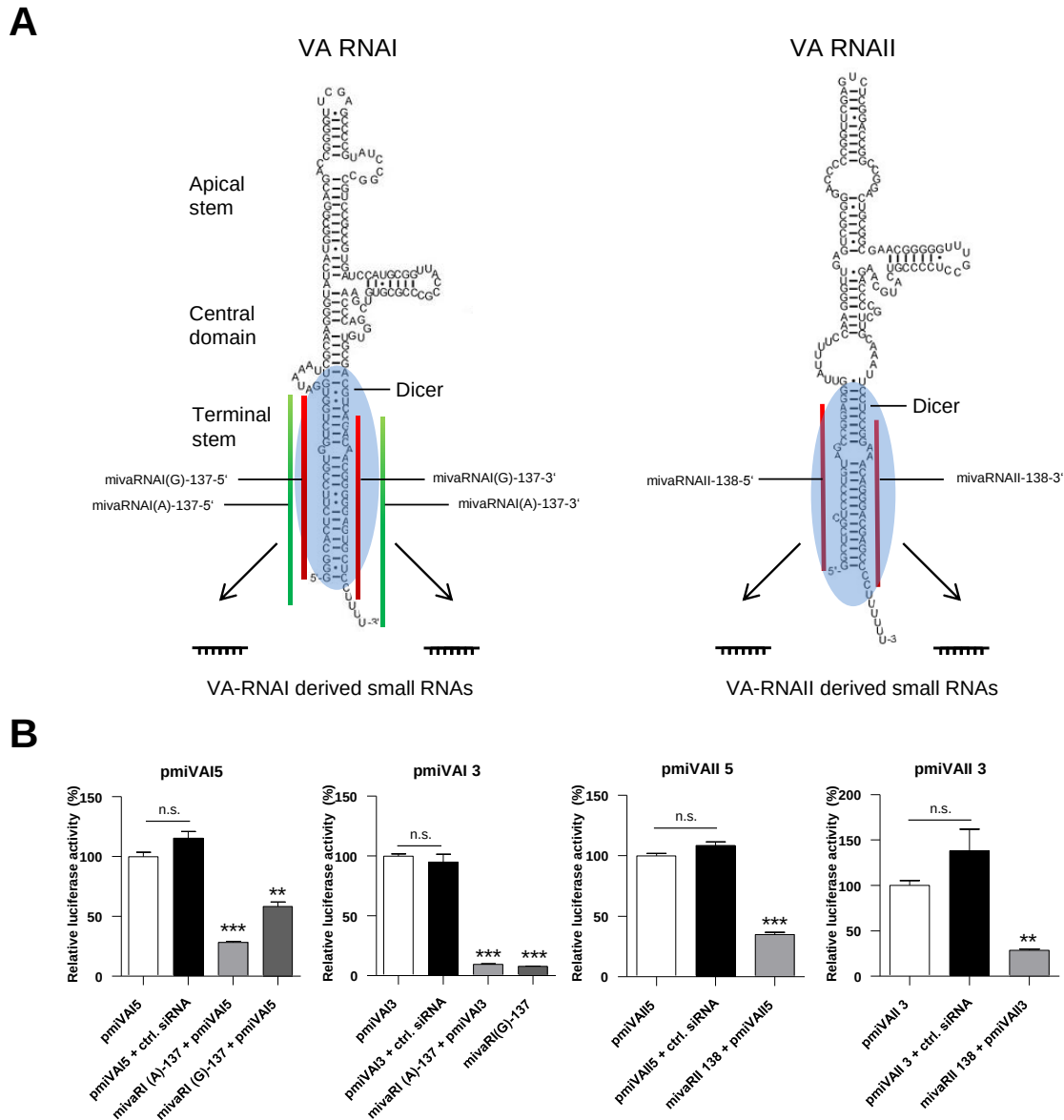


Figure 10: VA RNA mimic-mediated down regulation of target sequence. Adenovirus encodes two short RNAs termed VA RNAI and VA RNAII. During the infection cycle, they are processed into miRNAs by Dicer, and are incorporated into RISC (7). (A) (Adapted from reference 7). Secondary structure models of VA RNAI and VA RNAII are depicted. Sequences located next to the 5' end as well as the 3' end of both, VA RNAI and VA RNAII gives rise to mivaRNAs. Several mivaRNAs mimics were designed in analogy to these regions and are illustrated by the red and the green lines. (B) mivaRNA mimics promote Ago2-mediated cleavage of their target mRNAs. HEK293 cells were co-transfected with the mivaRNA mimic and a psiCHECKTM-2 vector carrying the corresponding target sequence inserted into the *Renilla* luciferase gene (pmiVAI3, pmiVAI5, pmiVAII3, pmiVAII5). A non-targeting siRNA was used as a negative control (ctrl. siRNA). *Renilla* and Firefly luciferase activities were determined 48 h post-transfection. Relative *Renilla* luciferase levels of cells only transfected with the target vectors were set at 100%. *Renilla* luciferase relative to Firefly

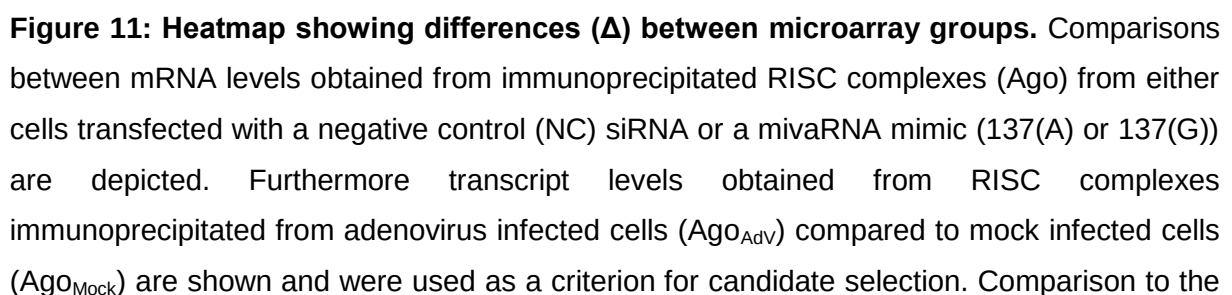
luciferase activities were calculated ($n = 3$; mean values $\pm$ SD; ** $p < 0.01$, *** $p < 0.0001$, n.s. = not significant).

6.2.6 Genome-wide screening for targets of virally-encoded miRNAs

The RIP-assay established was used to identify putative target genes of mivaRNAs. Since the mivaRNAs derived from VA RNAi showed better performance in luciferase assays, they were used for further experiments. A549 cells were transfected with mivaRNAI(A)-137 and mivaRNAI(G)-137 mimics as well as with a non-targeting negative control siRNA. At 24 hours after transfection, cells were harvested and RISC complexes including miRNAs and their target mRNAs were isolated using the anti-pan ago antibody as described in the previous sections. Additionally, lysates from A549 cells infected with Ad5 wild-type or mock for 30 hours were subjected to RISC-IP. RNA was purified from the immunoprecipitated complexes as well as from the whole cell lysates, amplified and treated as described in “Materials and Methods”. mRNA levels were quantified by microarray analysis.

After data normalization, mRNA levels obtained from the immunoprecipitated fractions were compared. Isolated RISC complexes from experiments in which mivaRNA mimics were transfected may contain their target mRNAs but additionally also contain mRNAs targeted by cellular miRNAs. In a first approach, to exclude these endogenous miRNA-mRNA interactions target mRNA levels obtained from mivaRNA-transfected cells were compared to targets from cells transfected with the non-targeting control siRNA. In comparison to the control, 4633 genes were found enriched in RISC complexes isolated from mivaRNAI(A) mimic-transfected cells. When RISC complexes isolated from mivaRNAI(G) mimic-transfected cells were compared, a list of 4920 enriched genes was obtained. 4966 genes were found enriched in RISC complexes isolated from Ad5-infected cells, compared to mock-infected cells. To reduce false positives stringent criteria for the selection of miRNA-targets were introduced. First, a transcript had to be enriched > 2.0 to be considered a target with a statistical significance of $p < 0.05$. 18 genes fulfilled this criterion. To define rules for the selection of putative candidate genes, the enrichment on beads over input was calculated. Finally, we used RIP-Chip-data from Ad5- or mock-infected cells and elaborated a list of differentially RISC-enriched genes. Genes enriched in virus-infected cells, were compared to the putative mivaRNA-targets

identified by RIP-Chip. Strikingly, most of the genes could be found enriched greater than 2-fold in the virus-infected fraction as well. Thus, the incorporation of this set of mRNAs into RISC does also occur during a natural adenovirus infection and is not an artifact of the particular assay setup. The enrichment values were visualized in a heatmap analysis (Fig. 11).



respective input levels are shown and were used as a further quality control. Arrows indicate the transcripts that were selected for further analysis.

The analysis revealed 18 putative miRNA targets which were selected for validation. Additionally, COMMD1 was included in the analysis, as it was the top-ranked gene in TargetScan analysis (Table 3 and Table 4). A subset of transcripts showed mimic-specific enrichment. For example CRIPAK was enriched over negative control only when the miRNA(A) was used. On the other hand GRP63 seemed to be targeted to a higher extend by miRNA(G) than by miRNA(A). This specific enrichment is presumably a result of targeting by the 5' strand of the distinct mimics. This strand is specific for each mimic and differs in its seed sequence.

Table 3: Putative targets of miRNA-137(G) identified by RIP-Chip. List of putative target genes selected for further validation sorted according to the enrichment following miRNA(G)-137 transfection.

Name	Ago NC - Ago 137 (G)	Ago Mock - Ago Adv	Enrichment over Input miRNA 137(G)	Enrichment over Input NC	Enrichment over Input Adv	Fold change Input
IL12A	5.66	2.43	8	1.4	2.68	0.99
ISOC1	4.76	8.75	9.19	1.29	14.83	0.67
TMEM222	3.01	4.5	4.96	1.3	3.03	0.79
NAT14	2.58	3.05	4.69	1.8	20.68	1
GPR63	2.55	5.7	2.97	1.17	9	1.01
SUPT7L	2.5	8.75	2.43	0.88	2.55	0.91
GUK1	2.43	18.51	3.97	1.54	30.91	0.94
PRR3	2.41	2.83	3.66	1.29	4.56	0.85
LY6K	2.3	3.56	5.78	2.04	4.86	0.81
COMMD9	2.23	3.1	2.41	1.05	2.13	0.97
LEPROTL1	2.03	12.13	1.16	0.54	5.28	0.94
BOK	1.93	1.52	4.76	1.87	8	0.75
SRI	1.92	3.03	1.01	0.48	1.18	1.99
RPL34	1.79	56.1	0.64	0.35	12.55	1.66
SMAGP	1.79	4.23	2.45	1.24	16.68	1.43
COMMD1	1.17	7.94	0.34	0.3	1.25	1.03
ZNF768	1.13	4.99	2.13	2.04	8.63	1.07
LGALS3	1.01	2.04	1.47	1.48	6.15	1.01
CRIPAK	0.91	8.51	0.72	0.82	3.68	1.02

Furthermore, consistent with our expectations the two distinct mimics shared a common group of putative targets. It has been shown, that mivaRNAs derived from the 3'-strand of the VA RNAI are loaded into the RISC complex with high efficiency. Moreover it has been demonstrated that Dicer cuts VA RNAI(A) and VA RNAI(G) at the same position. This implicates, that both mivaRNA(A)-137-3' and mivaRNA(G)-137-3' contain the same seed sequence (Fig. 10A; described in more detail in reference 113). Thus, the common group of targets will likely be targets of the 3'-derived strands.

Table 4: Putative targets of mivaRNA-137(A) identified by RIP-Chip. List of putative target genes selected for further validation and sorted according to the enrichment following mivaRNAI(A)-137 transfection.

Name	Ago NC - Ago 137 (A)	Ago Mock - Ago AdV	Enrichment over Input mivaRNA 137(A)	Enrichment over Input NC	Enrichment over Input AdV	Fold change Input
CRIPAK	3.89	8.51	3.86	0.82	3.68	0.82
IL12A	3.56	2.43	4.79	1.4	2.68	1.04
ISOC1	3.39	8.75	5.78	1.29	14.83	0.76
LGALS3	2.83	2.04	4.23	1.48	6.15	0.99
GUK1	2.69	18.51	4.38	1.54	30.91	0.95
TMEM222	2.6	4.5	3.97	1.3	3.03	0.85
NAT14	2.14	3.05	3.94	1.8	20.68	0.98
LY6K	2.13	3.56	5.24	2.04	4.86	0.83
ZNF768	2.11	4.99	4.26	2.04	8.63	1.01
LEPROTL1	2.1	12.13	1.19	0.54	5.28	0.96
SRI	1.99	3.03	1.08	0.48	1.18	0.87
PRR3	1.79	2.83	2.43	1.29	4.56	0.95
COMMD1	1.77	7.94	0.72	0.3	1.25	0.74
SUPT7L	1.74	8.75	1.66	0.88	2.55	0.92
GPR63	1.72	5.7	1.99	1.17	9	1.01
BOK	1.72	1.52	3.97	1.87	8	0.81
RPL34	1.66	56.1	0.59	0.35	12.55	0.98
COMMD9	1.51	3.1	1.62	1.05	2.13	0.97
SMAGP	1.43	4.23	1.97	1.24	16.68	0.9

6.2.7 Validation by using TargetScan

The putative targets were combined to TargetScan predictions, to possibly set an additional rule for selection of candidate genes. This algorithm searches for conserved matches of the seed region of a given miRNA. We used the seed

sequences of the distinct mimics and obtained a list of predicted target sites. The output was divided in sites located within the UTR and the coding sequence (CDS). Both possibilities were taken into consideration and we divided our analysis into genes with predicted UTR-hits and CDS-hits. For many genes, multiple hits within the same mRNA were predicted. Thus, we used the minimum score as a criterion to predict the targeting efficacy.

Next, these hits were combined to the genes identified by the microarray analysis. Strikingly, most of the candidates identified by RIP-Chip were among the predicted mivaRNA targets in the TargetScan analysis, substantiating their validity (Table 5).

Table 5: Predicted target sites on transcripts identified by RIP-Chip. Seed sequences of the distinct mivaRNA strands were used as input.

Name	minimal score cds	hitCount cds	minimal score utr	hitCount utr	Putative targets
BOK	-0.49	1	-0.47	1	mival137(A)-3'; mival137(G)-3'
COMMD1	-0.63	3	-0.62	1	mival137(A)-5'
COMMD9	-0.12	5	-	-	mival137(A)-3'; mival137(G)-3'
CRIPAK	-0.33	129	-	-	mival137(A)-5'; mival137(G)-5'
GPR63	-0.19	3	-	-	mival137(A)-3'; mival137(G)-3'
GUK1	-0.23	5	-	-	mival137(G)-5'
IL12A	-0.44	5	-0.45	1	mival137(A)-3'; mival137(G)-3'
LEPROTL1	-0.24	6	-0.08	1	mival137(A)-5'; mival137(G)-5'
LGALS3	-0.22	3	-	-	mival137(A)-5'; mival137(G)-5'
LY6K	-0.46	9	-0.35	3	mival137(A)-3'; mival137(G)-3'
PRR3	-0.29	119	-0.19	4	mival137(A)-3'; mival137(G)-3'; mival(A)137-5'
RPL34	-0.16	5	-	-	mival137(A)-3'; mival137(G)-3'; mival(G)137-5'
SMAGP	-0.18	7	-	-	mival137(A)-3'; mival(A)137-5'
SRI	-0.38	3	-0.18	2	mival137(A)-3'; mival137(G)-3'
SUPT7L	-0.24	9	-0.22	2	mival137(A)-5'
TMEM222	-0.32	23	-0.32	4	mival137(A)-3'; mival137(G)-3'; mival(G)137-5'
ZNF768	-0.31	4	-	-	mival137(A)-5'
ISOC1	-	-	-	-	-
NAT14	-	-	-	-	-

However, the accuracy of some predicted sites has to be carefully re-evaluated. In-depth analysis of the predicted regions revealed redundant predictions, resulting in an overestimation of the number of predicted hits. Furthermore, a region complementary to mivaRNA137(A)-5' in the NAT14 mRNA, a putative target which is

not among the predicted transcripts, could be manually identified. Furthermore, the occurrence of several transcript variants is not taken into consideration by the algorithm. Thus, additional binding sites may be missed. Nevertheless, some of the putative targets were predicted correctly. COMMD1, which has been found enriched in the presence of mivaRNA137(A) but not of mivaRNA137(G) is indeed predicted to be targeted by the 5' strand of mivaRNA137(A).

6.2.8 Validation of RIP-Chip data by RT-qPCR

The putative miRNA targets identified by RIP-Chip were validated using RT-qPCR assays. Therefore, levels of transcripts isolated from RISC of the mivaRNA mimic-transfected cells were determined by RT-qPCR and compared to levels of transcripts isolated from RISC of the control siRNA-transfected cells (Fig. 12).

In summary, I could reproduce most of the enrichment values obtained from microarray analysis. The enrichment over the negative control condition was statistically significant in most cases. Also the mimic-specific enrichment for the transcripts ZNF768 and CRIPAK could be shown. Nevertheless, some transcripts such as COMMD1 and COMMD7 revealed no significant enrichment over the negative control using the RT-qPCR assays. This might be either due to suboptimal priming conditions or due to false positive array data. Thus, re-evaluation of the dataset by an additional independent experimental setup is required.

Furthermore, the enrichment of putative target mRNAs in adenovirus-infected cells compared to mock-infected cells was assessed as well (Fig. 13). The enrichment obtained was generally concordant with our previous data. Although some enrichment values were not statistically significant, they tended to reflect the same pattern obtained in previous experiments. In some cases, the missing significance can be explained by the detection limit of RT-qPCR. Some transcripts could not be measured in both replicates isolated from the mock-infected cells. In these cases, a C_T -value of 40 was used to calculate the theoretical minimum amount. The differences between the replicates may arise when slightly different amounts of RNA are used for the initial RNA amplification. Since this method relies on a minimum amount of RNA for each transcript, especially low abundant mRNAs may not be efficiently amplified and thus fall below detection limits in subsequent assays.

Interestingly, the enrichment values tended to be generally higher than in the experiment where the mimics were used. This might be due to the high abundance of mivaRNAs at this stage of infection, suggesting less molecules per cell in the transiently transfected cells. Moreover additive effects implicating other virally encoded miRNAs sharing the same targets are conceivable.

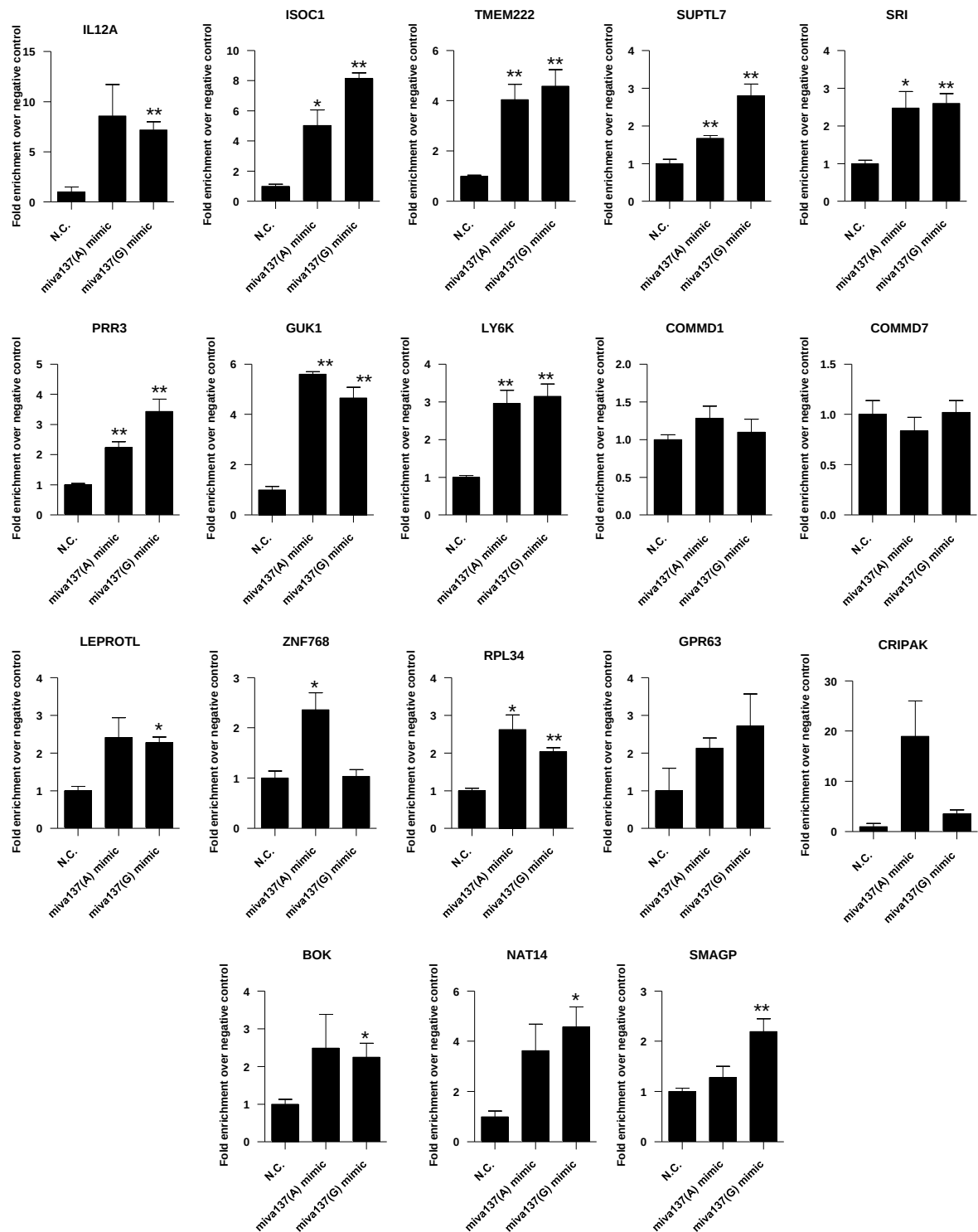


Figure 12: Validation of microarray data by RT-qPCR. Fold enrichment between RISC-isolated transcripts from cells transfected with a negative control siRNA or a mivaRNA mimic respectively were determined by RT-qPCR. Data was normalized to GAPDH (n = 3; mean values $\pm$ SD). Statistical evaluation of data was performed using the unpaired Student's t-

test, considering a p-value less than 0,05 as statistical significant (*) and less than 0,01 as highly statistical significant (**).

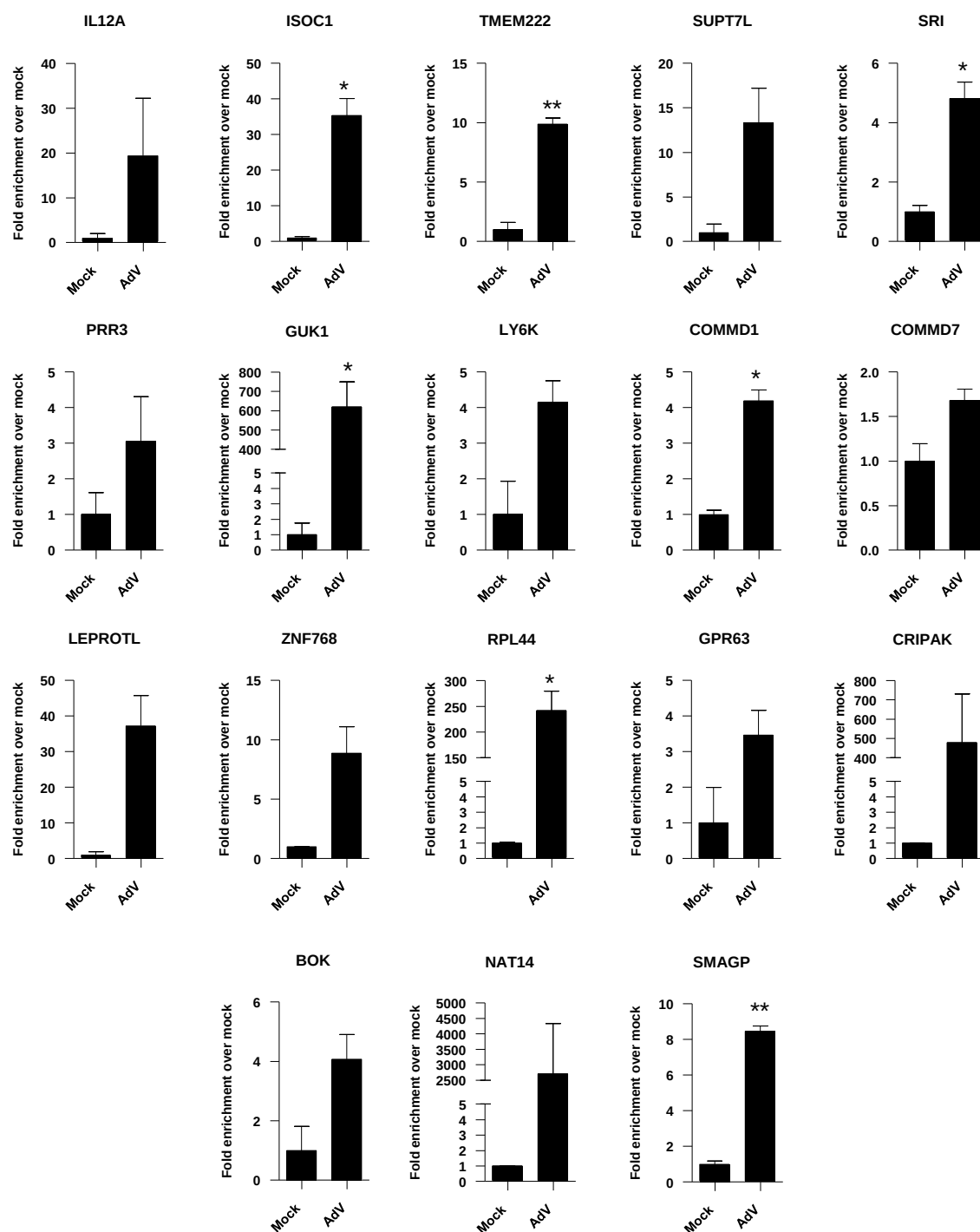


Figure 13: Validation of microarray data by RT-qPCR. Putative miRNA targets were quantified using RNA isolated from RISC-complexes adenovirus-infected cells. Mock-infected cells were used as a control. All data were normalized to GAPDH housekeeping gene (n = 2; mean values $\pm$ SD). Statistical evaluation of data was performed using the

unpaired Student's t-test, considering a p-value less than 0,05 as statistical significant (*) and less than 0,01 as highly statistical significant (**).

Together, these results provide additional evidence for the biological function of mivaRNAs to act as viral miRNAs. The RIP-Chip analysis revealed a set of putative genes related to distinct cellular functions which might be targeted by these small RNAs. This interaction may constitute a virus-host interaction, advantageous for the virus in terms of protection from cellular antiviral responses, prolonging the infection cycle, or establishment of a latent state.

6.3 Identification of viral gene products targeted by cellular miRNA

Adenovirus modulates the RNAi pathway by expressing its VA RNAs. The latter are oversaturating Dicer, Exportin-5 and RISC, and thus interfere with the host RNAi pathway. One suggestion is that such a mechanism may have evolved under selective pressure to counteract cellular miRNAs which would provide a cellular defense mechanism against viruses (7). In this last part of the project, I studied whether viral mRNAs are targeted by cellular miRNAs. This would help to elucidate the role of cellular RNAi as a general defense mechanism against viruses or as a regulator of the adenovirus replicative cycle.

6.3.1 Determining optimal conditions for adenovirus multiplication

In subsequent experiments different cell lines were used, which were chosen according to their properties to promote adenoviral replication. The human lung cancer epithelial cell line A549 was used as a reference, since it is commonly used to propagate different adenovirus serotypes (114). Furthermore, several leukocyte cell lines including the human monocyte cell line U937, the B lymphocytes-derived Burkitt's lymphoma cell line BJAB, as well as the human acute monocytic leukemia cell line THP-1 were used. These cell lines were selected based on previous studies showing the ability of adenovirus to establish latent infections in leukocytes (115, 116). As it was shown for other viruses, that latency can be accompanied by a

specialized miRNA expression pattern, these cell lines were of particular interest for these studies. A cell-type specific miRNA patterns could give rise to differences in the adenoviral infection cycle. Therefore, the establishment of a latent infection may be preferred. To optimize cell culture conditions for efficient virus replication, several parameters were considered.

In a series of experiments the different cell lines were infected with an EGFP gene-harboring adenoviral vector as described in the “Materials and Methods” section. At 24 hours post-infection, FACS analysis was conducted on mock- or EGFP transduced cells. The percentage of EGFP-positive cells directly correlates with the rate of infection. An obvious difference in the required MOI to obtain equal infection rates could be observed (Fig. 14). In A549 cells a MOI of 50 is high enough to infect almost the entire cell population. However this is different for the leukocyte cell lines BJAB and THP-1, since in these cases a much higher number of infectious particles per cell is required to obtain infection rates as high as in A549 cells. Furthermore, the U937 cell line could not be infected at all and was thus excluded from further analysis.

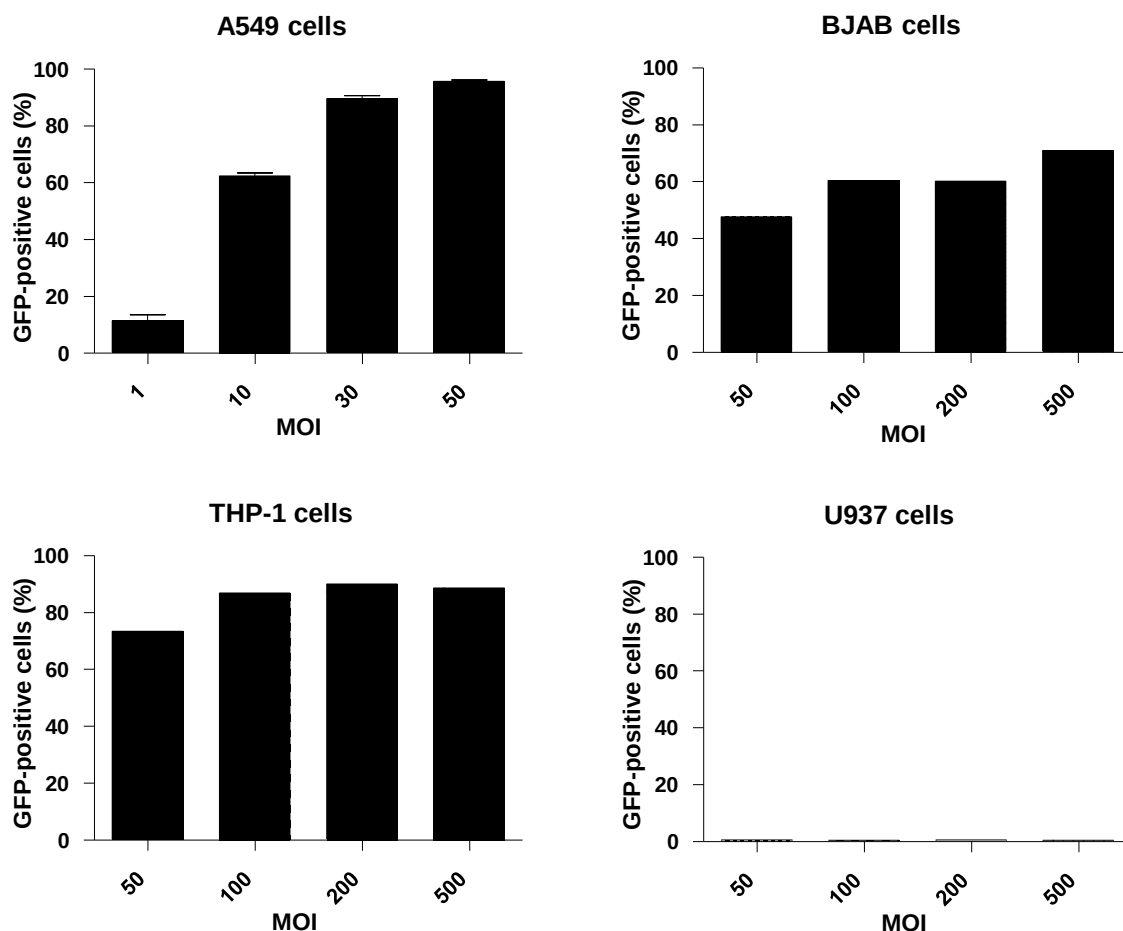


Figure 14: Flow cytometric analysis of different cell lines, infected with an EGFP gene-carrying adenoviral vector. All measurements were performed 24 hours post-infection. The infection was carried out at different multiplicities of infection (MOI) and the percentage of EGFP-positive cells indicates the infection efficiency. The figure shows infection efficiencies of adenovirus 5 in A549 cells (upper left panel), in the B cell lymphoma cell line BJAB (upper right panel), in the human acute monocytic leukemia cell line THP-1 (lower left panel) and in the pro-monocytic cell line U937 (lower right panel).

6.3.2 Infection kinetics of adenovirus type 5 wild-type and Δ VA RNA

Since expression of VA RNAs would presumably diminish miRNA-mediated silencing of adenoviral gene products I used a double mutant of Ad5 lacking the VA RNAI as well as the VA RNAII genes. This mutant is referred to as dl-sub720 (Ad5 Δ VA) and has been described previously (60, 117, 118). In order to examine the differences in the gene expression pattern between Ad5 wild-type and Ad5 Δ VA, I performed analysis of their infection kinetics in the different cell lines. This experiment allowed to

monitor changes in steady-state mRNA levels of adenoviral transcripts derived from either Ad5 wild-type or Ad5 Δ VA.

A549, BJAB, and THP-1 cells were seeded and infected as described in “Materials and Methods”. At different time points after infection, samples were taken and the expression rates of the adenoviral genes coding for E1A 13S, E1A 9S, E1B RNA4, E1B RNA5, E3, E4, IVa2, Hexon, pTP, DNA-binding protein (DBP), precursor terminal protein (pTP) and DNA polymerase (Pol) were determined by RT-qPCR. Due to the onset of a strong cytopathic effect, which eventually results in cell lysis, the latest time point for harvesting of Ad5 wild-type infected A549 cells was restricted to 48 hours post-infection. The onset of cytopathic effect was delayed when Ad5 Δ VA was used. Even 96 hours post-infection, most of the A549 cells were attached to the surface of the cell culture dish. Accordingly, obvious differences in the infection kinetics were observed (Fig. 15). The increase of steady-state levels of viral transcripts was slowed down when the mutant was used. Furthermore the overall mRNA levels were substantially lower compared to the abundance of Ad5 wild-type transcripts, even during the late phase of infection.

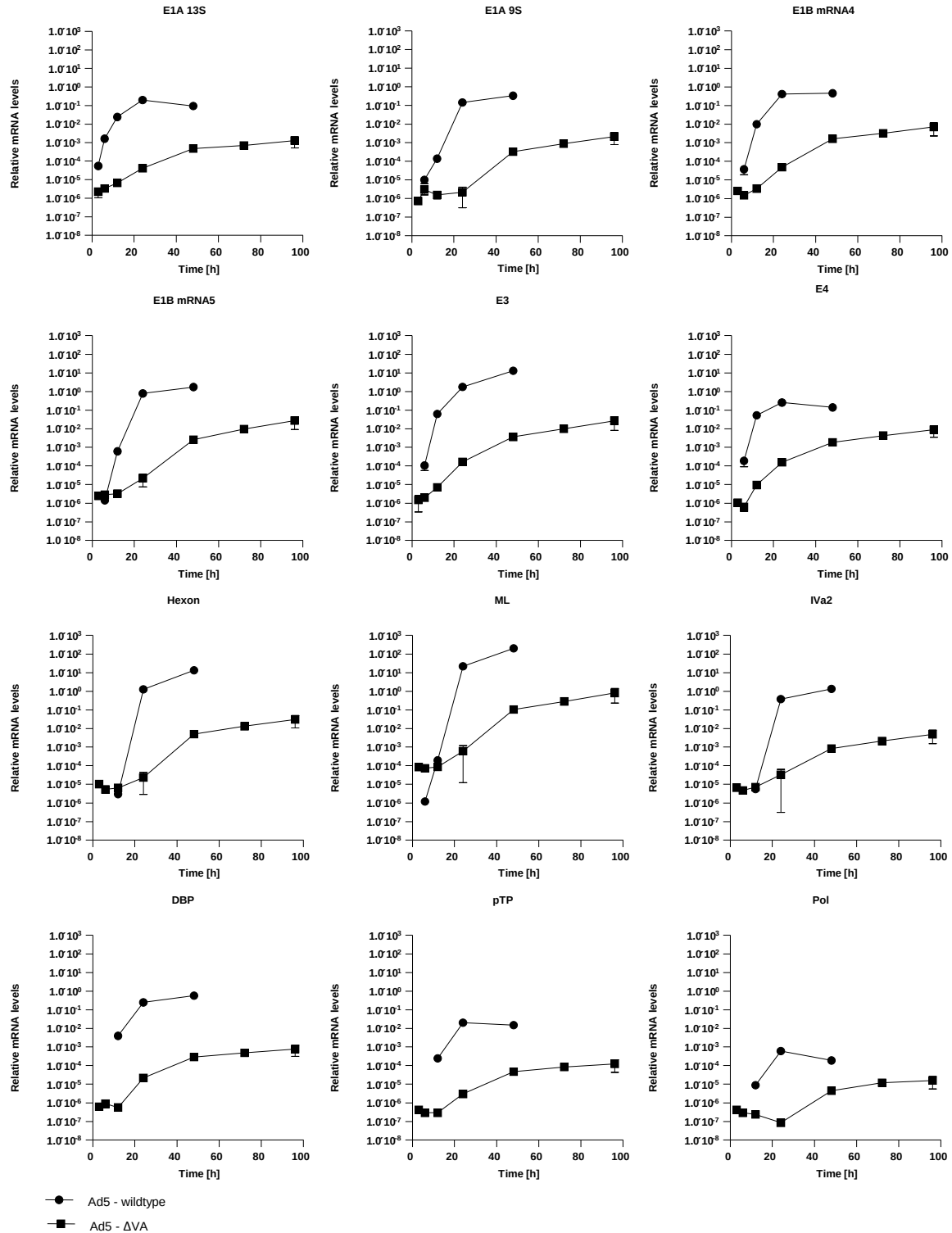


Figure 15: Expression kinetics of Ad5 wild-type and Ad5 Δ VA in A549 cells. mRNA levels are shown as the relative amount of the housekeeping gene GAPDH. E1A 13S, E1A 9S, E1B RNA4, E1B RNA5, E3, E4, IVa2, Hexon, pTP, DNA-binding protein (DBP), precursor terminal protein (pTP) and polymerase (Pol) mRNA levels were determined by RT-qPCR. (n = 2; mean values $\pm$ SD)

Although infected at a higher multiplicity of infection, the steady state mRNA levels of all transcripts were slightly lower compared to A549 cells, when THP-1 and BJAB cells were used. However, the infection kinetics in BJAB and THP-1 cells were not remarkably different from A549 cells with the exception that the increase in steady-state levels of the transcripts was slowed down (Data not shown). I measured RNA levels of E1A13S and hexon as representative genes to assess the differences between Ad5 wild-type and Ad5 Δ VA. When BJAB cells were infected with Ad5 Δ VA, the steady-state mRNA levels of viral transcripts were even lower. In THP-1 cells, the increase of transcript levels was delayed, however reaching comparable amounts at the end of the measurement series (Fig. 16).

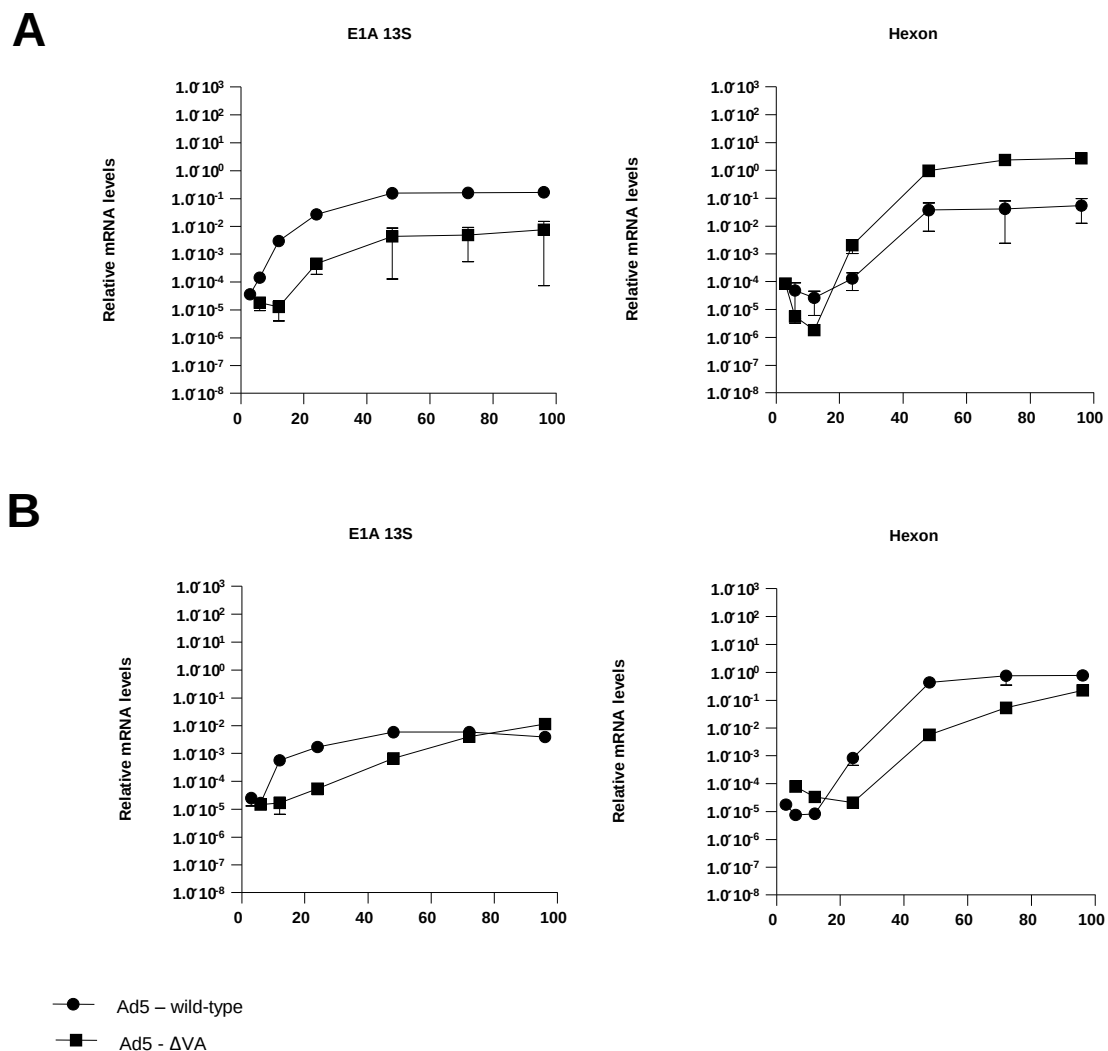


Figure 16: Infection kinetics of Ad5 wt compared to Ad5 Δ VA. BJAB (A) and THP-1 (B) cells were used. Amounts of E1A 13S and Hexon mRNA were normalized to housekeeping gene GAPDH. (n = 2; mean values $\pm$ SD)

6.3.3 Prediction of target sites for cellular miRNAs in the adenoviral genome

To screen for putative binding sites of cellular miRNAs in the human adenovirus type 5 genome (NCBI Reference Sequence: AC_000008.1) I used the TargetScan prediction software. For identification of miRNA target sites located on viral transcripts, such an approach is disadvantageous, as it can neither account for actually transcribed regions, nor different transcript variants, such as differentially spliced mRNAs. Nevertheless, this kind of analysis provided a first impression of the distribution of target sites throughout the genome. To filter the software output, a histogram-based threshold was introduced ("Appendix"). Predictions below a context score of 0.15 were selected for further analysis. To assess the distribution of the predicted target sites, the context scores were plotted against their respective position in the genome (Fig. 2B). This revealed an unequal distribution of the target sites, showing the majority of hits located in proximity to the terminal repeats on both sides of the genome. However, almost every transcription unit was predicted to be presumably subject to cellular miRNA targeting.

6.3.4 Adenoviral transcripts targeted by miRNAs in Ad5 Δ VA-infected cells

Next, I used the RIP-assay as an additional method to screen for viral mRNAs targeted by cellular miRNAs. Therefore, A549, BJAB and THP-1 cells were infected with Ad5 Δ VA. Media was exchanged at 4 hours post-infection and cells were subjected to a RIP-assay at 48 hours post-infection. This time point was chosen in accordance with the infection kinetic studies, expecting adequate mRNA levels for each viral transcript present in infected cells to not fall below the detection limit of the RIP-assay. After RNA amplification, RT-qPCR assays were performed to determine the enrichment of mRNAs as described previously.

Interestingly, I was able to observe enrichments for several mRNAs, most of them encoding proteins expressed during the late phase of infection (Fig. 17). This finding would support the hypothesis, that adenoviral mRNAs are indeed targeted by cellular miRNAs. Since a number of target sites identified by TargetScan were distributed over the region transcribed from the major late promoter, this result would also be in line with the bioinformatics approach.

Furthermore, the pattern of enriched mRNAs was different in the distinct cell types. For example, the pTP mRNA was found ~8-fold enriched in A549 cells compared to the input steady-state level. This enrichment was significantly lower in BJAB and THP-1 cells. Furthermore, in THP-1 cells, the hexon mRNA showed the highest enrichment. This was not true for BJAB cells, in which the hexon, the DPB and the IVa2 transcripts were equally enriched as DBP and IVa2. These different levels of enrichment in distinct cell types may be simply explained by their different miRNA expression pattern.

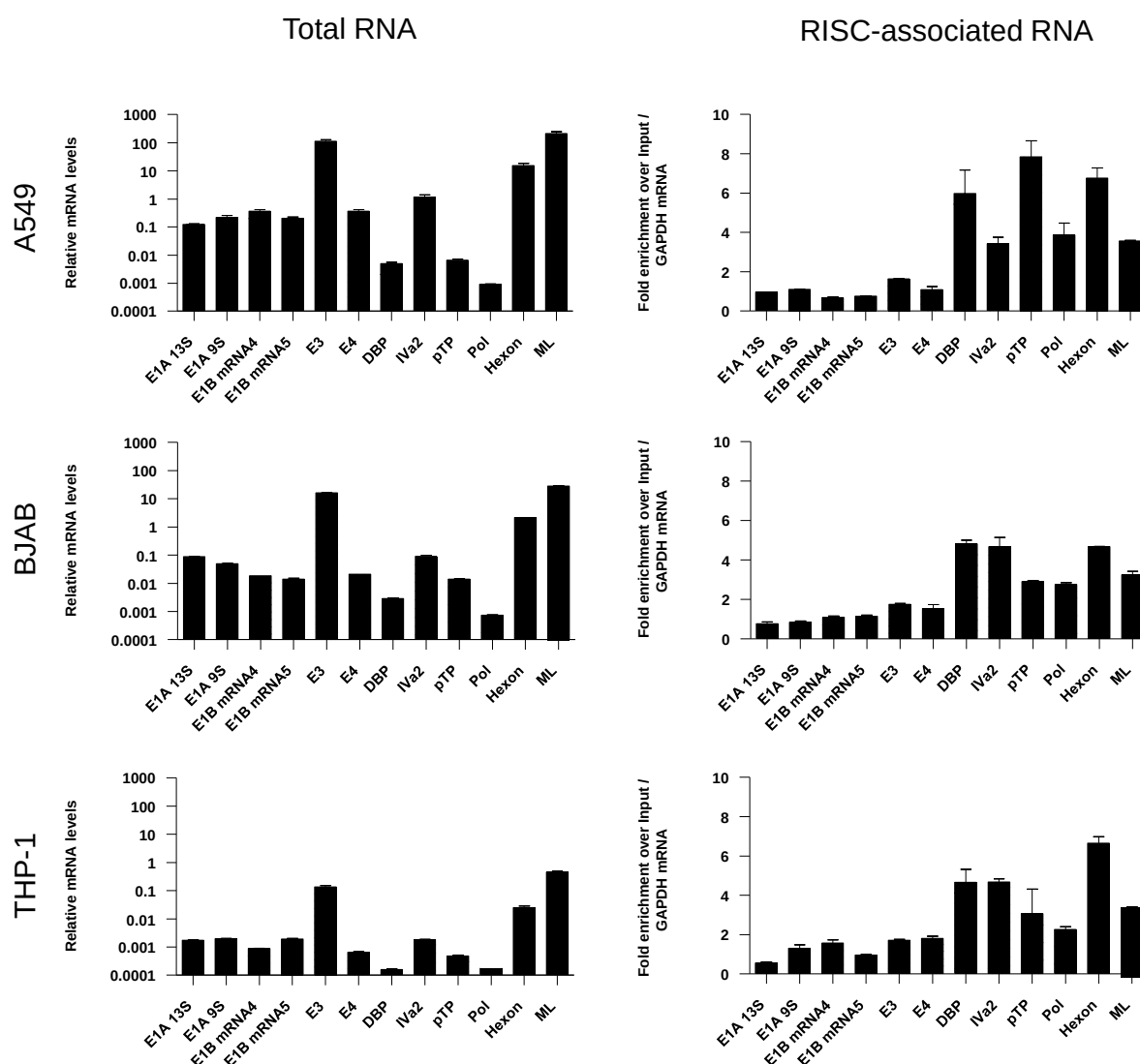


Figure 17: RIP-assay analysis of A549, BJAB and THP-1 cells infected with Ad5 Δ VA. 24 hours post-infection cells were subjected to RIP-assay. The left panels show relative mRNA levels of distinct adenoviral gene products in the total cell lysate determined by RT-

qPCR. Fold-enrichments of mRNA in the IP fraction over mRNA levels detected in total cell lysates are depicted in the right panel. All C_T values were normalized to the respective GAPDH values ($n = 2$; mean values $\pm$ SD).

6.3.5 Adenoviral transcripts targeted by miRNAs in adenovirus type 5 wild-type-infected cells

Finally I wanted to test whether adenoviral transcripts are targeted by miRNAs in the presence of VA RNAs. Therefore I repeated the experiment using Ad5 wt and A549 cells. At 30 hours post-infection, RIP-assays were performed and mRNA enrichments were determined by RT-qPCR (Fig. 18).

I could observe moderate enrichments over input for E1B mRNA4, E1B mRNA5 and IVa2. However, the enrichment of other mRNAs such as pTP, Hexon and the ML transcripts could not be reproduced. This outcome is highly interesting, since it is in line with the hypothesis, that the VA RNAs may provide a molecular shield to protect adenoviral mRNA from RNAi-mediated silencing. It is conceivable that in particular the late transcripts will benefit from such a protective function. Interestingly, some of them (*i.e.* hexon, ML, DBP, pTP) were found higher enriched, compared to RNAs encoded by early genes, when the virus lacking the VA RNAs was used. No such difference in the enrichment rate between early and late transcripts was observed for Ad5 wild-type.

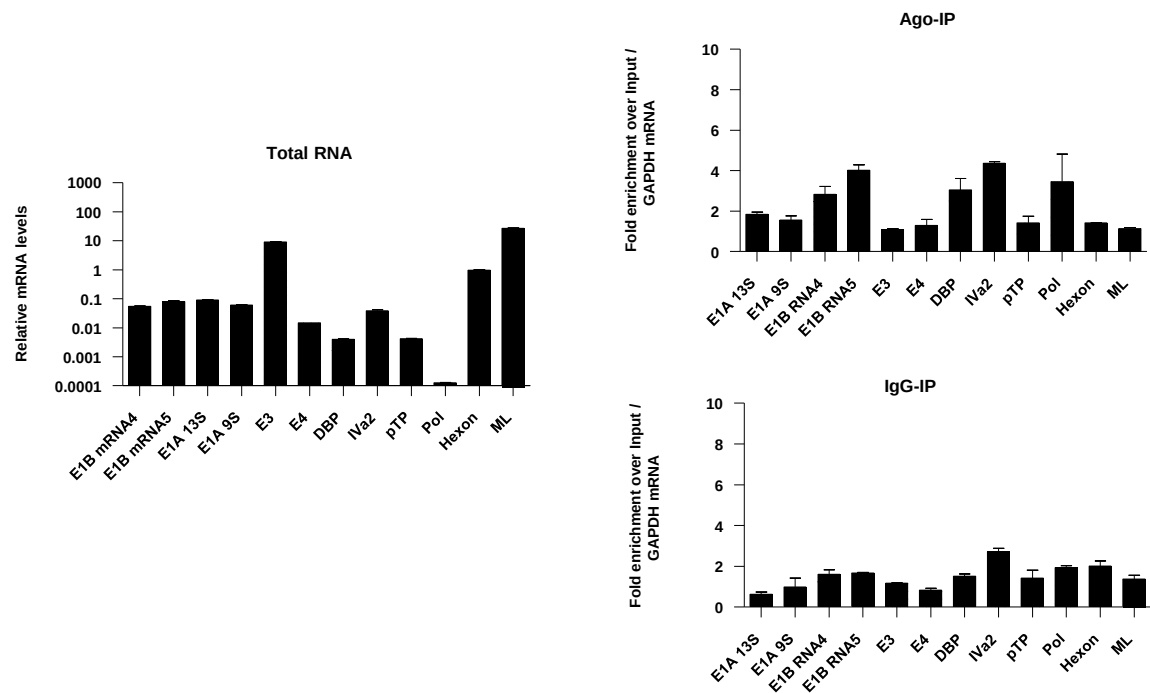


Figure 18: RIP-assay analysis of A549 cells infected with Ad5 wild-type. 30 hours post-infection cells were subjected to RIP-assay. The left panel shows relative mRNA levels of distinct adenoviral gene products in the total cell lysate determined by RT-qPCR. Fold enrichments of mRNA in the IP fraction over mRNA levels obtained from total cell lysates are depicted in the right panel. All C_T values were normalized to the respective GAPDH values. (n = 2; mean values $\pm$ SD)

7 Discussion

This study provides a comprehensive *in vitro* analysis of the molecular interactions between adenovirus-encoded RNAs and components of the host cell RNAi pathway. I made use of several methods to shed light on this poorly understood interplay.

The studies were initiated by the observation that the replication of adenovirus is disturbed when lacking genomic regions encoding the VA RNAs (59). The reason for such an impairment of the reproductive cycle might be multifactorial. Cellular antiviral defense mechanisms such as the induction of PKR-mediated inhibition of translation can be overcome by VA RNA expression (57, 62, 63). Furthermore, oversaturation of Dicer, Exportin-5 and RISC, the central components of RNA interference may be of particular importance for a successful virus replication (7). Since it is known that cellular RNAi can induce the degradation of viral mRNAs (82), antagonizing this other branch of antiviral defense would be reasonable (Fig. 19).

The saturation of RISC implies an efficient incorporation of VA RNA-derived mivaRNAs. This renders it likely that these miRNA-like molecules can also act as effectors of RNAi by targeting cellular mRNAs. Evidence for such mechanism was provided by Aparicio *et al.* (2010). Microarray expression analysis revealed a distinct set of genes downregulated upon transfection of the VA RNA-expressing vector pAdVantage (64). The authors could demonstrate the interaction between the mivaRNAs and selected candidate mRNAs. However, given the fact that miRNA-mediated gene silencing occurs not only by mRNA reduction, but also by translational repression, several mivaRNA targets may have been missed (98).

To unravel the implication of mivaRNA expression, I have employed an innovative biochemical approach. First, I transfected A549 cells with mivaRNA mimics, and by using a monoclonal antibody against cellular Argonaute proteins, I performed immunoprecipitation of RISC complexes. This approach allows direct isolation of co-immunoprecipitating miRNAs and their target mRNAs. Microarray analysis of captured mRNAs revealed a set of target mRNAs involved in various biological functions.

The putative mivaRNA target lymphocyte antigen 6 complex, locus K (LY6K), identified by Aparicio *et al.* (2010) could be confirmed in this study; however the list of top candidate genes showed only little overlap.

Although its functions are not entirely understood, it is anticipated that members of the LY6 family are involved in the susceptibility to adenovirus infections in mice. LY6 expression can be induced by Interferons and high levels are found in infected tissue, suggesting an immunomodulatory function (119 and references therein). Furthermore, genetic heterogeneity of the LY6 locus was linked to HIV-1 (120) and West Nile virus (121) susceptibility. Taken together, a specific pattern of Ly6 proteins may be beneficial for virus replication. Our data suggest that targeting of the member LY6K may be particularly advantageous for the promotion of the adenovirus infection cycle.

Another target which I was able to identify in this study, and which is particularly interesting is the alpha subunit of Interleukin 12. This immunoregulatory cytokine is produced by antigen presenting cells and is involved in a broad range of biological mechanisms. Its first proposed function was to activate the cytotoxic function of Natural Killer (NK) cells. However, it has later also been found to activate the production of Interferon- γ in T cells and NK cells (122, 123). Another function concerns the differentiation of T helper (T_H) cells. The latter can differentiate into distinct cellular subsets. Two major forms are termed T_H1 and T_H2 cells and are characterized by differences in cytokine production (124, 125). The functions of T_H1 cells involve the promotion of cell-mediated immunity and the coordination of immune responses against intracellular invaders such as viruses. On the other hand T_H2 cells are promoting humoral immune responses, directed against extracellular pathogens (125 and references therein). IL-12 encourages the development of T_H1 cells from precursor cells and thus acts as a master regulator of the appropriate immune response to pathogens (126). It has been shown by transcriptome analysis, that IL-12A expression is downregulated in the presence of adenovirus (127). The current study suggests that this downregulation can be at least partially attributed to gene silencing mediated by miRNAs. This could be interpreted as a mechanism to counteract host defense mechanisms by attenuating the cellular-immune response.

The putative target Galectin-3, which is encoded by the LGALS3 gene has multiple extracellular and intracellular functions and a control of its expression might constitute an additional possibility for the virus to influence the regulation of cell cycle, signaling, apoptosis and immune response. A detailed review on the spectrum of function of this master-regulator can be found in reference 128.

Other genes such as the guanylate kinase 1 (GUK1) or the N-acetyltransferase 14 are involved in metabolic processes. The deregulation of the host cell metabolism during infection has already been identified in other studies and may enhance the efficiency of viral replication. The same is true for molecules functioning in signaling pathways such as the G-protein signaling molecule GPR63 or CRIPAK and SRI, which may be targeted to deregulate the cellular gene expression (127). The regulation of the expression of these genes by miRNAs is an interesting finding, as it apparently represents an additional layer of complexity in terms of host-virus interactions.

Little is known about the function of other targets. For example the transmembrane protein 222 which is encoded by the TMEM222 gene is not well characterized. However, the finding that it possibly represents a target of miRNA may be valuable piece of information for subsequent studies.

In summary, our findings suggest that miRNAs have a broad range of cellular target genes. This RNAi-mediated regulatory mechanism might aid the establishment of an adenoviral infection. During the fight between the virus and its host cell, the virus strives to gain control over different biological processes such as the immune response, cellular metabolism and different signaling pathways. This goal may be in part achieved by expressing miRNAs and is reflected by the variety of the proposed functions for the putative target genes. A challenge for the future will be their further validation and a deepened analysis of their functions in the virus replicative cycle. This study can serve as a basis for further investigations of these molecular mechanisms.

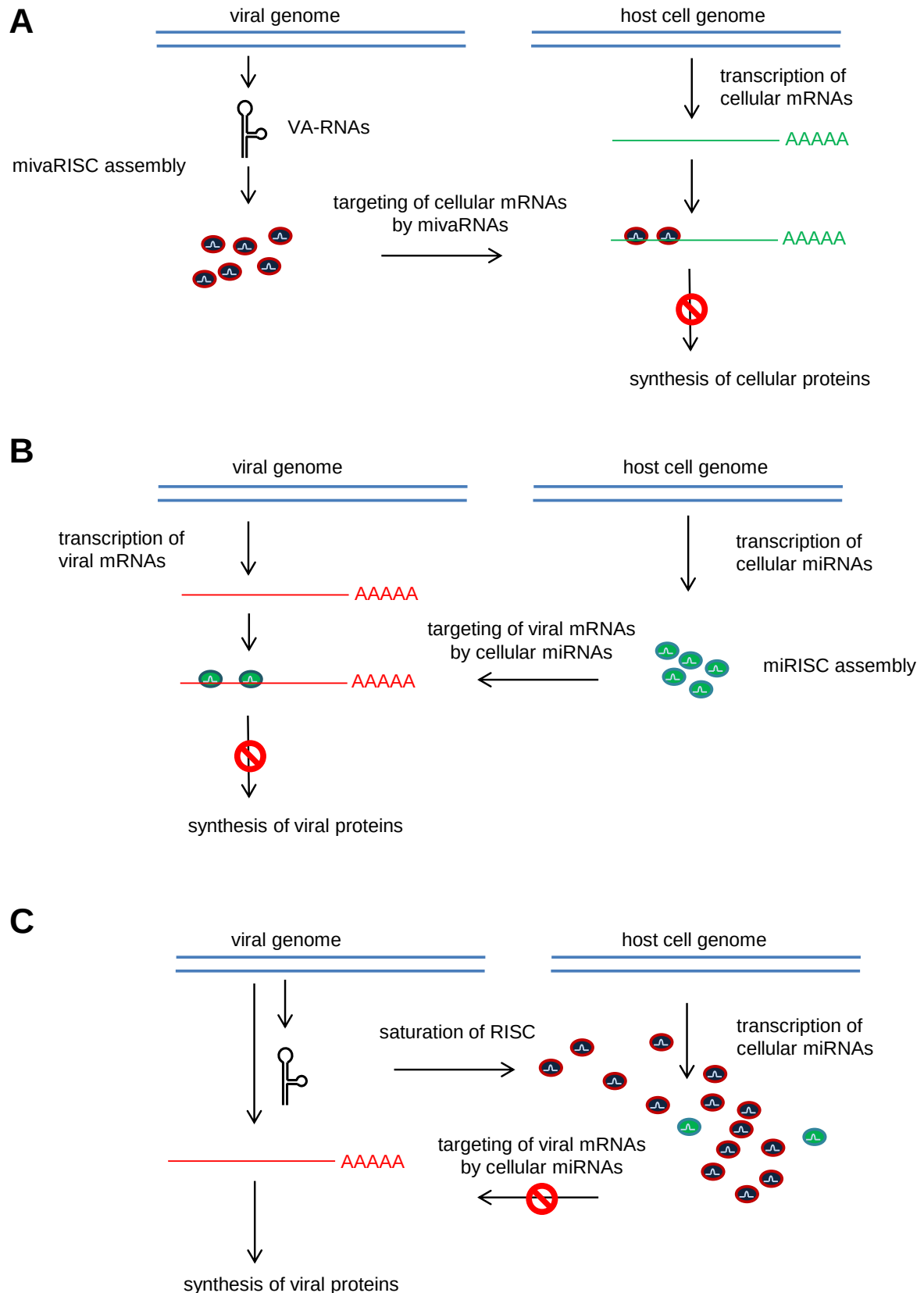


Figure 19: Summary of the proposed functions of VA RNAs. (A) VA RNAs are transcribed and give rise to different mivaRNAs which are incorporated into RISC (mivaRISC). These complexes can target cellular mRNAs and finally repress their translation or induce their degradation. (B) In the absence of VA RNAs, viral mRNAs are targeted by

cellular miRNAs which may result in an impaired synthesis of viral proteins. (C) mivaRNAs are saturating RISC which may protect viral mRNAs from cellular miRNA-mediated targeting. This may enhance the translation efficiency of viral proteins and promote virus replication.

In the last part of the study I aimed at the elucidation of the protective function of VA RNAs on viral mRNAs by using a mutant virus lacking the VA RNA genes. The infection rate of different cell lines was obviously decreased compared to the wild-type virus and analysis of its infection kinetics revealed diminished steady-state mRNA levels of distinct viral gene products. This indicates that an inhibitory and probably cellular mechanism affects the abundance of adenoviral transcripts. It is conceivable that these differences in infection kinetics arise at least partially due to a loss of protection against targeting by cellular miRNAs.

I employed bioinformatic analysis to screen for putative target sites of cellular miRNAs in the adenovirus genome. Some of these predictions could be confirmed by using the biochemical RIP-assay combined with RT-qPCR. When comparing Ad5 wild-type and Ad5 Δ VA, several mRNAs were found to be enriched in RISC. Strikingly, when the mutant virus was used, mRNAs transcribed during the late phase of infection were predominantly enriched. This effect was not observable when the wild-type virus was used. This finding would be in line with the literature, as it was shown that especially during the late phase of infection, expression of the VA RNAs is required to allow efficient translation of the viral mRNAs (59)

Interestingly, the pattern of viral transcripts associated with RISC was slightly different in distinct cell types. This might be due to a different, cell-type specific miRNA expression pattern.

Conducting the same experiment on cells infected with Ad5 wild-type revealed no enrichment for the hexon and the major late transcripts compared to early transcripts. This may have various reasons. For example, since E1B gene expression is initiated early during infection, translational repression of E1B gene expression by cellular miRNAs could occur even before the VA RNAs are efficiently expressed. In this case, translation of these mRNAs would be silenced already during the early phase of infection. Another explanation may be the activity of highly abundant miRNAs, which may still be present in active RISC during late phases of infection and target viral mRNAs. Finally, a third explanation may be that viral mRNAs are targeted by mivaRNAs themselves in a regulatory manner. Such an interaction would also lead to an enrichment in RISC and can thus not be excluded by the approach I have

used. Nevertheless, these suggestions are rather speculative and the results have to undergo further validation.

The major bottleneck of this approach is its limited comprehensiveness. Viral primary transcripts, such as the major late transcript, are processed into several mRNAs. Restricting the analysis to RT-qPCR assays used in this study, does not allow distinguish between all distinct mRNA species generated from the primary transcripts. Furthermore, several low abundant transcripts would presumably fall below the detection limit. The application of a combined approach including computational methods, RIP-assays coupled to RT-qPCR and deep sequencing will be necessary to re-evaluate and to further interpret the data presented here.

Taken together, the results of this work provide further insight into the interplay between adenovirus and the cellular RNAi machinery. The expression of VA RNAs presumably act as molecular shields to protect viral transcripts from miRNA-mediated decay, and additionally may also actively interfere with a variety of cellular processes such as the immune response to contribute to a successful infection of the host organism (Fig. 19).

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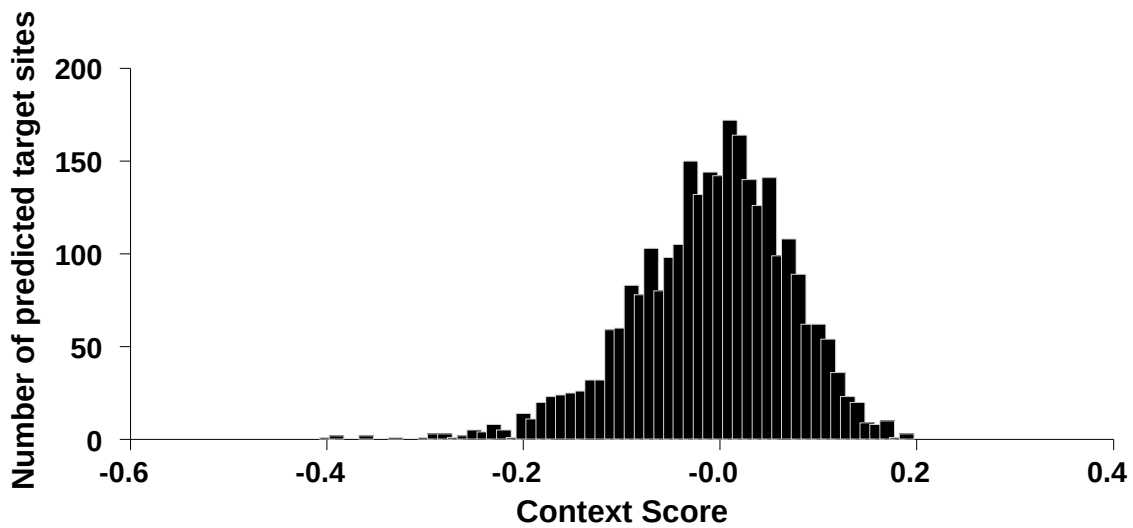
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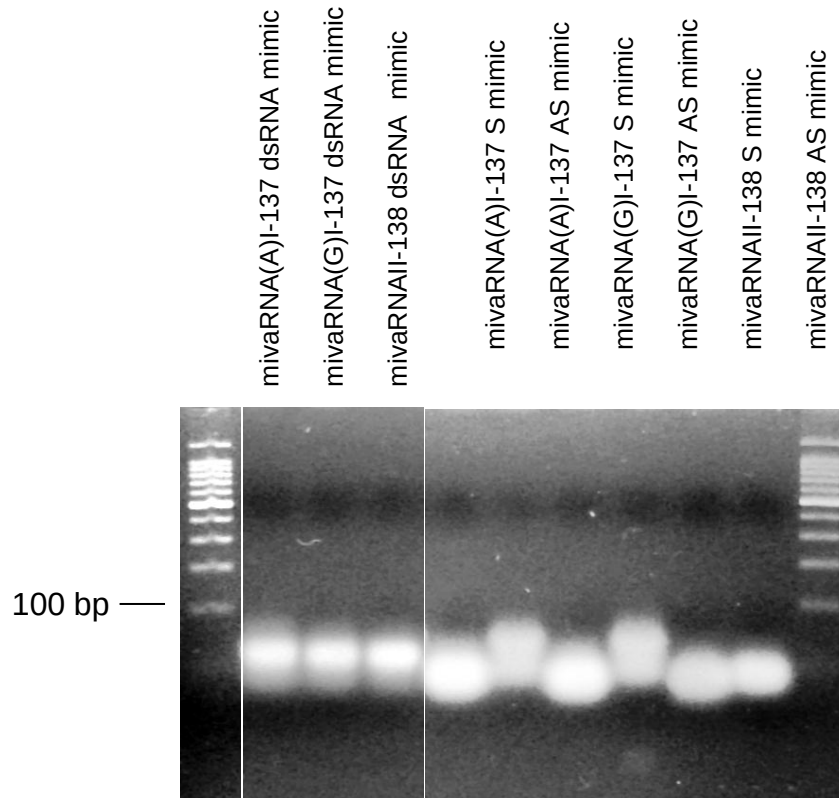
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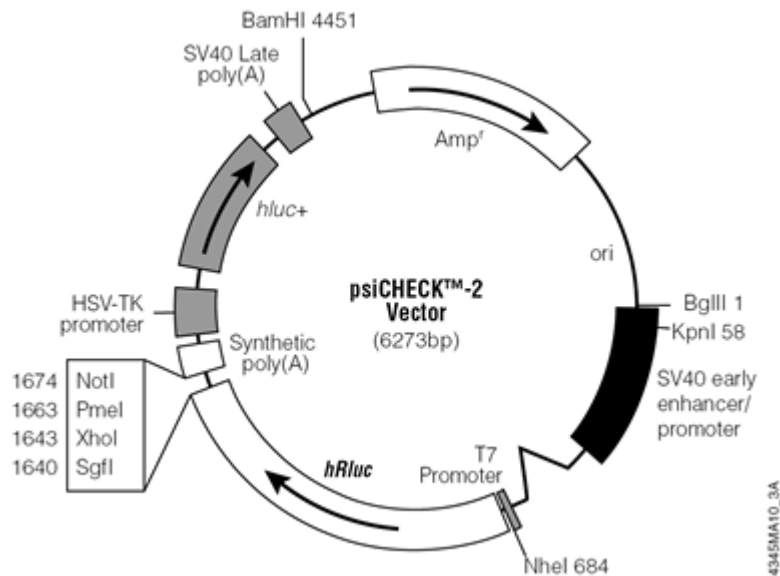
10 Appendix



Predicted cellular miRNAs targeting the adenoviral genome. Histogram showing the distribution of context score values for predicted target sites of cellular miRNAs in the human Ad5 genome calculated by TargetScan. A class width of 0.01 was used. A context score below -0.16 was assigned to 5 % of the putative binding sites. These were plotted against the relative position in the genome (Fig. 2B).



Annealing of mivaRNA mimics. After annealing of the single stranded RNA oligonucleotides as described in „Materials and Methods“, the migration behavior was assessed by agarose gel electrophoreses using a 4% agarose gel. The single stranded RNA oligonucleotides mivaRNA(A)I-137 AS and mivaRNA(G)I-137 AS showed slower migrations, which may be due to secondary structure formation. A different migration behavior was observed after annealing indicating successful annealing.



mivaRNA target vectors. The target sequences of the mivaRNA mimics were inserted into *SgfI* and *NotI* sites of the psiCHECK™-2 vector. The exact sequences are listed below:

pmiVAI5-T

GCGATCGC CATATGACCGGT ACCAGACCACGGAAGAGTGCCCGCT ACCGGT GAGCTC CTCGAC GCGGCCGC

pmiVAI3-T

GCGATCGC CATATGACCGGT AAAAGGAGCACTCCCCGTTGTCTG ACCGGT GAGCTC CTCGAC GCGGCCGC

pmiVAI5-T

GCGATCGC CATATGACCGGT TCCGGCTACAGGGAGCGAGCC ACCGGT GAGCTC CTCGAC GCGGCCGC

pmiVAI3-T

GCGATCGC CATATGACCGGT GGGGCTCGTCCCTGTTTCCG ACCGGT GAGCTC CTCGAC GCGGCCGC

Yellow: *SgfI*

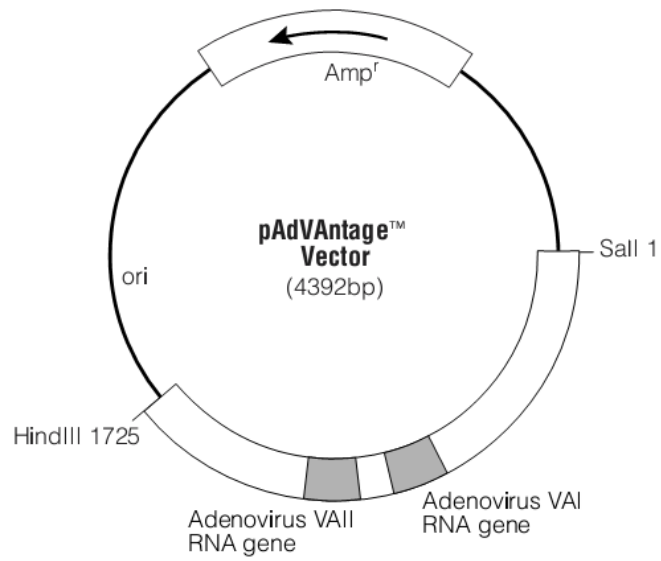
Red: *NdeI*

Pink: *AgeI*

Light blue: *SacI*

Dark green: *Sall*

Light green: *NotI*



Map of the pAdVantage™ Vector. The pAdVantage™ vector was used for transfection of mammalian cells. It contains the VA RNAI and the VA RNAII genes.

11 Curriculum Vitae

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Vienna, August 2012