The adenoviral control of the RNAi-microRNA pathway: How and why

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Summary
MicroRNAs constitute a large class of recently discovered short regulatory RNAs that control gene expression post-transcriptionally by binding to the complementary mRNAs and repressing their translation. The importance of the microRNA pathway for regulating gene expression in mammalian cells is already established. Moreover, it has been shown recently that many mammalian viruses can suppress the microRNA pathway, which could suggest that this pathway interferes with virus growth either directly or indirectly.

In this project I attempted to understand and characterize the interaction between adenovirus and microRNA pathway in human cell lines. Upon investigating adenovirus growth, by looking at production of late viral proteins and RNA using western and northern blotting, in cell lines with compromised microRNA pathway, my results showed a moderate enhancement in virus production in cell lines with compromised microRNA pathway. This could indicate the presence of, direct or indirect, inhibitory effects from the microRNA pathway on adenovirus growth. Also by investigating the levels of Argounate 2 (AGO2) and Dicer, the main components of the microRNA pathway, using western blotting during adenovirus infection I showed that adenovirus infection lead to rapid reduction in AGO2 levels; however the levels of Dicer remained fairly stable. This could suggest a novel mechanism for a mammalian virus to interfere with the microRNA pathway by degrading AGO2.

It had been shown previously that adenovirus produces large amount of the viral associated I RNA (VAI RNA), which is processed into small RNAs called the mivaRNAIs and saturate the microRNA pathway. However it is not known if the mivaRNAIs can function as microRNAs and target cellular or viral mRNAs modulating their expression. In order to investigate the function of mivaRNAIs, I constructed recombinant viruses in which I changed the seed sequence (2\textsuperscript{nd} to 8\textsuperscript{th} nucleotides, this sequence was shown to be essential for a microRNA to recognize its targets), of the mivaRNAIs. Upon investigating production of the viral proteins from the recombinant viruses no detectable change was observed, which might indicate that the mivaRNAIs have no significant targets mRNAs, but rather their main function is to block the microRNA pathway by out competing the cellular microRNAs.
Introduction

MicroRNA pathway in mammals

About one decade ago, a new family of gene expression regulators was discovered and assigned the name microRNA. A microRNA (miRNA) is a 21-22 nucleotides long, non-coding RNA. In mammals, miRNA genes are initially transcribed as long RNA molecules (pri-miRNAs) by either RNA polymerase II or III (Kim et al., 2009). Then this pri-miRNA, a few hundred nucleotides long with several local stem loops flanking ssRNA sequences, undergoes two processing steps mediated by two RNase III endonucleases, Drosha and Dicer respectively (Hutvagner et al., 2001; Lee et al., 2003) (FIG.1).

Fig 1. Biogenesis of microRNAs and their assembly into microribonucleoproteins. Adapted with permission from (Filipowicz et al., 2008)
The first processing step takes place in the nucleus by the so-called microprocessor complex, which consists of Drosha together with a cofactor called DGCR8 (Digeroge syndrome critical region 8). This complex interacts with the stem-loop together with the ssRNA sequences flanking it on both sides and cuts 11 base pairs (bp) away from the ssRNA-dsRNA junction, creating a precursor microRNA (pre-miRNA). Then the pre-microRNA, a stem loop with two nucleotides overhangs at the 3′ end, is exported to the cytoplasm via Exportin 5, a RanGTP-dependent dsRNA Binding protein (Yi et al., 2003). In the cytoplasm, this pre-microRNA undergoes the second processing step, in which Dicer with its cofactor TAR RNA binding protein (TRAP) cleaves the pre-microRNA near its terminal loop to generate a 22 base pairs microRNA duplex. Then the microRNA strand with less thermodynamically stable 5′ end will be loaded into the miRNA induced silencing complex (miRISC), whose core components are the argonaute family of proteins (AGO1-4), while the other strand (miRNA*) probably will be degraded (Peters and Meister, 2007).

**Target recognition, mode of action and function.**

The miRNA functions as a linker molecule guiding the miRISC components to the target mRNA. The target recognition depends mainly on the Watson-Crick base pairing between the 5′ end of an miRNA (especially its 2nd to 8th nucleotides, the so called seed sequence) and the 3′ UTR region of an mRNA. However, central mismatches or bulges often occur with some compensatory base pairing between the 3′ end of the miRNA and the mRNA (Lewis et al., 2005). As a consequence of this flexibility in target recognition, one miRNA can bind to a few hundred mRNAs, and an mRNA can be targeted by several miRNAs. After the miRNA-mRNA interaction is established, the miRISC downregulates the expression of the target mRNAs in either of two different ways, either through direct endonucleolytic cleavage or through translation repression of the mRNA (Bartel, 2009). The direct cleavage of the mRNA takes place when there is perfect complementary between the miRNA and its target. AGO2, the only argonaute with endonucleolytic activity, mediates this cleavage (Yoda et al., 2010). The translation repression can take place in different ways, for instance, inhibition of translation initiation or degradation of newly synthesized proteins by recruiting proteases (Filipowicz et al., 2008). The microRNA pathway regulates the expression of around 50 % of human genes, and as a consequence, it is involved in most cellular activities such as proliferation, morphogenesis, apoptosis and differentiation (Carthew, 2006).

**RNA interference and microRNA pathways**

In insects and plants, a mechanism similar to the microRNA pathway exists called RNA interference (RNAi). The RNAi is an antiviral mechanism and it is not involved in regulation of endogenous gene expression (Ding and Voinnet, 2007). The stimulus to initiate the RNAi is the introduction of an exogenous dsRNA of around 500 bp. Then it is processed by Dicer into small dsRNAs of ~21–25 bp in length, designated as small interfering RNA duplex (siRNA duplex). One strand of the siRNA duplex is incorporated into RISC and directs the cleavage of complementary mRNA targets. The miRNA and siRNA are similar in terms of biogenesis and mode of action, and the only clear distinction between them is the origin of the dsRNA (FIG.2) (He and Hannon, 2004).
RNA interference and microRNA pathways as antiviral mechanisms

In plants and invertebrates, the role of RNAi as defense mechanism against both virus and transposable elements is well established and conserved. This antiviral function mainly depends on formation of viral small RNAs, originated from long virus-derived dsRNA cleaved by Dicer, that trigger direct cleavage of the complementary viral RNA, in similar way as described previously for miRNA. These viral-derived dsRNAs can be the genome of dsRNA viruses or replication intermediates of ssRNA viruses or transcribed viral RNA with secondary structure similar to miRNA precursors in case of DNA viruses (Ding and Voinnet, 2007).

In mammals, the direct antiviral role of the microRNA pathway remains controversial due to the presence of an effective antiviral mechanism called the interferon (IFN) pathway (Cullen, 2006). The interferon (IFN) pathway is also induced by viral dsRNA, however its activation leads to a complete shut off of the translation machinery in infected cells (Levy and Garcia-Sastre, 2001). In addition, even though many mammalian viruses can produce viral small RNAs, for instance adenovirus (Xu et al., 2007) and herpesviruses (Cai et al., 2005), there is no evidence showing that these small RNA can target viral RNAs leading to their degradation like in plants or insects. On the other hand, several lines of evidences show that cellular miRNAs can target and downregulate expression of viral genes; for instance, miRNA-32 can reduce significantly the accumulation of the retrovirus primate foamy virus type 1 (PFV) in human cell lines (Lecellier et al., 2005).
Viral suppression of RNA interference and microRNA pathways

Different viruses have developed various ways to suppress the RNA interference. In plant, tombusvirus and rice hoja blanca virus encode RNA silencing suppressors (RSS) P19 and NS3 respectively. Both proteins interfere with RNAi using similar mechanisms; the RSS bind to small RNA duplexes preventing RISC assembly (Scholthof, 2006; Hemmes et al., 2007). In Drosophila, flock house virus (FHV) produces B2, a multilevel RNAi suppressor protein. B2 binds to dsRNA in a sequence- and length-independent manner. As a result, it can interfere with RNAi at two steps: first when Dicer processes the dsRNA into siRNA duplexes and second when these siRNA duplexes are incorporated into RISC (Lingel et al., 2005).

In mammals, many viruses have been showed to encode RSS that can efficiently interfere with the microRNA pathway. However, most of these RSS also block the interferon pathway, for example E3L, dsRNA-binding protein, of vaccinia virus (Li et al., 2004) and VA RNA of adenovirus (Gale and Katze, 1998; Lu and Cullen, 2004). As a result, it becomes complicated to visualize the significance of the microRNA pathway as antiviral mechanism in mammals.

Adenovirus

Adenoviruses are widespread in nature, infecting a broad range of hosts including mammals (Mastadenovirus), birds (Aviadenovirus), reptiles (Atadenovirus), amphibians (Siadenovirus) and fish (Ichtadenovirus) (Ginsberg, 1962). There are about 100 different serotypes, with more than 50 serotypes isolated from humans. They infect the mucosal membrane in the respiratory and intestinal tracts, causing respiratory infection and gastroenteritis respectively. An adenovirus is characterized by its non-enveloped icosahedral capsid of 90 nm in diameter consisting of 240 hexons and 12 pentons, with a protruding fiber from each penton base. It has a double-stranded DNA genome of 30-38 kb in length, with inverted terminal repeat at each end, that encodes 40-50 proteins (SHENK, 1996).

An adenovirus enters the cell in two steps: first the fiber knob binds to the coxsackievirus and adenovirus receptor (CAR) (Howitt et al., 2003). Then an exposed tripeptide arginine-glycine-aspartate (RGD) motif at the penton base portion of the fiber interacts with the α, integrins on the cell surface (Stewart et al., 1997). After binding to its receptor, the adenovirus is taken into the cell by receptor-mediated endocytosis in clathrin-coated pits. As the result of the low pH environment inside the endosome, the virus particles escape to the cytoplasm by a poorly understood mechanism. Once in the cytoplasm, the adenovirus finds its way to the nucleus though the network of microtubules (Kelkar et al., 2004). The viral capsid starts being partially degraded during the transport so that only the viral genome will be imported through the nuclear pore complex into the nucleus, where the viral early gene expression and DNA replication take place (Trotman et al., 2001). The adenoviral genome is divided into several transcriptional units according to their expression time during viral life cycle. There are five early genes (E1A, E1B, E2, E3 and E4), two delayed early genes (pIX and Iva2) and one late major transcriptional unit. All adenoviral genes are transcribed by RNA polymerase II except viral-associated (VA) I and II genes are transcribed by RNA polymerase III (McConnell and Imperiale, 2004) (FIG 3).
The E1A region proteins have two main functions. The first is to trans-activate other early transcriptional units and the second is to induce the host cell to enter S phase allowing viral DNA replication (Berk, 1986). E1B proteins, 55k and 19k, inhibit apoptosis in the infected cells either by binding to p53 or to proapoptotic proteins, Bcl-2–associated X protein (Baxi et al.) and Bcl-2 homologous antagonist-killer protein (Bak), allowing the infection cycle to be completed (Sundararajan et al., 2001). E2 proteins, adenovirus-encoded DNA polymerase, preterminal protein and DNA binding protein, are responsible for the viral DNA replication (de Jong et al., 2003). In order to maintain the viral infection, the E3 region proteins act to neutralize the host antiviral defense array: E3-gp19K sequesters the major histocompatibility complex MHC class I protein in the endoplasmic reticulum, preventing their translocation to the cell surface. As a result, it inhibits the presentation of the viral antigens in the MHC class I pathway and subsequent recognition by cytotoxic T cells and lysis of the infected cell (Burgert et al., 1987). E3 receptor internalization and degradation (RID) complex is an integral protein that consists of two E3 region products E3-10.4k and 14.5k. This complex inhibits apoptosis induced by chemokines tumor necrosis factor alpha (TNF-α), Fas ligand (FasL) and tumor necrosis-related and apoptosis-induced ligand (TRAIL) (Benedict et al., 2001). This inhibition takes place by inducing clearance of the chemokines from the cell surface and promotes their degradation in the lysosome. In addition, E3 RID together with E3 14.7K blocks TNF-induced secretion of inflammatory mediator arachidonic acid via preventing the translocation phospholipase A\textsubscript{2} to the cell membrane. The E4 region codes for seven proteins with wide activities involved in viral DNA replication, viral mRNA splicing and transport, shut-off of cellular protein synthesis and regulation of apoptosis (McConnell and Imperiale, 2004).

The transcription of late genes reaches a high level with the onset of the viral DNA replication. The primary transcript from the major late promoter is about 30.000 nucleotides
long. It is polyadenylated at one of five sites and undergoes multiple splicing events to generate five families of late mRNAs (L1 to L5) with more than 18 distinct mRNAs (Shaw and Ziff, 1980). All late Ad mRNAs have 200 nucleotides leader sequence at their 5’ end, which is referred as the tripartite leader. The tripartite leader sequences establish efficient translation of the late adenoviral mRNAs independent of the host initiation factor eIF4F (Dolph et al., 1988). Since late during infection, around 24 hours post-infection (hpi), Adenovirus-encoded 100k protein displaces eIF4E kinase Mnk1 from eIF4E, which results in blocking the translation of both cellular mRNAs and adenoviral mRNAs lacking the tripartite sequences (Cuesta et al., 2000). Finally, the newly synthesized adenoviral proteins are exported back to the nucleus where the virion assembly takes place. The whole infection cycle takes 24-30 hour and ends with the lysis of the infected cell and release of $10^4$-$10^5$ new viral particles.

**Silencing of the antiviral mechanisms in adenoviral infected cells.**

During virus infections, the levels of type I interferon are significantly increased which in turn induces the expression of protein kinase R (PKR). PKR is activated upon binding to long dsRNA, more than 30 base pairs, followed by its homodimerization and autophosphorylation (Meurs et al., 1990). Activated PKR phosphorylates eukaryotic translation initiation factor 2α (eIF2α), leading to shut-off of the translation machinery in infected cell and inhibition of production any more viral proteins (Garcia et al., 2007). Adenovirus has developed a unique way to overcome this defense mechanism by production of the non-coding RNA, viral-associated I RNA (VAI RNA) (approximately $10^8$ molecules per cell). VAI RNA has a stable secondary structure that resembles microRNA precursors, and it is consisting of three major regions, a terminal stem with paired 3’ and 5’ ends, central domain and an apical stem (Coventry and Conn, 2008). The central domain together with the apical stem are involved in binding to PKR, inhibiting its activation and, as a consequence, ensuring high translation efficiency in late infected cells (Thimmappaya et al., 1982).

Moreover, VAI RNA suppresses the microRNA pathway in adenovirus-infected cells at three different levels; it blocks Exportin5-mediated nuclear transport, inhibiting the transport of per-microRNA (Lu and Cullen, 2004). Secondly, VAI RNA hijacks Dicer acting as a competitive substrate, and as a result its terminal stem is cleaved off as a small RNA duplex. This small RNA duplex consists of the complementary 3’ and 5’ ends of the VAI RNA, and it is around 22 base pairs, similar in structure to the cellular miRNA duplex and designated as mivaRNAI duplex. Finally, these generated mivaRNAI duplexes will be unwinded into 3’ end and 5’ end mivaRNAs, and then both strands will be separately loaded into RISCs, (Andersson et al., 2005; Xu et al., 2007). Due to the high production level of VAI RNA, the RISCs in late infected cells are almost completely saturated with the mivaRNAs (Xu et al., 2007). Some adenovirus serotypes code for another non-coding RNA, viral-associated II RNA (VAII RNA) (Ma and Mathews, 1996). VAII RNA has similar secondary structure to the VAI RNA and it is processed in the same way producing the mivaRNAIIs, however VAII RNA is produced in much less amounts compared to the VAI RNA (Andersson et al., 2005). It is not known the function of the VAII RNA, because it has been shown previously that adenovirus mutant lacking VAII can grow as the wild type virus (Kitajewski et al., 1986; Cascallo et al., 2006).
Aim
The main aim of this project was to characterize the interaction between adenovirus and the microRNA pathway in human cells. I attempted to achieve this goal in three different ways: First, I investigated the adenovirus growth in a cell line with compromised microRNA pathway to see if this pathway had any potential antiviral function that would inhibit the virus growth. Second, I wanted to find out whether the adenoviral infection negatively affected the protein levels of both Dicer and AGO2 as a way to overcome this potential antiviral function. Finally I constructed recombinant adenoviruses, in which the seed sequence and the thermodynamic stability of the mivaRNAIs duplexes were altered to find out if the predicted microRNA-like function of mivaRNAIs was significant for the adenoviral life cycle.
**Results**

**Characterization of Hct116 cell lines**

Hct116 dicer \textsuperscript{exo5/exo5} cells are variants of Hct116 colorectal cancer cells in which the N-terminus of Dicer, where the helicase domain is located, is disrupted by an in-frame insertion of the 43 amino acids. This mutation was created in exon5 of the \textit{dicer} gene that is why the cells are called dicer \textsuperscript{exo5/exo5} cells (Cummins \textit{et al.}, 2006). In this cell line, the mutated Dicer shows poor processing efficiency of most, but not all, cellular pre-microRNA (Cummins \textit{et al.}, 2006; Soifer \textit{et al.}, 2008). In order to verify the presence of the inserted sequence, reverse transcription-polymerase chain reaction (RT-PCR) was done on RNA samples from both cell lines, the wild type and the mutated, using primers binding on both sides of \textit{dicer} exon 5 (FIG. 3). The RT-PCR results showed that Dicer mRNA extracted from Hct116 dicer \textsuperscript{exo5/exo5} cells contain about 100 nucleotides more comparing to that extracted from Hct116 wt cells.

![Fig 4. Characterization of Hct116 cells. One µg cytoplasmic RNA from both Hct116 wt and dicer \textsuperscript{exo5/exo5} cells were subjected to RT-PCR with dicer reverse and forward primers (Table 4), and then the PCR products were separated on 2% agarose gel.](image)
Adenovirus growth in Hct116 Cell lines

Hct116 cells (both wt and dicer\textsuperscript{exo5/exo5}) were infected with 50 virus particles (vp)/cell of adenovirus wt900. Cytoplasmic RNA and proteins were harvested at 12, 24, and 48 hours post infection (hpi) and used for northern and western blots, respectively. The northern blot showed that during adenovirus infection, the accumulation of both the full length VAI RNA and the processed mivaRNAI started earlier in Hct116 dicer\textsuperscript{exo5/exo5} cells compared to the Hct116 wt cells (FIG.5). Moreover, the production of mivaRNAII, the processed form of the VAI RNA, was also significantly increased in the Hct116 dicer\textsuperscript{exo5/exo5} cells (FIG.6).

![Image](https://example.com/image.png)

Fig 5. Production of mivaRNAI from VAI RNA (160 nt) in wt and dicer\textsuperscript{exo5/exo5} Hct116 cells upon adenovirus infection. A, structure of VAI RNA, showing in blue position of the probe (3’end mivaRNAI wt) (Table 4) at its 3’ end; B, northern blot of RNA from infected cells. 15 μg cytoplasmic RNA from wt and dicer\textsuperscript{exo5/exo5} Hct116 cells 10, 24 and 50 hours after infection with adenovirus wt900 was separated on a 12% polyacrylamide gel, blotted and probed with 3’end mivaRNAI wt probe (Table 4).
Fig 6. Production of mivaRNAII in wt and dicer^{exo5/exo5} Hct116 cells upon adenovirus infection. A, structure of VAII RNA, showing in blue position of the probe (3’end mivaRNAII s wt) (Table 4) at its 3’ end; B, northern blot of RNA from infected cells, 15 μg cytoplasmic RNA from wt and dicer^{exo5/exo5} Hct116 cells 10, 24 and 50 hours after infection with adenovirus wt900 was separated on a 12% polyacrylamide gel, blotted and probed with 3’end mivaRNAII s wt probe. The upper part of the membrane, where the full length VAII was located, was damaged and the star sign indicated the position of non-specific band.
Next, I wanted to investigate if the enhanced production of adenoviral RNA from Hct116 dicer$^{exo5/exo5}$ cells was accompanied with enhanced production of adenoviral protein by using western blotting. However it was difficult to obtain a clear blot from protein samples from wt and dicer$^{exo5/exo5}$ Hct 116 cells infected with adenovirus wt900.

The same experiments were repeated but with ad5, instead of wt900. The results showed that the production of viral RNA (VAI RNA and mivaRNAIs) was enhanced in the HCT116 dicer$^{exo5/exo5}$ cells (FIG.7). On the other hand, some viral proteins were up regulated in the dicer$^{exo5/exo5}$ Hct116 cells, while the majority of the viral proteins did not show any significant changes (FIG.8).

Fig 7. Northern blot showing the production of mivaRNAI from VAI RNA (160 nt) in wt and dicer$^{exo5/exo5}$ Hct116 cells upon adenovirus infection. 15 µg cytoplasmic RNA from both Hct116 WT and dicer$^{exo5/exo5}$ cells at time points 10, 24 and 50 hours after ad5 infection, were separated on 8% polyacrylamide gel, blotted and probed with 3’end mivaRNAI wt probe (Table 4).
Fig 8. Western blot showing the production of adenoviral proteins in both wt and dicer$^{exo5/exo5}$ Hct116 cells upon adenovirus infection. 20 μg of cytoplasmic proteins from infected cells 10, 24 and 50 hours after ad5 infection, were separated on 12% polyacrylamide gel, blotted and probed with antibodies against adenovirus structural proteins and actin (as a loading control) (Table 5).
The effect of adenoviral infection on Dicer and AGO 2 protein levels in 293 cells.

293 cells were infected with adenovirus wt900 50 vp/cell and cytoplasmic proteins were harvested at zero, 2, 6, 12 and 24 hpi. Immunoblotting with anti-Dicer antibodies showed that Dicer levels were fairly stable during adenovirus infection (FIG.9A). The same experiment was repeated with time points 15, 23, 24, and 48 hpi, similar results were obtained but Dicer levels were significantly reduced at 48 hpi (FIG.9B), in this particular experiment no loading control was used, but according to protein concentration measurements, equal amount of proteins were loaded to each well. On the other hand, Argonaute 2 (AGO2) levels showed rapid decline soon after adenovirus infection, about 6 hours post infection (FIG.9C).

Fig 9. Stability of Dicer and AGO2 upon adenovirus infection in 293 cells. A, 40 µg of cytoplasmic proteins from infected cells with wt900 6, 12 and 24 hours after infection, were separated on 12% polyacrylamide gel, blotted and probed with antibodies against Dicer and actin as a loading control (Table 5). B, the same as in A but the cytoplasmic proteins were harvested 15, 22, 24, 48 hour post infection, and no loading control was used. The star sign indicates the position of nonspecific band appeared above the Dicer band; C, 40 µg of cytoplasmic proteins from infected cells with wt900 6, 12 and 24 hours after infection, were separated on 12% polyacrylamide gel, blotted and probed with antibodies against AGO2 and actin as a loading control (Table 5).
Construction of recombinant adenoviruses with mutated mivaRNAIs.

In the last part of the project I mainly focused on understanding the function of the mivaRNAIs, the small RNAs generated from both 3' and 5' ends of VAI RNA that was why they were assigned the name 3’ and 5’ end mivaRNAIs. To test if the mivaRNAIs could function as miRNA and target any cellular or viral mRNAs modulating their expression levels, I constructed three recombinant viruses: In the first two, the seed sequences, the most essential part of a miRNA to find its target mRNAs, of both 3’ and 5’ end mivaRNAIs were changed, with a different sequence showed in (FIG.10) and Table 4, and assigned the names 3’mut and 5’mut viruses, the third recombinant virus was with the wild type VAI sequence, and assigned the name wt virus. The three recombinant viruses did not express VAI RNA.

293 cells were infected 50 vp/cell of each virus and after 24 hour after infection cytoplasmic RNA and proteins were harvested. Northern blot results showed that the 3’end mivaRNAI produced from the three recombinant viruses attained different sizes (FIG.11A). However, I could not find out what were the exact sizes the mutant mivaRNAIs had, also I did not know if the mutant VAI RNAs had different Dicer cleavage site other than the expected in (FIG.10). Western blot showed that the production of viral proteins from the three recombinant adenoviruses were almost at the same levels, slightly reduced in the 5’mut and 3’mut compared to the wild type (FIG.11B).

![Fig 10. Secondary structure of VAI RNA WT, 3’end mutant (3’mut) and 5’end mutant (5’mut). A, the secondary structure of VAI RNA showing dicer cleavage site, at the star sign, and the seed sequence of the processed mivaRNAI from both arms at 3’end and 5’end; B and C, the secondary structure of VAI RNA 3’end and 5’end mutant respectively, the mutated sequence indicated in bold letters.](image)
Fig 11. Production of adenoviral VAI RNA and proteins from the recombinant viruses in 293 cells. A, northern blot of RNA from infected cell with wt, 5’mut and 3’mut recombinant viruses showing both the full length VAI RNA and the processed mivaRNAI, 15 µg cytoplasmic RNA from 293 cells 24 hour after infection were separated on a 12% polyacrylamide gel, blotted and probed with 3’end mivaRNAI wt, 3’end mivaRNAI 3’mut and 3’end mivaRNAI 5’mut probes (Table 4); B, western blot against adenoviral proteins produced from the three recombinant viruses, 20 µg of cytoplasmic proteins from infected cell with wt, 5’mut and 3’mut recombinant viruses 24 hours after infection, were separated on 12% polyacrylamide gel, blotted and probed with antibodies against ad5 proteins (Table 5), no loading control was used.
Discussion

The first part of the project was concerned with studying the behavior of adenovirus in cells with a comprised microRNA pathway. In the original study where Hct116 dicer\textsuperscript{exo5/exo5} cell line was created, it has been shown that the production of many but not all miRNAs was significantly reduced comparing to the wild type (Cummins et al., 2006).

My results showed that the processing of VAI into mivaRNA\textsubscript{I} was not affected by mutating the helicase domain of Dicer protein (at its N terminus) in the Hct116 dicer\textsuperscript{exo5/exo5} cell line (FIG.4 and 6). However the virus growth, showed by the production of the full length VAI and a few viral proteins, was enhanced in the Hct116 dicer\textsuperscript{exo5/exo5} cell line. This enhanced virus production, even if it was moderate, in cells with a comprised microRNA pathway could indicate that there is, direct or indirect, antiviral function for the microRNA pathway against the adenovirus in mammalian cells. On the other hand, The observation that the majority of the viral proteins did not show any changes between the two cell lines could be due to that the microRNA pathway was partially but not totally impaired in Hct116 dicer\textsuperscript{exo5/exo5} cells. So in order to visualize more clearly the negative effects of the microRNA pathway on adenovirus growth, the microRNA pathway should be more drastically impaired, for instance by completely knocking down Dicer or AGO2.

The second part of this project focused on the interaction between adenovirus and Dicer and AGO2. The results obtained from 293 cells showed that the viral infection did not interfere with Dicer levels until the late period of infection cycle, 48 hours after infection. In addition to the previous published results (Andersson et al., 2005) that showed that Dicer activity was significantly reduced at 20 hours after infection, this can confirm that adenovirus interferes with the microRNA pathway by saturating Dicer with VAI and VAI\textsubscript{I} RNA acting as competitive substrates. On the other hand, AGO2 protein levels appeared to decline rapidly early during adenovirus infection, around 6 hours after infection. This result suggests that adenovirus might promote early degradation of AGO2 as way to block the microRNA pathway in the infected cells. However this needs to be further investigated.

The third part of this project was concerned with understanding the function of mivaRNA\textsubscript{Is} and if these mivaRNA\textsubscript{Is} have any important cellular or viral target mRNAs. Since the seed sequence of an microRNA is essential for recognizing it targets, so by changing the seed sequence of the mivaRNA\textsubscript{Is}, I changed the set of target mRNAs the mivaRNA\textsubscript{Is} would recognize. These introduced mutations to the mivaRNA\textsubscript{Is} seed sequences could compromise the virus growth if suppression of the corresponding target mRNAs is important for the adenovirus infection. The northern blot results showed that the mutant VAI RNAs expressed from the recombinant viruses were processed into mivaRNA\textsubscript{Is} with different lengths. These obtained results were unexpected, because Dicer cleaves long dsRNA, in sequence independent manner, into 22 bp small RNA duplexes (Zhang et al., 2002). So it was expected that the mutant VAI RNAs would attain the same Dicer cleavage site and processed into mivaRNA\textsubscript{Is} of the same length. It might be complicated to explain what happened, and more experiments need to be done to understand how the introduced mutations caused the variation in the processing site of VAI RNA. It was clear that even by changing the length and the seed sequence of the processed mivaRNA\textsubscript{Is}, the virus production was not significantly affected (FIG.11.B). This can indicate that mivaRNA\textsubscript{Is} do not have any significant target mRNAs and they only act to saturate the microRNA pathway in the host cells. However this will need to be further investigated by repeating the experiment with different vp/cell and in different cell lines.
**Materials and methods**

**Cell lines**
The 293: cell line originated from human embryonic kidney cells transformed with adenovirus 5, stably expressing adeno viral proteins E1A and E1B. It is routinely used to propagate recombinant adenoviruses missing the endogenous E1 region. 293 cells were routinely used in our lab that is the reason most of my experiments were carried out using these cells.
The Hct116: cell line originated from human colorectal cancer cells, the Hct116 Dicer\textsuperscript{exo5/exo5} cell line is a variant from Hct116 in which the N-terminus of Dicer is disrupted with in frame insertion of the 43-amino-acids (Cummins *et al.*, 2006).

**Adenovirus**
In this study, different variants of adenovirus serotype 5 were used (Table 1). In order to infect mammalian cells, the virus stock was thawed and mixed briefly. An appropriate volume (calculated from desired number of virus particles per cell and the titer of the virus stock) was diluted in 1 ml Dulbecco's modified eagle medium (DMEM), with no serum (Invitrogen). The medium from the plate/s (6 cm\textsuperscript{2}) was removed from the plate(s), and then 1 ml of the diluted virus was added to the plate(s) which was incubated for one hour in a CO\textsubscript{2} incubator (37ºC, 7% CO\textsubscript{2}). After the one hour incubation the medium was removed and 4 ml DMEM, supplemented with 10% newborn calf serum (NCS) (Invitrogen), were added and plates were returned back to the CO\textsubscript{2} incubator.

**Table 1. Adenoviruses.**

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Relevant properties</th>
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<tbody>
<tr>
<td>Ad5</td>
<td>Adenovirus serotype 5</td>
</tr>
<tr>
<td>Wt900\textsuperscript{1}</td>
<td>Adenovirus serotype 5 with two base pair deletion (nucleotide -25 and -26) upstream of the VAI gene.</td>
</tr>
<tr>
<td>Wt</td>
<td>Adenovirus serotype 5, VAI wt, ΔE1A and ΔE1B and ΔVAII.</td>
</tr>
<tr>
<td>3’mut\textsuperscript{2}</td>
<td>Adenovirus serotype 5, VAI 3’end mut, ΔE1A, ΔE1B and ΔVAII.</td>
</tr>
<tr>
<td>5’mut\textsuperscript{3}</td>
<td>Adenovirus serotype 5, VAI 5’end mut, ΔE1A, ΔE1B and ΔVAII.</td>
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</tbody>
</table>

\textsuperscript{1} Wt900 does not produce functional mivaRNAI from 5’end of VAI RNA (5’end mivaRNAI), however the production of mivaRNAI from 3’end of VAI RNA (3’end mivaRNAI) is not affected by this deletion (Xu *et al.*, 2009). I used wt900 in the experiments in the beginning because the stock concentration of wt900 was higher than that of ad5, and it was available in larger amounts.

\textsuperscript{2} the seed sequence of mivaRNAI generated from the VAI RNA 3’end is mutated (Table 4), with compensatory mutations in the 5’end, since both ends of (3’ and 5’ ends) VAI RNA binds to each other forming dsRNA (Ma and Mathews, 1996).

\textsuperscript{3} the seed sequence of mivaRNAI generated from the VAI RNA 5’end is mutated (Table 4), with compensatory mutations in the 3’ends, since both ends (3’ and 5’ ends) of VAI RNA binds to each other forming dsRNA.
Bacterial strains and propagation of bacteria

Different *E.coli* strains were used in this study (Table 2). Bacteria were grown either in a lysogeny broth (LB) liquid culture (1 % Bacto-tryptone, 0.5 % yeast extract and 1 % NaCl) or on agar plates (LB medium supplemented with 1.5 % agar).

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Genotype</th>
<th>Vendor</th>
</tr>
</thead>
<tbody>
<tr>
<td>XL-1 Blue</td>
<td><em>RecA1</em></td>
<td>Aligent technologies</td>
</tr>
<tr>
<td></td>
<td><em>endA1</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>gyrA96</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>thi-1</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>hsdR17</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>supE44</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>relA1</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>lac</em></td>
<td></td>
</tr>
<tr>
<td>Bj5183</td>
<td><em>EndA1</em></td>
<td>Aligent technologies</td>
</tr>
<tr>
<td></td>
<td><em>sbcBC</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>recBC</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>galK</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>met thi-1</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>bioT</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>hsdR(Sty)</em></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td><em>FhuA2</em></td>
<td>Aligent technologies</td>
</tr>
<tr>
<td></td>
<td><em>(argF-lacZ)U169</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>phoA</em></td>
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</tr>
<tr>
<td></td>
<td><em>(glnV44)</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Φ80</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>(Δ(lacZ)M15)</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>(gyrA96)</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>(recA1 relA1 endA1 thi-1 hsdR17)</em></td>
<td></td>
</tr>
</tbody>
</table>

1 *E.coli* strain routinely used in transformation and amplifying plasmids with prokaryotic origin of replication.

2 routinely used to generate recombinant adenoviruses.

Preparing of electrocompetent cells

Single colony of Bj5183 was inoculated into 5 ml LB and incubated overnight at 37 °C. Then 2.5 ml of the culture were inoculated into 200 ml LB and allowed to grow at 37 °C to an OD 600 (optical density) of ~0.5 to 0.7. Then the culture was transferred into pre-chilled 1 liter centrifuge bottle and allowed to stand on ice for 10-15 min. Next, the culture was centrifuged for 20 min at 5000 g at 2 °C. After that the supernatant was poured off and the pellet was suspended in 500 ml ice-cold water, mixed well and centrifuged as described previously. This washing step was repeated one more time. Then 40 ml ice-cold 10% glycerol was added to the cells, mixed well and centrifuged as described. Finally the pellet volume was estimated and an equal volume of ice-cold 10% glycerol was added to resuspend cells (on ice). 300 µl aliquots of cells were transferred into prechilled microcentrifuge tubes and frozen on dry ice and stored at -80°C.

Electroporation

Plasmid DNA was extracted from different *E.coli* strains (Table 2) using nucleic acid purification kit (Macherey-Nagel). 0.5 µg plasmid DNA was mixed with 300 µl thawed electrocompetent cells in an electroporation cuvette (Bio-Rad). Then the cuvette was placed in the sample chamber of the electroporation apparatus (Bio-Rad), which was adjusted to 2.5 kV, 25 µF and 400 ohms, and the pulse was applied. After that 1 ml LB medium was added and the cells were incubated at 37 °C for one hour. Then 200 µl was plated on LB plates, supplemented with an appropriate antibiotic (kanamycin or ampicillin 25 mg/ml).
**Chemical transformation**

0.5 µg DNA plasmid was mixed with 100 µl thawed chemocompetent cells (XL-1 Blue or DH5α) in an ice cold tube and was incubated for one hour at 4 °C. Then the tube was placed at 42 °C for 90 seconds then back 4 °C for two minutes. After that 1 ml LB medium was added into the tube, followed by one incubation at 37 °C. Then 200 µl was plated on LB plated supplied with an appropriate antibiotic (Kanamycin or Ampicillin 25 mg/ml).

**Molecular cloning**

**Construction of pShuttle VAI wt, 3’mut and 5’mut plasmids.**

3 µg pUC57 VAI wt (Table 3) (the accession number for pUC57 vector in the GenBank is Y14837 and the gene ID for VAI gene is 573997) was cleaved with XhoI and BglII generating two bands; the empty vector (2716 bp) and the ad5 VAI gene with extra up and downstream sequences (410 bp). Then the smaller fragment was extracted and purified form the gel, using a gel extraction kit (Macherey-Nagel). Then the purified fragment was ligated into an XhoI and BglII linearized, gel purified pShuttle vector (the accession number for pShuttle vector in the GenBank is AF334399). The ligation products, pShuttle VAI-WT (Table 3), were chemically transformed into XL-1 Blue (Table 2), plated on LB plates supplemented with Kanamycin 25 µg/ml and incubated at 37°C overnight. Then single colony was restreaked on LB plates supplemented with Kanamycin 25 µg/ml and incubated at 37°C overnight. After that a single colony was grown overnight in LB liquid culture supplemented with kanamycin 25 µg/ml. finally the pShuttle VAI-WT was extracted from the overnight culture, using nucleic acid purification kit (Macherey-Nagel).

The pShuttle VAI-3’mut and 5’mut were constructed in the same way. 3 µg pUC57 VAI-3’mut (or VAI-5’mut) (Table 3) were doubly digested with XhoI and XbaI releasing the VAI-3’mut (or the VAI-5’mut) fragment which was then gel extracted and purified. Then the pShuttle VAI-wt was cleaved with XhoI and Xbal releasing the VAI-wt fragment (232 bp) and the empty pShuttle vector (6763 bp). This empty pShuttle vector was ligated with XhoI and XbaI generated VAI 3’mut (or 5’mut) fragment. Then the ligation products were transformed, cultured, extracted as described before. The VAI 3’mut gene had two unique BpmI restriction sites, one at each end, while the VAI 5’mut gene had two unique BsmAI restriction sites, these unique sites were used to verify the construction of pshuttle VAI-3’mut or 5’mut. In addition, the plasmids were sent for sequencing at (Eurofinsdna) with pShuttle forward primer (Table 4) that bound to the pShuttle sequence upstream to the inserted sequence. The sequencing results were analyzed, using EMBOSS pairwise alignment algorithms tool (Lombard et al., 2002), and showed that the constructs were correct.

**Construction of pAdeasy WT, pAdeasy 3’mut and pAdeasy 5’mut plasmids.**

The construction of recombinant adenoviruses was done as described before (Luo et al., 2007). PAdeasy-11 that codes for ampicillin resistance, was first transformed into electrocompetent Bj8351, transformatants were then plated on LB plates supplemented with ampicillin 25 mg/ml and incubated overnight at 37°C. Selected colonies were grown overnight in LB plates supplemented with ampicillin 25 mg/ml. After that the pAdeasy-11 transformed Bj8351 were made electrocompetent as described before and assigned the name Bj8351-pAdeasy-11.

The pShuttle- (wt or 3’mut or 5’mut), codes for kanamycin resistance, was linearized with PmeI and transformed into the Bj8351-pAdeasy-11 and plated on kanamycin (25 µg/ml) plates. On the next day, 50 colonies were selected, grown overnight in 2 ml LB supplemented
with kanamycin 25 mg/ml and followed by plasmid extraction. The screening of potential recombinants was done using XhoI, in case of the correct recombinant, the restriction digestion would generate five bands (16119, 14482, 2466, 1445 and 595 bp), while in the case of only pshuttle the restriction digestion would generate only one band (6995bp). In addition, the potential recombinants were also digested with PacI, this restriction digestion would generate two bands (30527 and 4580 bp) in case of the correct recombinant, while with pshuttle would generate two bands (4061 and 2934 bp) as described before in (Luo et al., 2007). Based on the screening results, the right recombinants were chemically transformed into DH5α (Table 2), plated on LB plates supplemented with Kanamycin 25 µg/ml and incubated at 37°C overnight. Then single colony was restreaked on LB plates supplemented with Kanamycin 25 µg/ml and incubated at 37°C overnight. After that a single colony was grown overnight in LB liquid culture supplemented with kanamycin 25 µg/ml. finally the the recombinant pAdeasy (pAdeasy VAI wt or 3’mut or 5’mut) was extracted from the overnight culture, using nucleic acid purification kit (Macherey-Nagel).

### Table 3 plasmids.

<table>
<thead>
<tr>
<th>Plasmid name</th>
<th>Properties</th>
<th>Vendor</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUC57 VAI wt</td>
<td>Amp&lt;sup&gt;R&lt;/sup&gt;, VAI wt</td>
<td>Genscrpit</td>
</tr>
<tr>
<td>pUC57 VAI 3’mut</td>
<td>Amp&lt;sup&gt;R&lt;/sup&gt;, VAI 3’mut&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Genscrpit</td>
</tr>
<tr>
<td>pUC57 VAI 5’mut</td>
<td>Amp&lt;sup&gt;R&lt;/sup&gt;, VAI 5’mut&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Genscrpit</td>
</tr>
<tr>
<td>pShuttle</td>
<td>Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Aligent technologies</td>
</tr>
<tr>
<td>pShuttle VAI wt</td>
<td>Kan&lt;sup&gt;R&lt;/sup&gt;, VAI wt</td>
<td>This investigation</td>
</tr>
<tr>
<td>pShuttle VAI 3’mut</td>
<td>Kan&lt;sup&gt;R&lt;/sup&gt;, VAI 3’mut</td>
<td>This investigation</td>
</tr>
<tr>
<td>pShuttle VAI 5’mut</td>
<td>Kan&lt;sup&gt;R&lt;/sup&gt;, VAI 5’mut</td>
<td>This investigation</td>
</tr>
<tr>
<td>pAdeasy-11</td>
<td>Amp&lt;sup&gt;R&lt;/sup&gt;, adenovirus&lt;sup&gt;3&lt;/sup&gt; ΔE1, ΔVAI, ΔVAl</td>
<td>Akusjärvi laboratory</td>
</tr>
<tr>
<td>pAdeasy VAI wt</td>
<td>Kan&lt;sup&gt;R&lt;/sup&gt;, pAdeasy-11, VAI wt</td>
<td>This investigation</td>
</tr>
<tr>
<td>pAdeasy VAI 3’mut</td>
<td>Kan&lt;sup&gt;R&lt;/sup&gt;, pAdeasy-11, VAI 3’mut</td>
<td>This investigation</td>
</tr>
<tr>
<td>pAdeasy VAI 5’mut</td>
<td>Kan&lt;sup&gt;R&lt;/sup&gt;, pAdeasy-11, VAI 5’mut</td>
<td>This investigation</td>
</tr>
</tbody>
</table>

<sup>1</sup>the seed sequence of 3’end mivaRNAI was mutated (Table 4).

<sup>2</sup>the seed sequence of 5’end mivaRNAI was mutated (Table 4).

<sup>3</sup>the whole genome of adenovirus ad5 cloned in a plasmid called pAdeasy (Luo et al., 2007).

The lab technician did the part concerning the production and amplifying of the recombinant viruses. Briefly, the plasmid constructs of the recombinant viruses (pAdeasy VAI wt, 3’mut and 5’mut) were transfected to 293 cells using turboFect™ in vitro transfection reagent according to the manufacturer’s instructions (Fermentas). After transfection the expression of viral proteins took place, followed by viral DNA replication, the original template was the transfected plasmids, and adenovirus life cycle continued leading to cell lysis and release of the assembled progeny virions. After one week of transfection, the cell lysate, which contained the progeny virus particles, was collected and added again to 293 cells. The same step was repeated several times using increasing amounts of cells to amplify the amount of the virus per lysate. Finally the cell lysate was titrated and used for the subsequent experiments.
**Cell lysis**
From a 6 cm$^2$ plate, cells were collected into 15 ml Falcon tube, centrifuged for 5 min at 2000 g, and then the pellet was washed with 1 ml 1X Phosphate Buffered Saline (PBS) (Invitrogen), and centrifuged again as described. 200 µl of isOB-NP40 lysis buffer (10 mM Tris-HCl (pH 7.9), 150 mM NaCl, 1.5 mM MgCl$_2$ and 1% (v/v) Nonidet P-40) was used to resuspend the pellet followed by 30 minutes incubation on ice. Finally the content of the tube was transferred into 1.5 ml eppendorf tube and centrifuged at 12000 g for 3 min then the supernatant was used for either RNA extraction or protein analysis.

**RNA extraction**
After cell lysis, 130 µl 5X RPS (0.5 M Tris-HCl (pH7.9), 0.4% sodium dodecyl sulfate (SDS), 50 mM EDTA) and 600 µl phenol:chloroform (1:1) were added to the supernatant in a separate tube followed by mixing and centrifugation for 5 min at 14000 g. Then the supernatant was taken out and added to 600 µl phenol:chloroform (1:1) followed by mixing and centrifugation as before. Then the RNA in the supernatant, 200 µl, was precipitated with 600 µl isopropanol and 30 µl 3 M sodium acetate at -20 ºC overnight.

**Electrophoresis**
Agarose
2% agarose in 1X TBE (90 mM Tris, 90 mM boric acid pH8.3, 2 mM EDTA) was used. The DNA bands were separated at 150 V for 1 hour.

**Protein gels**
Proteins were separated on 12% polyacrylamide gel (40% acrylamide (37:1 acrylamide:bis-acrylamide), 1.5 M Tris-HCl (pH 8.8), 0.4% SDS, 20 µl TEMED Tetramethylethylenediamine (TEMED) and 300 µl ammonium persulfate) with stacking gel 5% polyacrylamide gel (40% acrylamide (37:1 acrylamide:bis-acrylamide), 0.5 M Tris-HCl (pH 6.8), 0.4 % SDS, 20 µl TEMED and 90 µl ammonium persulfate) in 1X running buffer (2.5 mM Tris- HCl and 19.2 mM glycine) at 150 V.

**RNA gels**
RNA was separated on 12% denaturing polyacrylamide gel (8 M urea, 12% acrylamide (29:1 acrylamide:bis-acrylamide), 1X TBE, 20 µl TEMED and 300 µl ammonium persulfate) in 1X TBE at 250 V.

**Oligonucleotides**
In this study oligonucleotides were used for different applications as mentioned in (Table 4).

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence 5’ to3’*</th>
<th>Application</th>
<th>Vendor</th>
</tr>
</thead>
<tbody>
<tr>
<td>3’end mivaRNAI Wt</td>
<td>AAAAAAGGACACTCCCCCGTTGTCTGA CGT CGCA</td>
<td>Northern blot</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>3’end mivaRNAI 5’mut</td>
<td>AAAAAGGACACTCCCCCGTTGTCTGA CGT CGCA</td>
<td>Northern blot</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>3’end mivaRNAI 3’mut</td>
<td>AAAACACCAGTCTCCCGTGGAGCGACGTCGCA</td>
<td>Northern blot</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>3’end mivaRNAII</td>
<td>AAAAAGGACACTCCCCCGTTGTCTGA CGT CGCA</td>
<td>Northern blot</td>
<td>Invitrogen</td>
</tr>
</tbody>
</table>
| **Dicer forward** | CTGTTTCCGGAGG  
| | GTTTCATTATGACCTTG  
| | CTATGTGC  
| **Dicer reverse** | CTCGATAGGGGTGGTC  
| | TAGGATT  
| **PShuttle forward** | GCCATTTTCGCGGG  
| **blot** | RT-PCR  
| **Invitrogen** |  
| **RT-PCR** | Invitrogen  
| **Sequencing** | Invitrogen  
| **VAI** |  
| | AGCGGGCACCCTTTCCG  
| | TGGTCTGTTGGATAAA  
| | TTCGCAAGGGGTACAT  
| | GGCAGGACGGGGGGGT  
| | TCGAGCCCCTATCCG  
| | GCCTGCCGGCTGATCC  
| | ATGCAGGTACCCACCGG  
| | CAGTGCGGAGTGCTCCTT  
| **3’mut** |  
| | AGCGGGCACCCTTTCCG  
| | TGGTCTGTTGGATAAA  
| | TTCGCAAGGGGTACAT  
| | GGCAGGACGGGGGGGT  
| | TCGAGCCCCTATCCG  
| | GCCTGCCGGCTGATCC  
| | ATGCAGGTACCCACCGG  
| | CAGTGCGGAGTGCTCCTT  
| **5’mut** |  
| | AAGAGGGGAGTGCTCCTT  
| **Molecular Cloning** | Genscript  
| **Genscript** |  
| **VAI wt** |  
| **Molecular Cloning** | Genscript  
| **Genscript** |  
| **TAI 1** |  
| **Molecular Cloning** | Genscript  
| **Genscript** |  
| **VAI 3’mut** |  
| **Molecular Cloning** | Genscript  
| **Genscript** |  
| **VAI 5’mut** |  
| **Molecular Cloning** | Genscript  
| **Genscript** |  

*all primers were designed manually except for Dicer forward and reverse were obtained from previous publication (Cummins et al., 2006)

1,2,3 the sequences of VAI wt, 3’mut and 5’mut genes that were synthesized by Genscript and inserted in pUC57 plasmid (Table 3). The bold italic letters indicates the position of the mutations in both the 3’and 5’ends of the VAI gene.
5' P\textsuperscript{32} end labeling

In order to prepare the probe for northern blotting, I did P\textsuperscript{32} 5'end labeling of oligonucleotides that were perfect complementary to the target RNA (Table 4). 40 pmole oligonucleotide was mixed with 5 µl 10 X polynucleotide kinase PNK buffer (Fermentas), 8 µl γ-P\textsuperscript{32} ATP (PerkinElmer), 10 units T4-PNK (Fermentas) and 31 µl H\textsubscript{2}O and incubated at 37°C for 2 hours. Then the labeled oligonucleotides were purified using Microspin\textsuperscript{TM} G25 column (Illustra\textsuperscript{TM}, GE Healthcare) according to the manufacturer's instructions.

Northern blot

For small RNA analysis, 5-15 µg RNA were separated on denaturing 8% or 12% polyacrylamide gel, transferred (Biorad semi-dry blotting chamber) onto Hybond XL membrane (Amersham Biosciences) and UV crosslinked at 1200 J (UVC 500, Amersham)

The Membranes were prehybridized in 5X Denhardt’s solution (0.1% Ficoll 400, 0.1% polyvinillypyrolidone and 0.1% bovine serum albumin (BSA)), 6X standard saline citrate SSC (diluted from 20X SSC, 3 M NaCl, 0.3 M sodium citrate) and 0.2% SDS for two hours at 42°C. After overnight hybridization with the probe, that was prepared as mentioned before, the membranes were washed in 3X SSC, 0.5% SDS 3 times for 10 min at 42°C and once with 1X SSC, 0.5% SDS for 15 min at 42°C. Signal was detected by exposure to Phosphorimager screen that was scanned afterwards by Phosphoimager (Phoaros Fx\textsuperscript{TM} plus).

RT-PCR

Two µg RNA were mixed with 200 ng random hexamers (Fermentas) and 2 µl dNTPs (10 mM each) (Fermentas) in a total reaction volume of 13 µl and incubated at 65 °C for 5 min. Then 4 µl of 5X first strand buffer (Invitrogen), 2 µl of 0.1M DTT and 2 µl (400 units) of reverse transcriptase (Invitrogen) were added to the reaction which was incubated for 50 min at 37°C followed by deactivation at 70 °C. Then 2 µl of the reaction were subjected to PCR with primers Dicer forward and Dicer reverse (Table 4) under the following cycling conditions: 95 °C, 5 min, once; 94 °C, 30 s, 55 °C, 30 s, 72 °C, 30 s, 30 cycles, and finally 72 °C, 10 min, once. PCR products were examined by electrophoresis on 2% agarose gel.

Restriction digestion reaction

0.8 µg DNA incubated with 0.5 µl restriction endonuclease (Fermentas), 1 µl 2 µg/µl BSA, 5 µl of restriction endonuclease buffer (Fermentas) and up to 20 µl water. After one hour, the digestion products were separated on 2% agarose gel.

DNA ligation reaction

The ligation reaction was prepared as follows: 0.5 µl T4 DNA ligase (Fermentas), 1 µl T4 10X ligase buffer (Fermentas), DNA (the insert and vector, with molar ratio of 4 insert:1 vector) and up to 10 µl water.

Western blot

Protein samples were separated on 8 or 12% polyacrylamide gel together with protein ladder (Fermentas). After that the proteins were electro-transferred (Biorad blotting chamber) onto Immobilon-FL western blot nitrocellulose membrane (Millipore) at 200 mA for 2-6 hours, using towinbin transfer buffer (25 mM tris, 192 M glycine). Then the membrane was blocked with Odyssey blocking buffer (Li Cor) for one hour at 4°C. The membrane was incubated
with primary antibody, diluted in the blocking buffer, overnight at 4°C and washed 4 times for 5 minutes in 1x PBS with 0.01% Tween-20. Then it was incubated with the secondary antibody, diluted in the blocking buffer, for one hour at room temperature and washed as mentioned previously. Finally the membrane was rinsed in tween-free PBS and scanned by Odyssey scanner (Li Cor).

**Table 5** Primary antibodies.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution used</th>
<th>Vendor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit polyclonal anti-ad5 (raised against adenoviral structural proteins)</td>
<td>1:500</td>
<td>Abcam</td>
</tr>
<tr>
<td>Mouse monoclonal anti-Dicer</td>
<td>1:100</td>
<td>Abcam</td>
</tr>
<tr>
<td>Mouse monoclonal anti-AGO2</td>
<td>1:500</td>
<td>Abnova,</td>
</tr>
<tr>
<td>Goat polyclonal anti-Actin</td>
<td>1:5000</td>
<td>Santa Cruz</td>
</tr>
</tbody>
</table>

**Table 6** Secondary antibodies.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution used</th>
<th>Vendor</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRDye$^1$ 800 nm polyclonal anti-rabbit IgG</td>
<td>1:10000</td>
<td>Li Cor</td>
</tr>
<tr>
<td>IRDye 800 nm polyclonal anti-mouse IgG</td>
<td>1:10000</td>
<td>Li Cor</td>
</tr>
<tr>
<td>IRDye$^2$ 680 nm polyclonal anti-goat IgG</td>
<td>1:10000</td>
<td>Li Cor</td>
</tr>
</tbody>
</table>

$^1$Infra-red dye with absorbance and emission maxima around 800 nm

$^2$Infra-red dye with absorbance and emission maxima around 680 nm
References


Ding, S.W., Voinnet, O., 2007. Antiviral immunity directed by small RNAs. Cell 130, 413-426.

