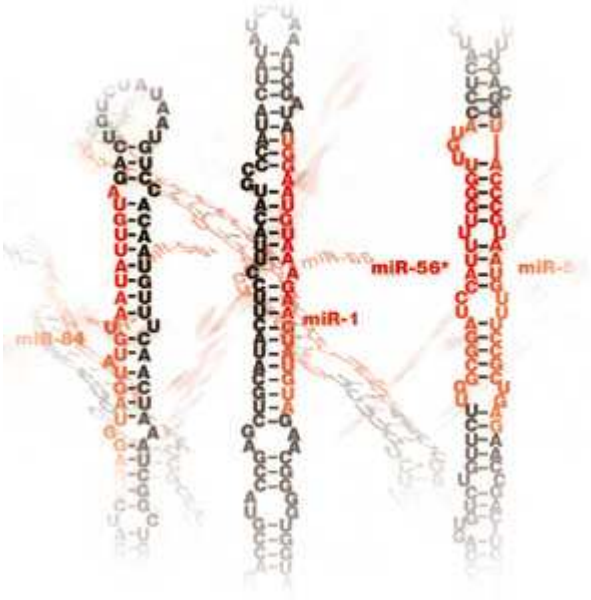
miRNAs in host–virus interactions



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Content

miRNAs in host–virus interactions

A new class of gene expression regulators has been identified[1], the so called microRNAs (miRNAs). miRNAs are small RNA transcripts encoded in genomes of helminths[2], plants[3], insects[4], etc. In mammals, their involvement has been suggested in many cell processes and also in anti-viral defence. Viruses also encode miRNAs[5] and these were shown to favour viral replication. This interaction will be subject of this review, discussing positive and negative aspects of both viral and host miRNAs in regard to virus-host interaction.

Cellular miRNAs

Biogenesis of miRNAs

MicroRNAs (miRNAs) represent a new class of ≈22 nucleotide long, non-coding RNA molecules found in genomes of many multicellular organimsm[6]. Firstly discovered in the nematode *Caenorhabditis elegans*[1], miRNAs were shown to play an important role in controlling gene expression.

Nearly ten years ago, predictions showed that 1% of the human genome encodes miRNAs[7], which are estimated to regulate 30% of the human genome[8]. Nowadays, more than 1000 miRNAs were identified in humans (miRBase, release 15.0, http://www.mirbase.org), encoded in various loci in the genome, ranking from introns of protein-coding genes to non-protein coding regions and exons[9]. About half of all mammalian miRNAs are found in clusters and transcribed from a single polycistronic transcription unit (PU)[10].

The generation of mature miRNAs involves several endonuclease steps (Fig. 1), depending where they are located in the genome. In the nucleus, intronic miRNAs genes are transcribed by RNA polymerase II, generating several kilobases long primary miRNAs (pri-miRNAs). These pri-miRNAs contain several stem-loops and show a polyA-tail as well as a cap structure[11]. Alternatively, RNA polymerase III is required for the transcription of the other (exonic) pri-miRNAs due to Alu repeats[12]. The transcripts are then further cleaved by the microprocessor complex composed of Drosha (RNase III) and DGCR8 (RNA binding protein) into a ≈70 nucleotide long precursor miRNA (pre-miRNA) with a step-loop hairpin structure[13, 14]. DGCR8 recognises the pri-miRNAs via their single-stranded (ss) RNA segments and their

stem loop of about 33 base pairs, and helps Drosha to cleave the transcript around 11 base pairs further from the double stranded (ds)– ss RNA locus[15].

The pre-miRNAs are then exported out of the nucleus by exportin-5, a ds-RNA-binding protein receptor in the nuclear membrane, together with its GTP-binding cofactor Ran. It recognises the 1-8 nucleotides long 3' overhang and the dsRNA stem and following GTP hydrolysis in the cytoplasm, the pre-miRNAs are released in the cytosol[16].

Subsequently, cytoplasmic RNAse III enzyme Dicer cleaves the pre-miRNAs into ≈22 nucleotide long ds RNA transcripts[17, 18], which are loaded onto proteins of the Argonaute family (Ago1-4). Ago together with Dicer and TRBP (TAR RNA-binding protein)[19] or PACT (a second RNA binding protein)[20] forms the RNA-induced silencing complex (RISC). Depending on thermodynamic stability of the two ends of the miRNA duplex, one strand remains bound to Ago in the complex (determined mature miRNA), whereas the complementary RNA strand is degraded (named miRNA*)[21].

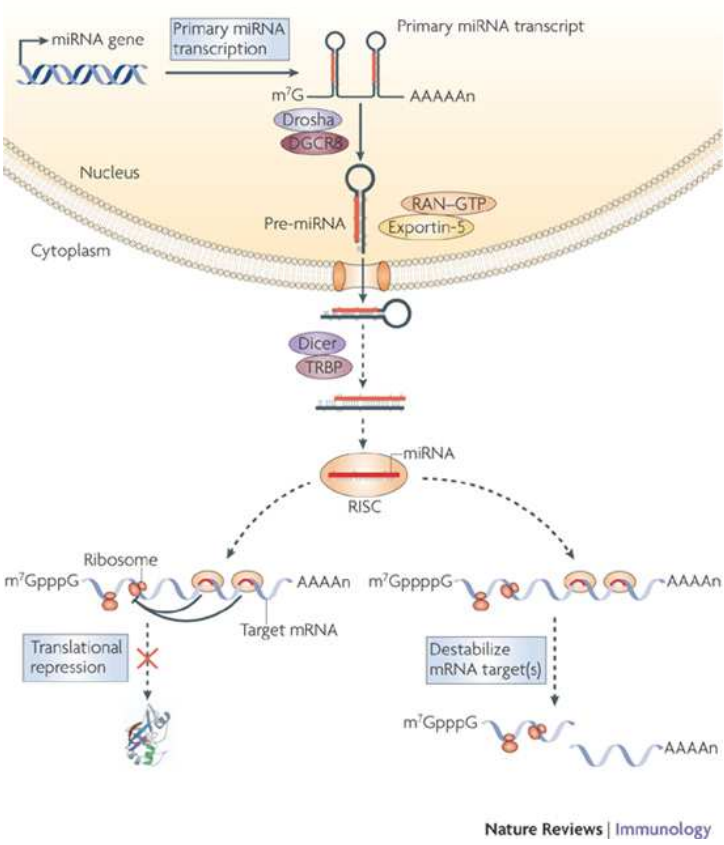

Fig. 1: miRNA biogenesis in humans. Adapted from Lodish et al. (2008)[22]

Function of cellular miRNAs

Firstly identified in nematode[1], small interfering RNAs (siRNA) were shown to suppress viral replication in helminths[2], plants[3] and insects[4]. In humans, miRNAs were shown to regulate many processes, ranging from cell differentiation and proliferation to cell death, and antiviral defence. Regulation by miRNAs is accomplished by targeting messenger RNAs (mRNAs). Specific sequences, which are most of the times located in the mRNA 3' untranslated region (3'UTR), are recognised and the complementarity of the miRNA and mRNA strands determines the outcome. Often, in order to bind to the target mRNA and exert its function, 6 nucleotides of the miRNA "seed region" (nt 2-7 from the 5' end) need to be complementary to the mRNA, but exceptions with less matching sequences also exist[8]. If the base pairing of the entire miRNA is a perfect match, this will result in mRNA degradation. In the case of incomplete complementarity, the mRNA is not destroyed but protein translation will be repressed. Several theories about the underlying mechanisms exist but the entire picture is not clear yet. So far, four main procedures can occur in each step of protein synthesis: initiation, elongation and termination. Firstly, the initial step of cap recognition or the joining of the 60S ribosomal subunit is blocked, resulting in inhibited start of protein synthesis. Secondly, the mRNA stand can be deadenylated (Fig. 1) and the mRNA is destabilised and degraded. Another way to block protein synthesis is the stop of the elongation step or the preliminary release of ribosomes. Finally, even after the synthesis start, proteins can be degraded by the miRNA complex either by direct interaction or by attracting other cell proteins[23]. Interestingly only the RISC complex containing Ago2 encodes a real endonuclease able to cleave the target mRNA, suggesting that miRNAs designated to destroy target mRNA are loaded onto Ago2[24]. However, despite the assumption that miRNAs only regulate mRNA translation by downregulating protein levels, it has recently been shown that a cellular miRNA is also able to upregulate translation. The cellular miRNA-369-3 is directing the association of translation activating signals, thereby inducing increased translation of cell cycle target mRNAs[25].

Viral miRNAs

Viruses have been shown to also encode miRNAs and hijack the RNA silencing machinery to interfere with the regulation of host and viral genes. Thus the biogenesis of viral miRNAs is usually the same as for cellular miRNAs, with some exceptions such as the miRNAs encoded by murine gamma-herpesvirus 68 (MHV68). These pre-miRNAs are first processed by tRNAse Z, due to their linked 5'tRNA moiety, and then by Dicer[26].

In 2004 the group of Pfeffer *et al*. firstly reported expression of viral miRNAs in Epstein-Barr virus infected human B-cells[27], nowadays more than 230 viral miRNAs are known (miRBase, release 15.0, http://www.mirbase.org). The advantage of miRNAs is their nonimmunogenicity compared to viral proteins for instance. Moreover, they can also be easily encoded even in small genomes due to their size. Thus miRNAs represent ideal tools for viruses to infect and persist in the host by targeting host and virus mRNAs.

Currently most miRNAs, their locations and functions were shown in Herpesviruses. These viruses are ds DNA viruses and consist of three subfamilies, of which 8 viruses are human specific (3 members of *Alphaherpesvirinae*, 3 of *Betaherpesvirinae* and 2 of *Gammaherpesvirinae*). Herpesviruses are able to establish and maintain latency, hence they require the ability to modulate gene expression of both the virus and the host. Depending of the viral life cycle, latent genes or lytic genes are expressed. Immediate-early genes regulate viral reactivation from latency, early genes are required for viral DNA replication and late gene expression is necessary for the accomplishment of viral particles[28]. It is thought that 25% of the herpes virus genome encodes proteins with immunmodulatory activity to evade the host's immune response and maintain latency[29]. However, considering the discovered viral miRNAs, the impact on host and viral protein modulation is even bigger and will be subject of this review.

Epstein-Barr virus (EBV)

EBV belongs to the subfamily *Gammaherpesvirinae* and is able to cause severe malignancies, such as Burkitt lymphoma, Hodgkin's disease and nasopharyngeal carcinoma. miRNAs were firstly identified in EBV-infected B cells[27], 25 encoding miRNA genes are known until now,

resulting in generation of 44 mature viral miRNAs[30]. They are primarily located in two clusters of the EBV genome. Three BHRF1 pre-miRNAs (miR-BHRF1-1 to miR-BHRF1-3) are located up and downstream the BHRF1 (BamHI fragment H rightward open reading frame 1) and result in four mature miRNAs. The second and bigger cluster is situated further upstream in the intronic regions of the BART (BAMHI-A region rightward transcript) gene and encodes at least 22 precurser miRNAs (miR-BART1 to miR-BART 22) leading to 40 mature miRNAs[31] (Fig. 2).

In order to get more insight in regulation of miRNA clusters, quantification of these miRNAs during the viral life cycle was performed. The BHRF1 miRNAs were only found in cells undergoing the lytic cycle or following to virus entry, whereas BART-miRNAs seem to be expressed during latency[32]. Interestingly, miRNAs are evolutionary conserved, implicating an important role for virus survival[5], and show homology with human miRNAs, suggesting that viruses hijacking this mechanism to control the host cell[31].

Kaposi's Sarcoma associated herpesvirus (KSHV)

Similar to EBV, KSHV is associated with lymphatic tumorgenesis, causing Kaposi's Sarcoma, primary effusion lymphoma and multicentric Castleman's disease. All 12 known miRNAs reside in the major latency-associated region in the genome. The transcription site of 10 pre-miRNA genes is located as a cluster within an intron lying between ORF71 and the protein encoding Kaposin (miR-K1 to miR-K9, miR-K11). The other two are encoded within the K12 open reading frame (miR-K12, miR-K10)[33] (Fig. 2). Expression patterns showed that the clustered 10 pre-miRNAs are not involved in the lytic cycle or viral replication, but more important for maintaining latency. In contrast, miR-K10 and miR-K12 are found increased in response to induction of the lytic cycle[33]. This shows that not only miRNA can modulate expression, but that also the miRNA expression is tightly regulated.

Human Cytomegalovirus (hCMV)

Human CMV can cause death in immunocompromised and in transplantation patients. It is also the leading cause of congenital birth defects. In contrast to the other viruses, the found miRNAs of CMV are scattered throughout the genome and were initially detected in

fibroblasts undergoing lytic replication. Firstly only 9 precursor miRNAs were identified[34], then additional two were found by Grey *et al*[35]. From these 11 pri-miRNAs, 14 mature miRNAs are generated (Fig. 2)[36]. Most of them are expressed as immediate early or early genes, suggesting that the function of these miRNA is associated with the lytic cycle and not latency.

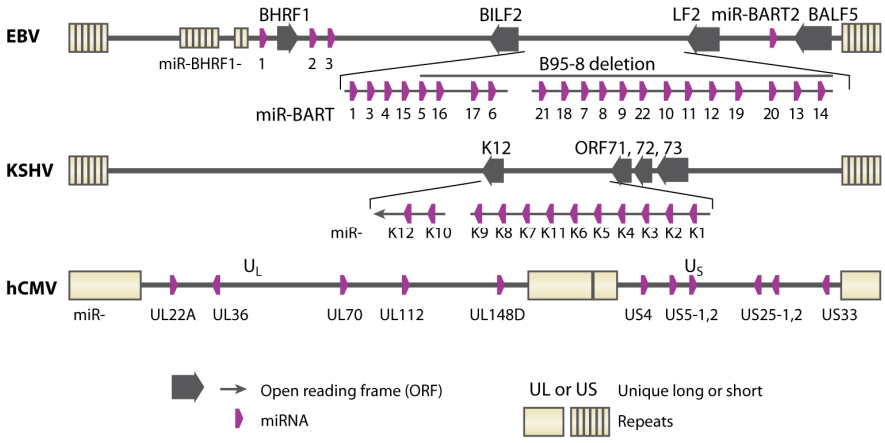

Fig. 2: Locations of miRNA genes in human Herpesviruses. Adapted from Skalsky and Cullen 2010[37]

Herpes simplex virus (HSV)

HSV-1 and HSV-2 belong to the group of *Alphaherpesvirinae* and their pathogenesis is due to cell damage of the lytic cycle and the resulting immune response. These viruses are also able to induce latency for a long time primarily in neurones. It has been hypothesised that miRNAs contribute to maintenance of latency[38], thus it is not surprising that most of the known miRNAs are encoded in genes associated with latency, i.e. noncoding latency-associated transcripts (LATs). Deep-sequencing revealed the existence of 16 and 17 miRNAs expressed by HSV-1 and HSV-2 respectively (Fig. 3). Interestingly, 9 of those miRNA sequences were found conserved between the two viruses, suggesting an important role in viral pathogenicity. Moreover a second family of miRNAs (miR-H11 to miR-H13) was found, encoded within the origins of replication[39]. However, the functions of the miRNAs still need to be further elaborated.

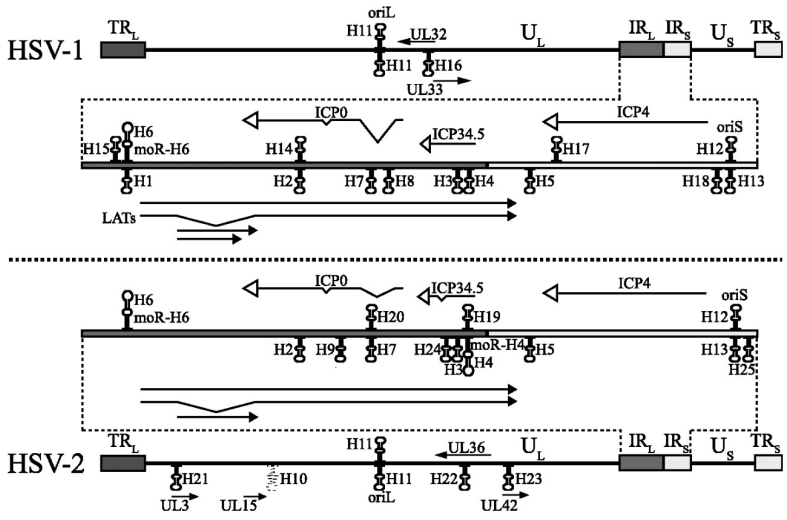

Fig. 3: miRNAs located within the HSV genome. Adapted from Jurak et al. 2010[39]

miRNAs as a tool for immune evasion

Considering the abundance of encoded miRNAs in viruses and humans, many of them have properties leading to viral replication and survival.

Prevention of apoptosis

Apoptosis represents one mechanism for the host's immune system to avoid viral spread and thus clearing of the infection. When cells undergo apoptosis, intracellular pathways lead to the programmed death of the cell and thus viral replication is shut off. To avoid this, viruses produce numerous proteins that target proapoptotic proteins of the host. Recently, the involvement of miRNAs has also been shown, further improving the immune evasive power of viruses.

Firstly, viral miRNAs were shown to specifically target cellular mRNAs to prevent apoptosis. EBV was shown to inhibit apoptosis by abundantly expressing miR-BART5. Bioinformatics analysis determined many cellular mRNA as targets. However, only p53 up-regulated modulator of apoptosis (PUMA) was suppressed by miR-BART5 in functional reporter assays. PUMA is a pro-apoptotic protein, which belongs to the "BH3-only" group of the Bcl-2 family, and it is in involved in p53-induced apoptosis. Hence, by targeting the mRNA of PUMA, miR-BART5 facilitates cell survival[40]. Moreover, the miRNAs from the BHRF1 locus seem to

protect from apoptosis early after infection. Infection with EBV with deleted BHRF1 miRNA cluster (as well as all miRNA loci) resulted in less cell survival compared to wild type[41].
KSHV miRNA miR-K10a was shown to target the 3'UTR of TWEAKR (TNF-like weak inducer of apoptosis receptor) and to downregulate directly its expression. TWEAKR is abundantly expressed on many cell surfaces. Binding of TWEAK (TNF-like weak inducer of apoptosis, also known as CD255) can lead to induction of apoptosis. In miR-K10a knock-down assays, TWEAKR protects cells from TWEAK-induced apoptosis. However this was not the only effect, additionally decreased expression of proinflammatory cytokines was observed, further reinforcing the immune evasive effect of viral miRNAs[42].

Retroviruses have been shown to encode only three miRNAs (miRBase, release 15.0, http://www.mirbase.org), but it should be noted that identification of those miRNAs could not be repeated by other groups. One of the miRNAs is located at the 5' end of the TAR transcript and has been shown to protect infected cells from apoptosis. It down-regulates cellular genes involved in the apoptotic process, among others ERCC and IER3[43]. However, no direct 3'UTR binding was detected by the miRNAs in this study, so further evidence is needed to prove that also HIV-1 miRNAs are directly involved in suppression of pro-apoptotic protein expression, thus favouring viral infection.
So it can be concluded, that viral miRNAs target mRNA of proteins important in pro-apoptotic signalling, thereby preventing apoptosis. On the other hand, viral infection can result in a different pattern of cellular miRNAs, which may also be beneficial for the virus by preventing apoptosis.
The profile of miRNA expression in human T-cell leukaemia virus-1 (HTLV1) infected cells and HTLV1 patients revealed the upregulation of many cellular miRNAs. miR-93 and miR-130b were both found increased in the cell line as well as in the patient samples. Bioinformatics approaches showed that the 3'UTR of the tumor suppressor protein TP53INP1 (tumor protein 53-induced nuclear protein 1) is targeted at two sites by miR-93 and miR-130b. Knock-down of both miRNAs resulted in increased TP53INP1 protein levels and increased apoptosis[44]. So by creating an environment with increased cellular miR-93 and miR-130b, HTLV1 succeeds in preventing apoptosis.
In opposite to just mentioned HTLV1, it is known how human papilloma virus (HPV) prevents induction of apoptosis. It expresses the viral oncoprotein E6, which decreases the cellular

miR-34a expression by destabilising p53, a tumor suppressor protein leading to apoptosis. Induced ectopic expression of miR-34a resulted in decreased cell growth and increased apoptosis[45]. So here two effects can be observed. The viral production of E6 directly targets pro-apoptotic protein and also modulates the cellular miRNA profile in order to avoid apoptosis.

Prevention of Natural killer (NK) cell and cytotoxic T-cell (CTL) recognition and killing

Another efficient mechanism to clear virus infection is the activation of two other cell types, NK cells and $CD8^+$ CTLs.

CTLs are activated via two signals, the T-cell-receptor binding to the antigen presented on MHC I and costimulation by the CD28-B-7 interaction. Activation results in release of cytotoxic enzymes, which enter the target cell and activate the caspase cascade leading to apoptosis. A second way to induce apoptosis is via FAS ligand-FAS binding. In opposite to CTLS, NK cells recognise their target cells by the "missing" MHC I molecule on the cell surface. They also contain cytotoxins and release these granzymes, which will kill the cell either by the formation of pores in the cell membrane and subsequent osmotic cell lysis, or by also inducing apoptosis. Thus recognition of infected cells by NK and CTLS represents a crucial step in clearing a virus infection.

Viral miRNAs were shown to inhibit recognition of NK cells and CTLs. Firstly in 2005, Sullivan *et al.* showed for the first time that miRNAs of Simian virus 40 (SV40) is able to reduce susceptibility to CTLs. The miRNAs are perfectly complementary to early transcribed viral mRNAs, which results in cleavage of the mRNAs and consequently less protein synthesis. This leads to reduced synthesis of viral large and small T cell antigens (LTAg, stAg), but did not affect the viral replication. Looking at CTL-mediated lysis of cells infected with the wild type virus and a miRNA-mutated version, a significantly lower susceptibility of the wild type was observed, suggesting that miRNAs protect from CTL recognition by inhibiting viral T cell antigen synthesis[46].

The hCMV encoded miRNAs target not viral but cellular mRNAs to prevent recognition of infected cells by NK cells. hCMV miR-UL112 was predicted to target the major histocompatibility complex class I-related chain B (MICB) gene. Functional analysis showed

that indeed MICB expression is downregulated during hCMV infection. Since MICB represents a ligand for NK cell receptor NKG2D, reduced binding and subsequent killing by NK cells could also been observed[47]. Confirming the efficiency of this mechanism, the same group discovered that miRNAs of other herpesviruses, such as KSHV (miR-K12-7) and EBV (miR-BART2-5p), also target MICB and prevent NK recognition and killing[48].
The two cellular miRNAs miR376a and miR-433 represent cellular players regulating MICB expression in cells. Interestingly, miR-UL112 and miR-376a were shown to act synergistically, probably by targeting the MICB mRNA at proximate sites and thereby inhibiting translation of MICB[49].

Establishment of latency

Finally, the establishment of latency is one of the pathogenicity factors of herpesviruses, such as HSV, which belongs to the family of *Alphaherpesvirinae*. Its life cycle is characterised by two phases, the lytic phase and the latent phase. In the lytic phase, the immediate-early (IE) genes are transcribed and expressed, stimulated by the tegument protein VP16. The IE proteins are then transported into the nucleus and induce the expression of early (E) genes, which are important for the viral DNA replication. This in turn drives late (L) gene expression and the synthesis of viral structure proteins[50]. Thus new viral particles are produced to infect more cells. After a primary infection, the virus can hide in the sensory neurons and no infectious virus levels are detectable. This stage is called latency and represents the other side of the HSV life cycle. During the stage of latency, the only locus transcribed is the LAT locus, which also encodes several miRNAs (Fig. 3). The exact functions of the LAT transcripts are not entirely known yet, but so far they are attributed to inhibition of apoptosis, repression of lytic gene products and chromatin assembly[39]. Under certain circumstances, HSV can reactivate and start the lytic cycle again, thereby causing recurrent infections.
HSV-1 expressed among others the miRNAs miR-H2-3p and miR-H6, which target both viral proteins ICP0 and ICP4, respectively. Both proteins are important for the exit of latency. miR-H2-3p is encoded in the antisense orientation of ICP0, which represents a transcription factor important for viral replication. Curiously, despite complete complementarity of mRNA and miRNA, expression of ICP0 is downregulated by inhibiting the translation but not cleavage of mRNA. The precursor RNA of miR-H6 lies upstream of the LAT locus and shows

extended complementary base pairing to ICP4, a second important transcription factor for viral replication, and interestingly also to lately expressed miR-H1[38]. Thus, viral miRNAs are important to inhibit the expression of certain viral proteins, in order to establish latency. Moreover, hCMV was shown to encode miRNAs suppressing immediate-early genes to enter and maintain the latent stage. Especially miR-UL112-1 seems to be involved by targeting the 3'UTRs of 14 potential viral transcripts[35]. Functionally it has been shown by two groups, that miR-UL112-1 inhibits the expression of immediate early protein IE72 (also called UL123 or immediate-early protein 1), which is important for the induction of early and late gene expression and required for replication[35, 51]. Additional miRNAs were identified to have similar effects. miR-US25-1 and miR-US25-2 were also shown to reduce viral replication of hCMV. Moreover, the ectopic expression of these miRNAs also resulted in lower viral titers of other CMV strains[52], suggesting that this represents a common efficient mechanism.
Additionally to modulating viral protein expression, viruses use cellular miRNA to induce latency to evade elimination. However, the underlying mechanism by which the virus (or the host) induces the transcription of those miRNAs remains unclear.
For instance cellular miRNAs of the miR-200 family (miR-200a,b,c ; miR-429 and mir-141) are regulating the induction into the lytic cycle of cells infected with EBV. In these cells, miR-200b and miR-429 were found downregulated, which negatively correlated with the levels of their target, the cellular transcription factors ZEB1 and ZEB2. Addition of those miRNAs led to lytic reactivation of EBV[53]. Thus, by inhibiting expression of cellular miR-200 family members, EBV induces expression of ZEBs, thereby inducing the maintenance of the latency.

Hepatitis C virus (HCV) uses cellular miRNA for efficient replication

Certainly another 'aim' of the virus in infecting cells is the production of viral particles, which then in turn can infect more cells. Cellular miRNAs were also shown to positively influence the viral infectivity and thus the viral spread. Hepatitis C virus (HCV) seems to be a master using this pathway to facilitate virus genome replication. miRNA-122 is abundantly expressed in liver cells and was found to bind the 5' noncoding region of the HCV genome. Surprisingly, activation of miR-122 resulted in HVC replication[54] and not, as assumed, in destruction or repression of transcription. This is so far the only report of a cellular miRNA directly facilitating virus genome replication and the mechanism behind it is still not entirely

understood. Suggestions include that the direct binding could stimulate internal ribosome entry sites (IRES)-mediated translation[55], however the first study concluded that miR-122 binding does not affect mRNA translation[54].

Another cellular miRNA is miR-141, which was shown to target the tumor suppressor gene DLC-1. miR-141 has been found upregulated in HCV infected cells, in accordance with found decreased levels of DLC-1. Depletion of miR-141 and respective increased DLV-1 levels, resulted in inhibited viral replication. Thus miR-141 repression is necessary for efficient HCV replication[56].

Finally, profiling cellular miRNAs in cells of acute HCV infection revealed that also miR-24, miR-149, miR-638 and miR-1181 were overexpressed in HCV infected cells and that they are involved in entry, replication and propagation of HCV[57].

Evasion of cell surveillance

The cytokine environment plays an important role in control and elimination of viral infections. Especially T-cells, but also other cells such as macrophages, neutrophils and B-cells, are attracted to site of infection by chemokines. Following a concentration gradient, the cells find infected cells and get activated. Evolutionarily seen, viruses found several ways to interfere with this process on a protein level, however recently also miRNAs were found to be involved. On one hand viral miRNA directly target chemokine mRNAs, on the other, the interferon stimulation due to the viral infection results in upregulation of cellular miRNA miR-146, thereby creating a favourable environment for the virus to evade immune surveillance.

For instance, EBV encodes miR-BHRF1-3, which was shown to target the mRNA of cytokine CXCL-11. CXCL-11, also called Interferon-inducible T-cell alpha chemoattractant (I-TAC), represents an important chemoattractant for T-cells. Inhibition of EVB-mir-BHRF1-3 using antisense oligos correlated with increased protein levels of the cytokine[58]. Despite no functional assays, this shows that EBV directly suppresses cytokine production and thus evades immune surveillance.

Another option is the activation of the NF-κB pathway by interferon stimulation, thereby inducing cellular miR-146a. Several viruses, such as EBV[59],KSHV[60] and HIV[61], infected cells show significantly upregulated levels of miR-146a. Interestingly miR-146a targets two

important cytokine players: the α-chemokine receptor CXCR4[60] and the cytokine CCL8/ Monocyte chemoattractant protein(MCP)-2[61]. CXCR4 is found on the cell surface of many blood cells and binding of chemokine CXCL12/SDF-1 results in attraction and activation of the cells. The biological consequences of such a receptor downregulation by overexpression of miR-146a have not been demonstrated yet in functional assays, but one could speculate that it could also result in impaired chemotaxis and thus increased virus survival. The chemokine CCL8/MCP-2 represents another target of the 3'UTR of miR-146a and overexpression of this cellular miRNA resulted in decreased secretion of MCP-2. Since MCP-2 is an important activator of leukocytes[62], again one could think about evasion of immune surveillance by inhibiting attraction and activation of host cells. Finally, CCL8/MCP-2 is also involved in inhibiting HIV entry and replication, thus its downregulation enhances infectivity[61].

miRNAs and tumorgenesis

Finally, viruses can ensure their persistence in the host by inducing cell survival and the development of immortal cells. The first evidence for involvement of miRNAs in the development of cancer came from a study by Calin *et al.* in 2002. They found that in cells of patients with chronic lymphocytic leukaemia (CLL) tumor-suppressive miRNAs miR-15 and miR-16 were significantly downregulated or deleted due to chromosomal deletions[63]. More recently, more miRNAs were identified and the most attention is turned to miR-155. Mapped within the B-cell integration cluster (BIC) on chromosome 21, miR-155 is not only involved in cancer but also in other biological processes such as inflammation, immunity and haematopoiesis (for details see review by Faraoni *et al*. 2009[64]). One important finding for the involvement of miR-155 in immunity is its target protein activation-induced cytidine deaminase (AID). This enzyme is responsible for high frequency mutations involved in somatic hypermutations and the development of a broad high-affinity IgG repertoire[65]. In miR-155 deficient mice, reduced amounts of IgM were produced and impaired capability of switching antigen-specific antibodies was observed, suggesting that miR-155 is required for normal immune functions[66].

In cancer cells, overexpression of miR-155 in pancreatic ductal adenocarcinoma results in a decrease of TP53INP1[67], a tumor suppressor protein, which is also targeted by miR-93 and

miR-130b. These two last miRNAs were found to be induced in HTLV1 infected cells[44], thereby suppressing apoptosis. Thus, viruses use several ways to induce a miRNA environment that is on one hand favourable to their replication and on the other hand contributes to the development of cancer.

Moreover, miR-155 was found increased in patients with Burkitt's Lymphoma[68], a B-cell cancer which development is also associated with the infection with EBV. Also other B-cell malignancies also come along with high miR-155 levels, such as Hodgkin's lymphoma and several types of Non-Hodgkin's lymphoma[64]. Even in bone marrow cells of patients with subtypes of acute myeloid leukaemia[69] and many solid tumors[64] this was observed, suggesting an important role of this miRNA in cancer. miR-155 directly targets many mRNAs of proteins involved in regulation of transcription, protein receptors, kinases, nuclear proteins and binding proteins[64], which could explain its strong oncogenic potential. Especially ten genes involved in myeloid hyperplasia and haematopoiesis were downregulated to only 30% expression levels in a myeloid cell line transfected with miR-155. These cells also displayed neoplastic features[69].

Furthermore, miR-155 was found increased expressed in primary B-cells after EBV infection, and Epstein-Barr virus (EBV)-encoded latent membrane protein-1 (LMP1) seems to be involved in its activation via the NF-KB pathway[70]. miR-155 and EBV directly target genes, but also act indirectly by altering gene expression by interfering with signal transduction pathways[71]. Suppression of miR-155 activity in two lymphoma cell lines resulted in attenuated growth, which is probably due to difficulties of the cells to go from the G1 to the S phase and further progression into apoptosis[72]. Thus miR-155 is important for cell cycle progression, which could also be an important step for the formation of cancer and viral replication.

Interestingly, several other viruses seem to encode orthologous miRNAs of cellular miR-155, which could have several reasons. It appears that the miR-155 pathway is favourable to the viral life cycle and the side effect is the formation of cancer of virus-infected cells. The oncogenic Marek's disease virus miRNA-M4 shows perfect seed sequence to the chicken miR-155 and targets the same genes, such as PU.1 and C/EBPβ, important transcription regulators[73]. KSHV encoded miR-K12-11 shares also perfect seed complementarity with cellular miR-155 and they were both shown to regulate a set of common genes[74, 75]. This

shows that viruses developed another strategy on a different level to interfere with the host cell regulating processes, which may lead to the formation of malignancies.

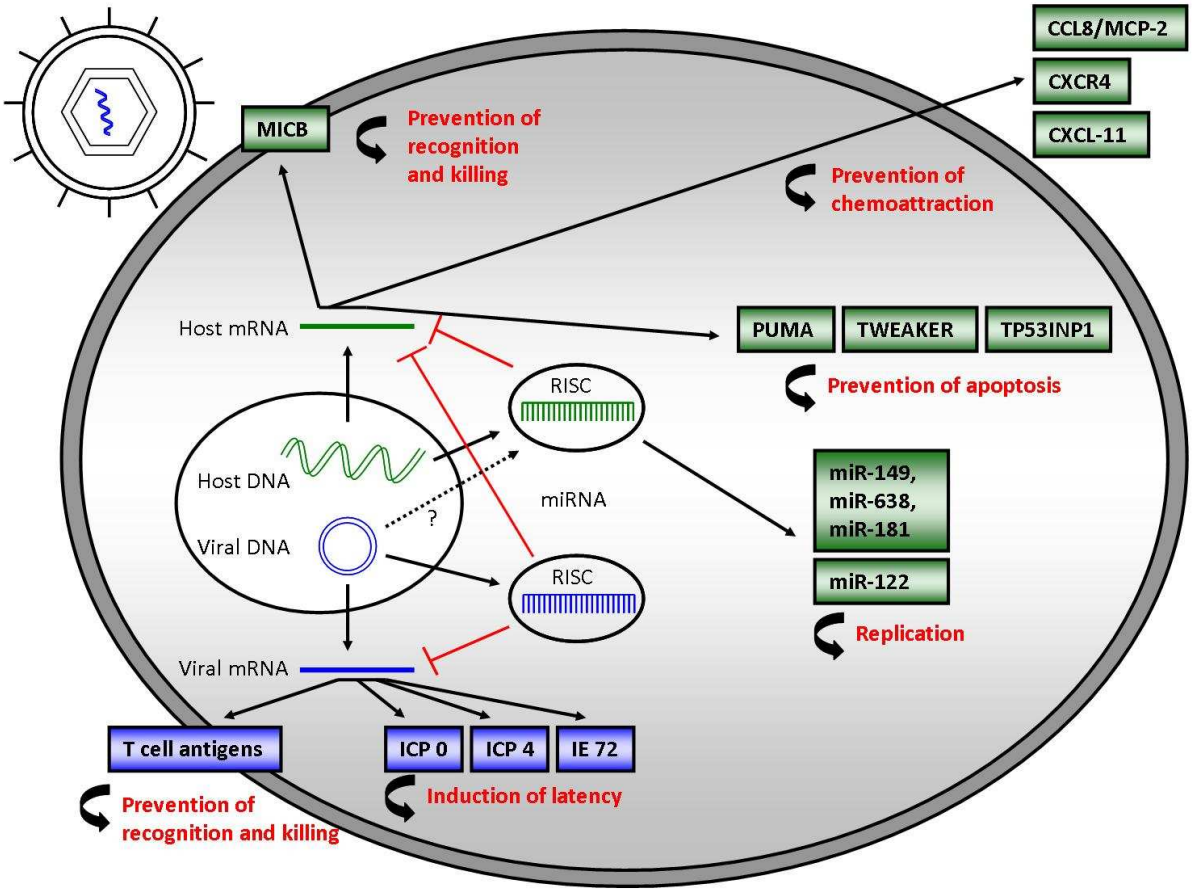

Fig. 4: Viral immune evasion mechanisms by inducing both viral and cellular miRNAs. Viral miRNAs target the host and viral mRNA or regulate expression of cellular and viral miRNAs in order to favour viral replication and evade recognition and killing. Adapted from Boss and Renne, 2010[76]

Summarising all the findings (Fig. 4) one can conclude that viral encoded miRNAs target either viral or cellular mRNAs, or induce expression of cellular miRNAs, which favour the survival of the virus. Moreover, viruses can also ensure their persistent presence by inducing immortality of cells by viral miRNAs. Moreover they use cellular pathways by mimicking cellular miRNAs to their benefits.

Detrimental cellular miRNAs – inhibition of viral replication

In general, the expression profiles and functions of cellular miRNAs are not entirely understood, which is also due to the complexity of cellular maintenance and regulation. Demonstrated so far, cellular miRNAs have important roles in cell development, apoptosis, cancer and especially the tool of RNA interference (RNAi) is very attractive to cure diseases[77]. Another interesting aspect of miRNAs is their involvement in the immune response, especially in regard to virus infections. Many studies tried to generate miRNA profiles[34, 78, 79], but despite found altered expressions of multiple cellular miRNAs, it seems that a virus infection does not result in a general anti-viral profile[80]. Using microarray techniques, it was found that the majority of both up- and down-regulated miRNAs target genes involved in inflammation and immune response, as well as genes of the nucleotide metabolism and cell cycle/cell death[79].

Direct targeting by cellular miRNAs inhibits viral replication

Despite the yet undiscovered complexity of cellular miRNA induction after viral infections, direct effects of cellular miRNAs on viral replication were shown for some miRNAs.
The first to prove the miRNA silencing effect was the group of Lecellier *et al.* in 2005. Using reporter assays with fused GFP proteins to the sequences of the viral genome and cell transfections, they identified the cellular miR-32 as a potent inhibitor of retroviral primate foamy virus type 1 (PFV-1) accumulation in human cells. miR-32 was strongly predicted to target a sequence that is found within the 3' UTR of all PFV-1 mRNAs. Its potential target sequence is located in the ORF2 of the viral genome, which is transcribed for expression of all viral proteins necessary for replication and viral structure (namely Gag, Pol, Env, EnvBet, Tas and Bet). Despite no further detailed analysis, silencing miR-32 using antisense locked nucleic acids resulted in enhanced virus production[81].
Several other miRNAs were shown to be important during infection with HIV. Huang *et al.* reported that miR-28, miR-125b, miR-150, miR-223 and miR-382 were upregulated in infected $CD4^+$ T-cells. These cellular miRNAs target the 3'end of HIV mRNA and thereby inhibit HIV production. Individual silencing of these miRNAs resulted in a rather modestly inhibited viral particle production, but a combination of all five inhibitors had a dramatic effect[82]. This suggests that the miRNA silencing network acts rather synergistically than

individually in order to elicit a strong response. Another cellular miRNA cluster was shown to be important in HIV infection. In opposite to above mentioned upregulated miRNAs, the miRNA cluster miR-17/92, comprising miR-17-(5p/3p), miR-18, miR-19a, miR-20a, miR-19b-1 and miR-92-1, is suppressed by HIV. It was shown that suppression was necessary for efficient viral replication, suggesting antiviral properties of the miRNA cluster. Interestingly those miRNA were not predicted to directly target the virus, but to have an indirect effect by targeting cellular mRNAs. Indeed, it was found that the miRNAs if this cluster inhibits protein synthesis of a histone acetylase, which is an important cofactor for TAT in HIV replication[83]. It should be noted that it is unknown how the expression of cellular miRNAs is induced and how it regulates and contributes the viral replication for many miRNAs. Especially for HIV, induction of inhibitory miRNAs could also be beneficial for the maintenance of viral latency[82].

Hepatitis virus infection is another example, where miRNAs were found to inhibit particle production. Using antisense oligonucleotides to known human miRNAs, silencing of miR-184, miR -185, miR -196a, miR -199a-3p, miR -210 and miR -217 showed enhanced expression of Hepatitis B antigens. Subsequent bioinformatics analysis revealed that miR-199a-3p and miR-210 feature binding sites in the HBV genome. miR-199a-3p and miR-210 both target the HBV surface protein and the polymerase and they both degrade and inhibit translation of these target mRNAs. Each miRNA effect alone is sufficient and knock-down of both miRNAs did not result in increased Hepatitis B virus expression[84], showing a strong direct cellular response against the virus. Interestingly, miR-199a-3p also plays an important role in Hepatitis C virus infection. The same effect, namely the inhibition of viral replication was also observed for HCV. Here it was shown that miR-199a-3p targets the internal ribosomal entry site (IRES) of two HCV genotypes[85], suggesting that this miRNA represents an important factor against several viral infections.

Cellular miRNAs miR-24 and miR-93 were shown to have binding sites in the genome of vesicular stomatitis virus (VSV). Overexpression of both miRNAs resulted in 50% suppressed VSV replication, miR-24 and miR-93 targeting the viral RNA-dependent RNA polymerase (L protein) and the phosphoproteins (P protein) genes, respectively. These genes encode elements essential for polymerase binding and for signalling replication and transcription. In mice infected with VSV lacking the binding sites for both miRNAs, increased pathogenicity was observed for the mutants compared to the wild type, further reinforcing the impact of

miRNAs in clearing infections *in vivo*[86]. It should be mentioned that this is one of the few *in vivo* studies with miRNA.

Recently, cellular miRNAs against influenza have been discovered. Using 3'UTR reporter assay, it was found that the PB1-5 gene, encoding a polymerase subunit, may be targeted by miRNAs. The three endogenously expressed miR-323, miR-491 and miR-654 showed the same binding sites for PB1, which represents a highly conserved region across many influenza strains. Despite incomplete complementarity of the miRNAs to their target, binding results in mRNA degradation. Antisense miRNA oligonucleotides against the miRNAs inhibited the viral replication. Again no difference was observed if the individual miRNAs were silenced or all three together[87].

In conclusion, it can be seen that cellular miRNAs can have direct detrimental effects on the virus replication.

miR-193b induces apoptosis

Another way to inhibit viral replication is the induction of apoptosis, because the cellular machinery cannot be used anymore for the production of new viral particles. Profiling of HCV infected cells revealed many up- and downregulated miRNAs. Among others miR-193b was found in four fold increased in HCV infected cells compared to control cells. miR-193b targets the anti-apoptotic protein Mcl-1 and indeed lower levels of Mcl-1 were found in miR-transfected cells. Moreover, these cells were more sensitive to drug-induced apoptosis, showing that miR-193b could potentially be used to combat viral infection[88].

Interferon-mediated signalling induces miRNAs, which inhibit viral replication

Interferons belong to the important group of cytokines, which are released by leucocytes upon infections. Due to their antiviral activity and the induction of apoptosis in virus-infected cells, the interferon pathway is very attractive for curing diseases. Upon interferon binding to the cell receptor, the JAK/STAT cascade is activated leading to transcription of many pro-inflammatory genes. Other pathways and signalling cascades are activated via Toll-like receptor (TLR) stimulation, but the entire mechanism and how the pathways are influencing each other, is not fully understood yet. The induction and influence of miRNAs in

this context is even a greater mystery, nevertheless their involvement in immune cell development and function has been accepted[89].

Pedersen *et al*. showed that interferon-β (IFN-β) treatment induces a different cellular miRNA profile. Especially miR-1, miR-30, miR-128, miR-196, miR-296, miR-351, miR-431 and miR-448 were significantly upregulated in human hepatoma cell line as well as primary murine hepatocytes. Interestingly, those miRNAs were predicted to have targets in the HCV genome by nearly perfect complementarity. Indeed miR-196, miR-296, miR-351, miR-431 and miR-448 decreased HCV replication by more than 50% in transfected cells. The mixture of all five miRNAs even reduced viral replication by more than 80%, suggesting that the inhibition is probably rather due to a common mechanism than an individual effect. miRNA kinetics demonstrated that induction of miRNAs occurs very fast, the peak is reached after 30 minutes after interferon treatment. Furthermore, interferon treatment also results in decreased expression of the pro-viral cellular miR-122[90]. These results show a rapid interferon-dependent induction of cellular miRNA expressions that leads to inhibited viral replication.

TLR and cytokine stimulation results in induction of cellular miRNAs, especially miR-155 and miR-146a were highly expressed in viral infected cells[91]. miR-155 was found ten fold increased in two different RNA virus infected splenocytes and bone barrow-derived dendritic cells. Upregulated miR-155 resulted in suppressed viral replication in macrophages and inhibition of miR-155 facilitated the VSV replication. Computational analysis revealed that miR-155 does not directly target VSV RNA directly, thus it was thought to act positively through the raised IFN signalling response. Looking at the interferon production on protein and mRNA level, no difference was seen if miR-155 was either overexpressed or inhibited. However, a significant induction of phosphorylation of downstream STAT1 was observed if miR-155 was overexpressed. Further investigations demonstrated that miR-155 targets SOCS1, a potent type I interferon signalling inhibitor and thereby indirectly enhances the anti-viral state of the cell[91].

Cellular miRNA	**Target**	**Biological significance**
miR-32	3’ UTR of PFV-1 mRNA	Inhibition of viral replication
miR-28, miR-150, miR-223, miR-382	3’ UTR of HIV mRNA	Inhibition of viral replication
miR-17/92	Cellular histone cyclase	Inhibition of viral replication

miR-210	HBV surface protein and viral polymerase	Inhibition of viral replication
miR-24, miR-93	VSV polymerase and phosphoprotein	Inhibition of viral replication
miR-323, miR-491, miR-654	Influenza polymerase	Inhibition of viral replication
miR-199a-3p,	HBV surface protein and viral polymerase, IRES of HCV mRNA	Inhibition of viral replication
miR-193b	Cellular Mcl-1	Induction of apoptosis
miR-196, miR-296, miR-351, miR-431, miR-448	HCV genome	(Interferon mediated) Inhibition of viral replication
miR-155	SOCS1	Enhance interferon signalling

Table 1: Anti-viral cellular miRNAs.

In summary it can be concluded that humans also developed miRNA strategies to fight a viral infection. By either direct targeting the viral mRNA of the viral genome or by downregulating cellular targets, the host creates an antiviral response, which finally leads to inhibition of replication (Table 1). However, there is not much known so far and this represents just the start of new discoveries.

Discussion

miRNAs are a small class of molecules found in helminths[2], plants[3] and insects[4]. These ≈22 nucleotides long RNAs are encoded in exons and introns of non-protein coding genes and protein-coding genes in humans, representing an important part of the genome[7]. During miRNA biogenesis, longer RNA transcripts undergo several enzymatic steps to finally be loaded into the RISC complex, where they can exert their roles, i.e. the regulation of gene expressions. If the miRNA is perfectly complementary to the target mRNA, the mRNA transcript is destroyed and in case of incomplete complementarity, the translation is repressed[23]. In any case the mRNA will not be processed for protein synthesis. Interestingly, recent discoveries show that cellular miRNAs are also able to upregulate translation of proteins, but further research is necessary to back up this hypothesis[25]. Many miRNAs have also been identified in viruses, especially DNA viruses. They use the human miRNA biogenesis machinery to silence either viral or human mRNAs in order to regulate the cell status for their benefits. The advantage of DNA viruses is the direct silencing of viral mRNAs

by encoding antisense miRNAs in the viral genome, which may be the reason for not many found RNA virus encoded miRNAs.

In virus-host interaction miRNAs play also an important role. Many viral miRNAs were found to target cellular mRNAs, which are involved in apoptosis, cell recognition and the establishment of latency. The translation of anti-apoptotic proteins inhibits induction of cell death and thereby favours the cell and thus the viral survival. Cellular receptors are less abundantly expressed, which prevents the recognition and killing by NK cells and CTLs. Furthermore, the cytokine environment is suppressed by viral miRNAs. This results in less cell attraction and again increases the chances of viral survival. Moreover, viruses, such as HCV regulate the expression of cellular miRNAs, which directly promote viral replication. Finally, viral miRNAs can also target viral mRNAs to silence expression of viral proteins. Thus less potential viral antigens, which may be presented at the cell surface, are present and targeting viral proteins is also a way for the virus to induce the state of latency. Due to no production of viral proteins, the virus evades recognition and killing. Finally, the immortalisation of cells is a way for long term viral persistence. By inducing expression of cellular miR-155, virus-infected cells may become cancer cells. Additionally, some viruses encode viral miRNAs owing the same seed sequence as miR-155. By mimicking the biological function of miR-155, the virus interferes with many pathways, which are beneficial for its replication.

However, some cellular miRNAs were found to counteract the viral infection. Despite not many studies, it seems that cellular miRNAs are induced upon virus infection that directly target viral mRNA or indirectly regulate cellular miRNA expression targeting cellular mRNAs. But it should be noted that it is often not known, how the expression of cellular miRNAs is induced or suppressed. On one hand this could be a direct effect induced by the virus, or more likely, is a consequence of a complex mechanism of induction/repression and up-/downregulation of many cellular genes and proteins. Which miRNA are involved in which pathways still remains to be investigated, which is not an easy task due to the complexcity. Thus only small pieces will be enlightened and it will probably take a lot of time to understand the entire picture.

The technique of microarray analysis represents one important research tool for profiling. In both studies of Huang[82] and Triboulet[83], HIV-1 infected cells were profiled for their miRNA

expression. Since two cell types were used it is not surprising that the profiles were different. However, if the virus directly affects cellular miRNAs, at least some similar miRNAs expressions should have been found but this was not the case. Thus it is more likely that not the virus itself regulates the cellular miRNA expression. This also shows that the entire profile is not virus-specific and that in fact not much is really known about cellular miRNAs in respect to virus infections.

Technically, the step after finding the significantly differently expressed cellular miRNAs is the computational analysis with different programs, which are developed to reveal target sites. Most of the time binding sites to many sites in the genome are predicted, but it is not sure whether they are biologically relevant and more research is necessary to define all targets in functional assays. If theoretically a miRNA targets a cellular mRNA, it is not certain that it does so *in vivo*. In fact this is another important factor to realise. There are only low amounts (only one found in this review) of *in vivo* studies and most experiments are transfection experiments into cells in order to find miRNA functions. Only in some studies the effects of naturally occuring miRNAs are proven *in vitro*. Another interesting technique is the miRNA silencing, which could indeed be used as a diagnostic tool to cure diseases. Hereby antisense miRNAs (antagomirs) are used to inhibit the activity of miRNAs.

Compared to cellular miRNAs, function and expression of viral miRNAs are a bit better defined and understood. This could be due to the smaller amount of found miRNAs and the miRNA knock-out viruses represent a feasible technique to study them.

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