MASTER THESIS

UMC Utrecht

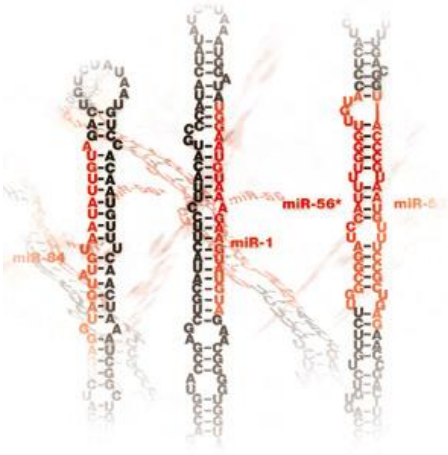
Universiteit Utrecht

Viral microRNAs

EBV and KSHV exploit microRNAs to modulate both viral and cellular processes

Florine Scholte

30-Jul-10



Florine Scholte
Student Biomedical Sciences
Biology of Disease
Utrecht University
Student nr: 0448303
F.E.M.scholte@students.uu.nl

Supervisors: prof. E. Wiertz
M. Hooykaas
Medical Microbiology
Experimental Virology
University Medical Center Utrecht
E.Wiertz@umcutrecht.nl
M.J.G.Hooykaas@umcutrecht.nl

Voorwoord

De scriptie die nu voor u ligt vormt mijn master thesis in het kader van de master Biomedical Sciences, masterprogramma 'Biology of Disease' te Utrecht.
Deze scriptie heb ik geschreven onder begeleiding van professor E. Wiertz en mevr. M. Hooykaas (aio) van de afdeling experimentele virologie, Universitair Medisch Centrum Utrecht, aan de universiteit Utrecht. Deze scriptie representeert 7.5 ECTS en vormt zo mijn master thesis. In deze scriptie beschrijf ik hoe de herpesvirussen Epstein Barr virus (EBV) en Kaposi's Sarcoma associated Herpes virus (KSHV) gebruik maken van zelfgecodeerde microRNAs om hun gastheer te beïnvloeden ten einde hun infectie en replicatie zo succesvol mogelijk te laten verlopen. Hierbij wil ik graag mijn supervisor, prof. E. Wiertz en Marjolein Hooykaas, bedanken voor de uitstekende begeleiding gedurende deze periode.

Florine Scholte

Utrecht, 30 juli 2010

Contents

MiRNAs are a class of small regulatory RNAs that have been shown to be utilized by plants and mammals as well as viruses. Via binding to the 3' UTR of target mRNA microRNAs can specifically induce degradation or translational inhibition. Gammaherpesviruses encode the most viral miRNAs, with 25 pre-miRNAs for EBV and 12 for KSHV, the latter being the best characterized at the moment. Viral miRNAs can target both cellular and viral transcripts. The viruses exploit miRNAs to optimize the cellular environment for infection and replication; miRNAs have been shown to play a role in maintaining the latent stage and modulating the host immune response. Viral microRNAs are thought not to be necessary for infection but are involved in protecting the infected cell. Viral targets include the EBV DNA polymerase BALF5 to prevent accidental induction of viral replication and the viral proteins LMP1 and -2a to promote cell survival and evasion of the immune system and the RTA latent-lytic switch of KSHV. Cellular miRNA targets include MICB, which plays a role in immune recognition of infected cells (EBV & KSHV), and IκBα, the 'brake' on the NFκB pathway (KSHV). Homology to cellular microRNAs is an additional method that viruses use to exploit the miRNA system.

1.1 MicroRNAs

MicroRNAs form a class of recently discovered regulatory RNAs. They are small, non-coding single stranded RNAs, 18-25 nucleotides (nt) in length. MiRNAs regulate gene expression posttranscriptionally in a sequence-specific manner, in every somatic cell of both plants and animals.
MiRNA precursors are found in the genome as part of introns or exons of both protein-encoding and non-coding genes. Some are located in polycistronic transcripts that often encode multiple, closely related miRNAs. Other miRNA encoding regions are found in genomic repeats or are of unknown origin. MiRNA sequences can be found both on the sense and the antisense DNA strand. They often have a tissue-specific or developmental expression pattern [7].

At present day, about 700 miRNAs are discovered in the human genome [8]. Each miRNA can target several mRNAs and a single mRNA can be targeted by a variety of miRNAs, thus providing a vast regulatory potential. MiRNAs are thought to regulate over 60% of the entire genome [9]. A high degree of conservation of many miRNAs across metazoan species indicates a strong evolutionary pressure and participation in essential biological processes. Indeed previous research indicates that miRNAs play fundamental roles in diverse biological and pathological processes as varied as cell proliferation, differentiation, apoptosis and carcinogenesis. These effects of miRNAs are found in species ranging from *C. elegans* to humans [10]. Single miRNAs have the

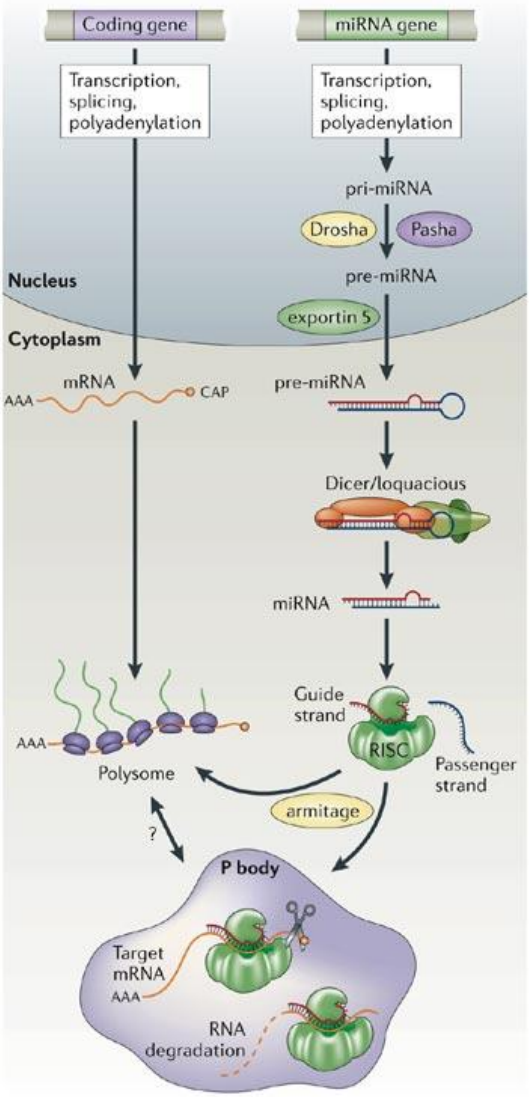


Figure 1-1: Generation of microRNA
RNA pol II generates a primary construct that is processed by Drosha and Dicer to yield a mature miRNA whose guide strand is incorporated into RISC. RISC binds to target mRNA and induces cleavage or translational inhibition.

potential to target over 300 different transcripts, thus their impact can be significant [11].

MiRNAs begin their lifecycle after transcription by RNA polymerase II as primary transcripts of several thousand bases long, called primary miRNA (pri-miRNA). Pri-miRNA has a stem-loop configuration, compromising a 5'cap structure and a polyadenylated 3' tail sequence. After transcription, this primary transcript is processed into a 70-100 nucleotide hairpin-shaped precursor by cellular RNAse III enzyme Drosha and cofactor DGCR8 (in vertebrates). This generates a ~60 nucleotide RNA hairpin intermediate with a characteristic 2-nt 3' overhang called pre-miRNA. After cleavage by Drosha, at least three segments remain: the pre-miRNA and its flanking 5' and 3' sequences. The flanking sequences are generally degraded in the nucleus, whereas the pre-miRNA is exported to the cytoplasm by a heterodimer formed by exportin-5 and the GTP bound form of its cofactor Ran. Together they recognize and bind the 2-nt 3' overhang and adjacent stem which are characteristics of pre-miRNA. In the cytoplasm GTP is hydrolysed and the pre-miRNA is released and subsequently bound by a second cellular RNAse III enzyme, Dicer. Dicer and its cofactor TRBP bind to the 2-nt 3' overhang and remove the terminal loop creating a second 2-nt 3' overhang, resulting in the miRNA duplex intermediate. Dicer enables the assembly of the miRNA guide strand into the RNA induced silencing complex (RISC), the passenger strand is released and degraded. The exact composition of RISC has not been defined yet but it contains an Argonaute protein and interacts with target mRNA (see fig. 1-1). The selection procedure is mainly based on the strength of basepairing at the end of the miRNA duplex. The RISC complex contains one of the four Argonaute proteins of which only Argo2 has endonuclease activity needed to cleave bound target mRNA [12]. Additionally to the 3' UTR miRNAs are also able to target mRNA coding sequences [11, 13].

1.2 Classic mechanisms of action

MicroRNAs are known to negatively modulate gene expression at the posttranscriptional level. This is achieved by base pairing of individual miRNAs at sites in the 3'UTR of target mRNAs. The binding specificity of individual miRNAs for their target mRNAs is presumed to be dictated by approximately 6-7 of the 18-25 nucleotides that compose a miRNA. This sequence, located at the 5' end of the miRNA molecule, is crucial for binding to the target RNA and is called the seed sequence. Additional sequence complementary and secondary structure of surroundings regions of the mRNA influences target recognition by miRNAs. MiRNAs function dose-dependent, 2-fold differences already have significant biological consequences.

The precise mechanisms through which miRNAs regulate gene expression are still not known exactly. However a simplified classic model states that depending on the overall degree of complementary with the target sequence, miRNAs will either inhibit translation or induce degradation of the target mRNA. Usually, the interaction of a miRNA and its target mRNA is characterized by extensive mismatches and bulges, which results in a reduced efficiency of translation. Imperfect base-pairing between miRNAs and their target mRNAs results in translational inhibition. MiRNAs can also inhibit expression of target mRNAs by 100% perfect base-pairing, which results in mRNA degradation mediated by cleavage by Argonaute II.

MiRNAs often have numerous target mRNAs. MiRNAs are therefore said to function according to a 'combinatorial circuitry model', whereby a single miRNA targets multiple mRNAs and several co-expressed miRNAs may target a single mRNA. MiRNA target sites that lie within 40 base pairs of each other can act cooperatively during silencing, further spaced out target sites act independent and can have an additive effect [14, 15]. This complicates research concerning microRNAs and their targets.

1.3 Novel mechanisms of action

As more is learned about microRNAs, additional modes of action are uncovered. For example, it has been found that a particular microRNA can have non-specific overall tissue expression of its pre-microRNA and very specific tissue restriction of the expression of its mature microRNA because of specific cell-selective inhibition at the processing step [16]. Adenosine-to-inosine editing through the action of adenosine deaminases is another method to alter the action of microRNAs [17]. New microRNA isoforms can be created this way, which subsequently interact with a different recognized set of mRNAs [18]. Similarly, subtle editing of the 3' UTR of mRNAs might be a mechanism whereby mRNAs are changed slightly to be recognized by different microRNAs, but experimental proof hereof is still lacking [19].

1.4 Inhibition of microRNAs

Inhibition of microRNA function by noncleavable, nonprotein-coding RNAs has been found in plants. These RNAs interact stably with complementary microRNAs, repressing their activity. This process is called target mimicry and is used frequently as a research tool to knockdown microRNAs when examining their targets and/or functions [20]. AntagomicroRNAs (antagomirs), for example, are anti-microRNA oligonucleotides synthesized with cholesterol-like molecules to permit entry into the cell and can be used to bind unwanted microRNAs [21]. They have been shown capable of silencing microRNA expression in the murine heart [22].

MiRNAs are linked to various diseases as cancer and hypertrophy of the heart muscle. There is a significant difference in the levels of distinct miRNAs in patients compared to healthy controls. For example, cellular miR-15a and miR-16 expression is often downregulated or deleted in B cell chronic lymphatic leukemia.
Selectively knocking down miRNAs might prove to be a powerful tool to help treat cancer, cardiac hypertrophy and viral infection.

1.5 Viruses exploiting miRNAs

Viruses have evolved to exploit RNA silencing for regulation of both host and viral genes [23]. Several viruses use miRNAs to their own advantage. They can manipulate the cellular miRNAs but some have also been found to encode their own miRNAs. The existence of viral miRNA was first reported in 2004 by Pfeffer *et al* who described 5 miRNAs produced by Epstein-Barr virus (EBV) infected B cells [10]. MiRNAs mediate posttranslational regulation. This provides opportunities and problems for viruses and it has undoubtedly had an impact on viral evolution [7].

Not all viruses encode miRNAs and there are several reasons why not all viruses can benefit from bringing their own miRNAs to the battlefield. Firstly, miRNAs need to be processed by Drosha in the nucleus, so viruses replicating exclusively in the cytoplasm, as most RNA and some DNA viruses, do not have access to the necessary machinery. Additionally, after Drosha cleavage only one of the three RNA-fragments is exported to the cytoplasm, causing cleavage and destruction of the viral genome. Furthermore, miRNAs mostly knock down protein expression, a process that takes time, varying from hours till days. This is convenient for dormant viruses but of little use for viruses undergoing fast lytic replication. So next to cytoplasmatic replicating viruses, viruses undergoing fast lytic replication are probably a second group of viruses that do not encode microRNAs. There are some tricks to get around the necessity to replicate in the nucleus for viruses that would like to encode miRNAs but this will not be discussed here.

The remaining group of viruses consists largely of nuclear DNA viruses with long-term latent or persistent infections, such as herpes viruses. All herpes viruses that are analyzed up till date encode several viral miRNAs. Herpes viruses that express miRNA include EBV, KSHV and hCMV. Gamma-herpes viruses are the group of viruses that encodes viral miRNA most abundantly. Rhesus lymphocryptovirus

(rLCV) leads the pack at the moment with 32 identified viral miRNAs. rLCV is closely related to EBV. EBV encodes 25 pre-miR and Kaposi sarcoma-associated herpesvirus (KSHV) 12 [4]. There are a couple of non-herpes viruses encoding a single miRNA, these are nuclear DNA viruses too (e.g. SV40 and adenovirus). Striking is that miRNAs of related viruses do not show sequence homology, except 7-8 miRNAs shared between EBV and rLCV. These two viruses have a common ancestor and have diverged over 13 million years ago [7].

Viruses can benefit from miRNAs to reshape their cellular environment to optimize viral replication in several ways. Firstly, it is a highly specific method to downregulate the expression of host cell gene products that could interfere with viral replication. Secondly, mature miRNAs are very small and do not take up a lot of space in the viral genome, which is convenient as it is restricted in size. Thirdly, microRNAs are not antigenic, so they will not elicit an immune response.

Virally encoded miRNAs are thought to play a role during the latent phase as they are expressed in latently infected cells. However, at least one KSHV and nearly all EBV miRNAs show an enhanced expression after induction of lytic replication. This could be functionally relevant or just a consequence of the increased genome copy number during lytic replication.

Viral miRNAs can exert their effect in a number of ways. It can target its own or the host mRNA and cellular miRNAs can be affected by viral infection as well. Viral mRNA targets are easier to identify than cellular targets because of the restricted viral genome size. However, most viral miRNAs probably target host mRNAs. This enables them to inhibit the host adaptive and innate immune system, to modulate aspects of signal transduction and regulate cellular proliferation. Additionally, viral miRNAs can serve as orthologs of cellular miRNAs and thus regulate the expression of several cellular mRNAs (e.g. KSHV miR-K12-11) [24].

It is thought that viral miRNA are not necessary for the viral lifecycle but for example play a role protecting the infected cells [7].

1.6 Viral miRNA targets

After the initial discovery of viral microRNAs not many of their targets have been described. The targets of KSHV are thought to be involved in angiogenesis, proliferation, immune evasion and repression of apoptosis [25-29]. For EBV cell survival promotion and targeting of cellular chemokines can be added to that list [30, 31].

The individual function of most miRNAs is not yet elucidated, but some are characterized. For example, human cytomegalovirus miR-UL112 targets genes of the host immune system, leading to reduced killing

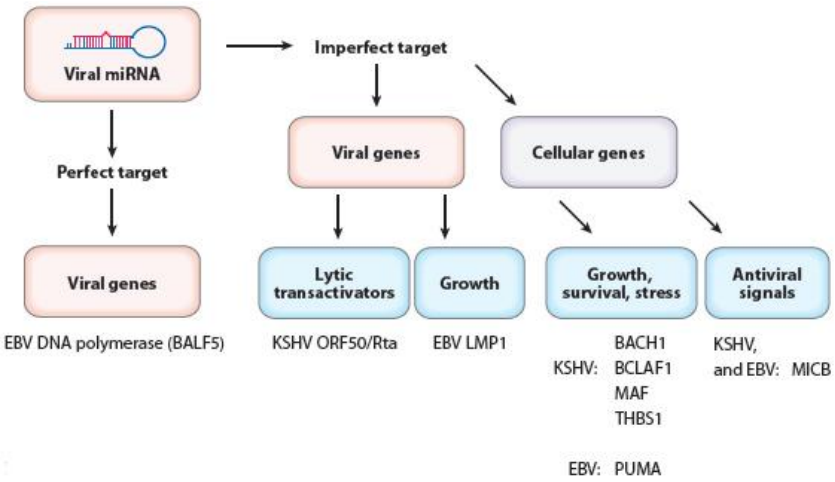


Figure 1-2: Overview of viral and cellular targets of EBV and KSHV microRNAs. Adapted from [4]

by natural killer cells. Another example is SV40 that expresses a miRNA that regulates viral gene expression to protect itself from NK cells by downregulation of the T antigen in a late stage of infection. KSHVs miR-K12-11 functions as an ortholog of cellular miR-155, exploiting preexisting pathways in B cells. HSV1 has been shown recently to express miRNAs that maintain and regulate the latent stage in neurons of the sensory ganglia [32].

In general, viral and cellular miRNA do not have similar seed sequences. An exception is KSHV miR-K12-11 which shares the first eight nucleotides with the human miR-155 [28]. MiR-155 is often upregulated in tumors such as lymphoma. Gottwein *et al* suggest that miR-155 and kshv-K12-11 regulate the same set of target genes which include genes playing a role in cell growth regulation [33]. For example, BACH-1 is a predicted target mRNA of both miRNAs. There is no known EBV ortholog for miR-155/kshv-K12-11.

Because of the lack of sequence similarity between viral and host miRNAs, new viral miRNAs are difficult to identify with software, which is often based on sequence homology. Preferentially, one should use software that predicts secondary structures [23].

1.7 Identification of miRNAs

Most miRNAs have been identified using a modified version of the RACE protocol (Rapid Amplification of cDNA Ends) [34]. The total RNA is size-fractioned by denaturing polyacrylamide gel electrophoresis, excised from the gel and eluted. Subsequently oligonucleotides are ligated to the 5' and 3' ends of the RNA. Ligated products are reverse-transcribed, amplified by PCR and digested with restriction enzymes. Next the products are concatemerized and sequenced. This approach proved very successful: most of the known miRNA are identified this way. The disadvantage is that the cloning is laborious and a lot of emplicons have to be sequenced to saturate the screen. So it is possible to miss miRNAs that are expressed at a low level. Another disadvantage is that a lot of starting RNA material is needed, which can be unpractical with patient samples.

Computational algorithms that use the characteristic hairpin structure of pri-miRNA and pre-miRNA precursor structures have been used to predict the sequence of miRNAs based on their primary sequence. A limitation is that this often relies on evolutionary conservation [35]. Candidate miRNAs need to be confirmed with cloning or northern blotting [36]. The cDNA cloning method has identified miRNAs in both EBV and KSHV [36].

2.1 Epstein Barr Virus

Humans are infected by two members of the γ-herpes family: EBV and KSHV. Both viruses can cause proliferative diseases and preferentially infect B cells, although they are able to proliferate in epithelial cells as well [37]. EBV was the first virus that was associated with cancer [30]. It was isolated for the first time from Burkitts lymphoma (BL), a B-cell derived tumor [38]. Additionally, EBV was the first human virus found to encode miRNAs [10]. EBV (human herpes virus 4 (HHV4) is a ubiquitous herpesvirus that asymptomatically infects the majority of the population (>95%)[23]. This infection is usually without serious consequences; however a small percentage of latently infected humans develop malignancies of lymphocytic or epithelial origin [30]. The virus is associated with several diseases and malignancies as infectious mononucleosis (IM), Burkitts lymphoma (BL), Hodgkin's disease (HD), gastric carcinoma (GC) and nasopharyngeal carcinoma (NPC) [39].

EBV has a high degree of B cell tropism. *In vitro* EBV is able to infect all resting B cells and turn them into activated proliferating lymphoblasts. In contrast, EBV is usually found in a quiescent state in resting memory cells which are present in the peripheral circulation *in vivo*. EBV is a 172 kb dsDNA virus. It enters B cells after binding to a B cell specific surface molecule CD21, which is a receptor for the C3d fragment of the complement system [40].

The virus is thought to use viral proteins LMP1 and LMP2a to transform an EBV infected B cell into a memory cell. EBV preferentially infects B cells but is able to infect other cell types as well, mostly epithelial cells. Virus-receptor interactions activate the host cell and infected cells can enter the mitotic cycle and turn into proliferating cell lines (e.g. lymphoblastoid cell line (LCL)).
During infection the viral genome is maintained in episomal form and nine viral proteins are expressed. The exact gene expression pattern depends on the target cell type, differentiation, activation state etc. About 100 genes are expressed during productive replication. During a primary humane infection EBV replicates in the oropharyngeal epithelium and establishes latency III in B lymphocytes [41] [42, 43]. There is a stable number of infected cells in the peripheral bloodstream (0.5-50 per million, depending on the patient) during a persistent infection [44]. In healthy individuals there is a constant shedding of the virus in saliva, even though the virus is under strict immunosurveillance [40]. The EBV genome found in virions is linear and it becomes circular (episome) after infection and locates in the host nuclei [6].

2.2 Latency

Herpesviruses can be present in infected host cells in two stages: latency or lytic replication. During latency there is a very restricted gene expression whereas during lytic replication all genes are expressed. Lytic replication eventually leads to the death of the host cell and the release of nascent virions. Latency is the default gene expression state of most γ-herpes viruses, but reactivation is occasionally needed for cell to cell or host to host spread [45].
There are 3 different stages of latency in EBV infected cells, characterized by the expression of various subsets of latency genes [10]. EBV encodes nine latency-associated proteins, six of these proteins are localized in the nucleus (EBV nuclear antigens (EBNAs)) and three are transmembrane proteins (latent membrane proteins 1-3 (LMP1-3)) [46]. During the latency phase EBV Cp or Wp EBNA promoters drive expression of the six nuclear antigens proteins (EBNA2, EBNALP, EBNA3a, EBNA3b, EBNA3c and EBNA1) from one alternative spliced transcript. Additionally, EBER 1 and 2 are present in latently infected cells, these are not proteins, but short, non-polyadenylated RNAs .

Latency stages

- **Type III latency (growth program)**
  Characteristic of the growth program is that all latency EBV proteins are expressed (in LCL cells) [46]. Two alternative viral promoters (Wp or Cp) regulate the gene expression of six EBNAs and three LMPs. The function of all these viral proteins is not completely elucidated yet. They interact with each other and with cellular transcription factors and co-activators. They are able to activate a large number of cellular genes.
- **Type III latency**
  Latency III is found only in B lymphocytes. LMP1 has a strong effect on the B-cell phenotype, it induces activation markers and co-stimulatory molecules. This enlarges the immunogenicity of these B-cells and thus they can only exist during the acute phase of a primary EBV infection, before the EBV-specific T cell response is developed. Additionally it can be seen in immunocompromised patients, post-transplantation for example. These patients are at risk to develop lymphoproliferative diseases.
- **Type I latency**
  Using this program the virus is able to persist in its host without risking killing it. In this latency phase only viral protein EBNA1 is expressed. The large Wp or Cp initiated transcript is not produced in these infected cells. EBNA1 is monocistronic and its mRNA is transcribed of the Q promoter [47, 48]. The type I latency program does not induce proliferation and the phenotype of EBV infected cell in this stage resembles non-activated B-cells. Type I infected B cells are found in the memory B cell population in healthy people. Without the simultaneous expression of costimulatory molecules they

are not recognized by EBV specific T lymphocytes. EBNA1 is not a target for CD8 positive cytotoxic T lymphocytes, possibly because of its long glycine-alanine repeats which prevents ubiquitin-proteosome dependent processing needed for loading of MHC 1 presentation [49]. This latency program has been observed in BL cells.

- **Type IIa latency**
  Viral proteins EBNA1, LMP1 and LMP-2 are expressed during type IIa latency. This has been observed first in NPC cells [50]. EBNA2-6 are not expressed in non-B cells because only B cells possess the specific transcription factors needed. EBNA2 is needed for the induction of proliferation of EBV infected B cells. The lack of EBNA2 in latency phase IIa infected cells prevents induction of proliferation, except in the presence of additional cellular changes and growth promoting signals from the micromilieu.
- **Type IIb latency**
  During type IIb latency all six EBNA proteins are expressed, but LMP1 is not, indicating that here too an important protein needed for B cell transformation is missing. This has been seen first in B-chronic lymphocytic leukemia cells (B-CLL) infected in vitro [51].
  B-CLL is the clonal expansion of long-lived resting B lymphocytes; its pathogenesis is not clarified. The absence of LMP1 is striking because this protein is normally activated by EBNA2. No tumors with the latency phase IIb expression pattern have been found to date, this pattern has only been observed *in vitro*.

Concluding, infected cells in stage III latency risk elimination by development of a host EBV specific immune response, whereas in stage I latency the virus can persist in the host cell without being detected. Type I latency does not seem to influence the differentiation of B cells [40].None of the genes

Genes that are expressed during latency

- **EBNA1** binds to the origin of replication of the EBV episome and this interaction is needed for the replication of the viral genome and the distribution to the daughter cells after proliferation. EBNA1 is not presented by MHC I so EBV infected cells only expressing EBNA1 are not recognized by cytotoxic T lymphocytes.
- **EBNA2** is a viral protein expressed after infection of B cells *in vitro* and is essential for cellular transformation. It is a transactivator that regulates several viral and cellular genes.
- **LMP1** inhibits apoptosis by upregulating anti-apoptotic proteins Bcl2 and A20 and induces an activated phenotype. LMP1 is an oncogene and most of its effects are caused by induction of the NFκB pathway, similar to CD40, which plays a key role in activation and differentiation of B cells. LMP1 resembles in several aspects a constitutively active CD40 receptor [3].
- **LMP2a** has immunoreceptor tyrosine-based activation motifs (ITAMs) in its cytoplasmatic domain. These are also present in B cell co-receptor CD79a and CD79b and they send activating signals after B cell receptor stimulation. LMP2a binds and sequesters tyrosine kinases and inhibits thus B cell receptor signaling, preventing unwanted antigen triggered activation of EBV positive B cells, causing them to enter the lytic cycle. LMP2a mimics a B cell receptor [5].
- **EBER 1 and 2** encode short non-polyadenylated RNAs that induce a plethora of effects as secretion of several interleukins, contribution to clonal proliferation of BL cells, resistance to apoptosis and inhibition of cytotoxic T lymphocytes[6].

expressed during latent infection is conserved between EBV and KSHV, every virus captures its own set of host genes [36].

2.3 EBV miRNAs

EBV is a 172 kb dsDNA virus that produces nuclear localized transcripts with partially dsRNA structures that are processed into smaller miRNA-like molecules [52]. It encodes 25 miRNAs that are found in two distinct clusters. The first cluster is located close to the BHRF1 gene and the other one is located in the BART gene (fig. 2-1). Sequence homology revealed that EBV miRNA can target several classes of cellular mRNA, including regulators of cell proliferation and apoptosis, chemokines, transcription factors and signal transduction components [52].
EBV microRNAs are important regulators of viral gene expression but probably also play a role in targeting cellular transcripts to facilitate viral persistence and oncogenesis.
Predicted targets of *in silico* analysis include but are not restricted to growth regulators, modulators of apoptosis, B-cell specific chemokines/ cytokines, transcriptional regulators and signal transducers. However, there is no experimental evidence to support this yet (table 1) [30].
There are 3 EBV miRNAs in the BHRF1 gene and 21 miR-BARTs divided over 2 clusters (except miR-BART2). These miRNAs enlarge the potentially biologically active molecules during latent infection enormously. The functions of these mature miRNAs in EBV are unfortunately not well understood yet.
The EBV and BART miR clusters are differentially expressed: the 3-member BHRF-1 miRNA cluster is expressed only in EBV infected B cells in stage III latency; whereas the 21-member BART clusters are mainly expressed in transformed cells undergoing EBV stage II latency [53]. MiRNAs seem to act at different stages of the EBV infection. The exact importance is not known yet [7].
Cluster 1 BART miRNAs are linked to downregulation of LMP1 whereas miRNAs in the BHRF1 region are associated with viral replication and regulation of chemokine CXCL-11 [54].
Some EBV miRNAs are very abundant: ~5-19% of the total miRNA is viral in NPC samples. High levels of ebv-miR-BART1, 4, 6, 7, 11, 12, 19, 21 and 22 are found in NPC samples, but no BHRF1 miRNAs could be detected. This might indicate that the BHRF1 miRNAs are not involved in NPC pathogenesis [32].

The enhanced expression of EBV miRNAs during lytic replication is one of the differences between EBV and KSHV, which only has a modest increase of miRNA levels upon the start of lytic replication [53, 55].

BHRF1

The BHRF cluster is located within the mRNA of the BHRF1 gene encoding a distant Bcl2 homolog and contains miR-BHRF1-1 to 1-3. BHRF1 miRNAs lie adjacent to the gene and are derived from an intron generated by splicing of viral EBNA transcripts that are solely expressed in latency III infected B cells [43]. The latency III primary transcript contains multiple ORFs that are probably the source of the three BHRF1 miRNAs [55]. MiR-BHRF1-1 is located in the 5' UTR whereas miR-BHRF1-2 and 1-3 are encoded in the 3' UTR of the BHRF1 mRNA [10, 55].

Barth *et al* found that in addition to miR-BART2 both the precursors and the mature miR-BART1 are downregulated upon induction of lytic replication. This is in line with previous work that showed that mature miR-BHRF1-1 and -2 are downregulated after TPA stimulation in EBV infected cell lines. They suggest that additional virus-encoded miRNAs may play a role in repression of the lytic replication cycle [56].

BART

The remaining miRNAs cluster in intronic regions of the BART gene [10]. BART miRs are a set of long, alternative spliced transcripts.These miRNAs were discovered by Pfeffer *et al* (2004) and Cai *et al* (2006) and are located in the BART (BamH1-A rightward transcript) gene, this indicates that the expression of

these miRNAs is related to the expression level of BART mRNA[53]. There are 2 clusters of BART miRNAs: 8 miRNAs lay in cluster 1 and 13 in cluster 2. MiR-BART2 is an individual located miRNA. The 22 BART miRNAs are located in the introns of viral BART transcripts. It is proposed that BART miRNAs like BART mRNAs are preferentially
expressed in infected epithelial cells. MiR-BARTs have been shown to regulate the expression of LMP1 viral oncoprotein [39]. BART miRNAs are present in all latent stages of infection [10]. EBV miR-BARTs were first identified in NPC as multispliced transcripts and were later found in a broad range of EBV-associated cancers. BART expression is low in B lymphocytes and high in epithelial tissues, this could point out that they are important in epithelial malignancies. BART miRNAs might provide more insight in this process.
Striking is that most EBV miRNAs are deleted in the lab strain B95-8 but this strain is still able to immortalize primary B cells in culture [53].This indicates that most BART miRNAs are not needed for EBV latency in B cells. It is possible that they do play a role in infected epithelial cells.
Other viral genes in the BART and BHRF1 region also have regulatory functions regarding apoptosis and cell signalling. For example, BHRF1 encodes a Bcl-2 homologue, which is anti-apoptotic and enhances cell survival. Additionally, some protein products of the BART region might modulate Notch signalling and RACK1 activities [57].

MiRNAs have been detected via cloning, by northern blotting or predicted by computational approaches. However, this does not say anything about the amount of molecules per cell. Pratt *et al* have quantified miRNAs via stem-loop-rtPCR and synthetic standards in 17 cell lines. Ebv-miR-BART7, -10 and -12 are detected the most in different studies and miR-BART15 and -20-5p are hardly found at all. In general all miRNAs in one cluster are expressed or none at all. The levels of individual miR-BARTs can vary enormously (up till 50-fold) between cells, this is conserved between cell lines. All miR-BARTs are derived from the same transcripts so it is likely that the changes occur after processing. Tertiary structure of the pri-miRNA is thought to play a role as this influences its accessibility for the microprocessor. Another factor might be the stability of the pre-miRNA. The absolute levels of miRs can vary up till 50-fold too between cell lines, which is important as miRNAs act with a dose-dependent effect [58].

2.4 EBV & rLCV

EBV and rLCV have diverged over 13 million years ago. Yet they share conserved miRNA, which could indicate a role in the viral life cycle. Both EBV and rLCV encode 2 miRNA clusters: the first close to the viral BHRF1 gene and the second in the BART gene. Some but not all miRNAs are highly conserved between these viruses.

EBV miRNA	Target	Involved in
miR-BART2	BALF5	DNA polymerase
miR-BART1-5p, 6 & 17-5p	LMP1	Cell proliferation, immune evasion
miR-BART22	LMP2a	Immune evasion
miR-BART5	PUMA	Apoptosis
miR-BART2	MICB	Antiviral signals
miR-BHRF1-3	CXCL-11/I-TAC	Chemokine modulation
miR-BART16	TOMM22	Protein import
miR-BART3	IPO7	Protein import
miR-K12-4-5p	Rbl2	DNMT regulation

Table 1: Identified targets of EBV encoded microRNAs.

The high degree of similarity between EBV and rLCV miRNAs could indicate a siRNA function, which requires full complementary. Another possibility is that multiple mRNAs are targeted and this degree of similarity is needed to cover all targets. Extensive conservation points towards cellular targets since the sequence of the two viruses are far from similar, especially concerning the latent genes [53, 55].

7 of the rLCV miRNA are largely conserved between EBV and rLCV in terms of sequence and relative genomic position, the others are not. This is easy to explain as the miRNA sequences are very short and some sequence difference can be tolerated. This leads to a potential very fast evolution of viral miRNA with a selection of novel cellular mRNA targets whose downregulation is beneficent for the virus.

22 of the 25 EBV pre-miRNAs share some degree of conservation with rLCV miRs, but there are also seed regions that are different, suggesting an evolutionary relationship but divergent functions [53, 59]. The genomic locations of the miRNAs between diverse herpesviruses are largely conserved; difference in seed sequence does not automatically mean that the miRNAs have different targets as well.

3.1 KSHV

Kaposi sarcoma-associated herpesvirus (KSHV) is the second γ-herpes virus able to infect humans. Another name for KSHV is HHV8 and the virus is like EBV linked to several malignancies as Kaposi's sarcoma, a cancer commonly occurring in AIDS patients as well as primary effusion lymphoma (PEL) and some types of multicentric Castleman's disease. Similar to EBV and all other herpes viruses KSHV has a multi-stage viral replication cycle. Initial acute infection is followed by a long lasting latent period from which KSHV is able to reactivate.

KSHV is present during latent infection as a circular multicopy minichromosome retained in the nuclear compartment. Gene expressing is restricted to viral genes encoding the latency-associated nuclear antigen (LANA) ORF73, viral cyclin (vCyclin; ORF72) vFLIP (ORF71) kaposin (K12) and a cluster of 12 miRNAs.

During the primary infection KSHV is actively replicated by its viral machinery and host cell lysis leads to the release of nascent virions. In this stage the genome is linear. Lytic gene expression starts with the expressing of immediate-early genes (IE) which regulate the expression of subsequent genes. The latent-lytic switch of KSHV is regulated by RTA (replication and transcription activator), which is encoded by ORF50 of the KSHV genome [60]. ORF50/RTA expression is repressed during latency, but can be induced by hypoxia or pharmaceutical agents [61]. Stimulation of RTA expression induces lytic replication by directly transactivating various KSHV promoters. KSHV can establish long term latency in B cells after initial infection [62] and during this phase only a few genes are expressed. The latent genome is circularized by joining of GC rich terminal repeats at the ends of the viral genome, forming an extrachromosomal circular episome [63].

Viral protein LANA (latency associated nuclear antigen) is expressed in latent infected cells and binds

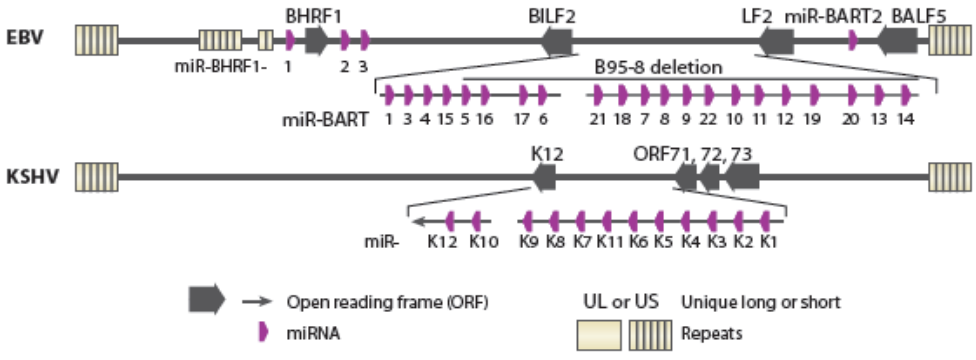


Figure 2-1: Localization of pre-miRNAs in the EBV (25) & KSHV (12) genome. The viral miRNAs are primarily found in latency-associated clusters [4].

this episome to a host chromosome. LANA is also involved in the regulation of the episome replication by the host cell machinery [64].

3.2 KSHV miRNAs

Samols *et al* showed in 2005 that KSHV encodes miRNAs too, till date 12 pre-miRNAs have been identified [45, 65]. All cloned and predicted KSHV miRNAs are within a 5kb region of the 140kb viral genome, which also encodes the transforming protein kaposin [66]. Kshv-miR-K12-10 lies in such a manner in the ORF12 that its excision coincidences with the destruction of a part of the kaposin mRNA. Kshv-miR-K12-10 lies in the kaposin mRNA transcript in a position overlapping the K12 ORF. Excision of the K12-10 pre-miRNA probably abolishes the protein expression of the remaining fragments which are devoid of a cap or poly(A)tail. The other KSHV miRNAs are located in the intronic region of the larger kaposin primary transcript (fig. 2-1).
The best characterized cellular targets of virally encoded miRNAs are those of KSHV (table 2) [4]. The virus encodes 17 miRNAs which are the products of 12 pre-miRNAs. Of the 17 mature miRs 14 are co-expressed in a cluster [23, 67]. The KSHV miRNAs are latently transcribed and expressed in KSHV infected cells and KS lesions [68, 69]. They are all expressed during latency, indicating that they are involved in the control or adjustment of the latency-lytic switch [45].
The function of most KSHV miRNAs is poorly characterized but there are some identified targets (table 2). The KSHV miRNAs are clustered in the latency-associated region and are highly expressed in latently infected PELs. 10 pre-miRs (K1-9+11) are clustered within a viral intron between ORF71 and kaposin, the remaining two are located in de coding region (MiR-K10) of the 3' UTR (K12) of KSHV miR-K12 gene [23, 36, 65]. The expression of the pri-miRNAs is regulated by one latent and two lytic RNA polymerase II promoters [55, 70]. Deletion of the 10 miRs cluster does not abolish lytic replication [70] and induction of lytic replication does not enhance expression of the miR-cluster, which seems to play a role during latency. Upon lytic induction miR-K10 and miR-K12 are upregulated [67].

Regulation of lytic reactivation is thought to be controlled partially by epigenetic factors. Viral genomes can be reactivated by inhibitors of histone deactelylases and by inhibitors of DNA methyltransferases (DNMTs). Viral genomes are split into active and inactive chromatine domains which causes certain areas to be accessible and others not.

Pre-miR-K10 is partially modified by a cellular adenosine deaminase that edits an adenosine to an inosine on the 2nd position of the seed region. This has to affect the specificity of the miRNA but the functional consequences have not been found yet [71]. There are two 1-nucleotide variants of miR-K12-10, they differ with an A or G on the 2nd position and are named miR-K12-10a or -b respectively. In genomic RNA only the K12-10a variant can be found, which indicates that the K2-10a variant partially is getting edited by a dsRNA specific deaminase that the adenosine on position 2 substitutes for an inosine. It remains unclear whether this has a regulatory function has because it lies in a region which is

KSHV miRNA	Target	Involved in	
miR-K12-11	BACH1	growth, survival, stress	[25, 28]
miR-K5	BCLAF1	growth, survival, stress	[29]
miR-K9*	ORF50/RTA	lytic transactivator	
mir-K12-11 & **K12-6**	MAF	growth, survival, stress	
miR-K12–1, -K12-3-3p, -12-6-3p & **-K12–11**	THBS1	growth, survival, stress	[27]
miR-K12-7	MICB	antiviral signals	
Kshv-miR-K6 kshv-miR-K1	IκBα	inflammation	

Table 2: Identified targets of KSHV encoded microRNAs.

most critical for target recognition. Editing of cellular miRNAs has been reported before but on a much lower frequency than this viral miRNA. Mir-K12-10 is specifically upregulated during lytic replication induced by TPA.
As EBV BHRF1 gene and its miRs are upregulated upon induction of the lytic stage are kaposin and its imbedded K12-10 upregulated after induction of lytic replication. This indicates that viruses are able to express both coding and non-coding sequences simultaneously. Depending on the expression levels a part of the transcripts can escape miRNA processing to serve as a template for protein synthesis [23].

4. Viral targets of viral miRNA

Only a handful viral targets of viral miRNAs are found till date. The first described target was the EBV encoded DNA polymerase (BALF1) that is transcribed from the strand opposite ebv-miR-BART2, which is thus fully complementary and leads to its degradation
The second viral target of a viral miRNA that has been identified is encoded by SV40 which targets early viral mRNA and protects against cytotoxic T cells [7].

4.1 BALF5

Ebv-miR-BART2 is encoded antisense to the 3' UTR of DNA polymerase BALF5 and is thus perfectly complementary leading to cleavage and destruction of the mRNA. During latent infection only low levels of miR-BART2 are present, these low levels are needed to inhibit aberrant transcribed BALF5 mRNA preventing accidental induction of viral replication. The EBV DNA polymerase is cleaved at the predicted position (10nt from the 5' end of the region of homology to miR-BART2) [56]. Upon induction of lytic replication there are reduced miR-BART2 levels and thus less cleavage of BALF5 3' UTR. This suggests that miR-BART2 is involved in maintaining latency [24].

4.2 LMP1

LMP1 is a constitutively active viral mimic of the TNF receptor family and induces cell proliferation during latency. LMP1 induces NFκB activity and suppresses cisplatin induced cytotoxity in LMP1 expressing NPC cells. LMP1 can transform rodent fibroblasts and induce a wide range of phenotypic changes in epithelial cells and B cells in vitro. It activates the NFκB, JNK, JAK/STAT, p38/MAP and Ras/MAPK pathways and alters cellular gene expression. LMP1 is a candidate for driving NPC development [39]. However, overexpression of LMP1 inhibits growth and stimulates apoptosis. Finetuning this balance can promote the proliferation of infected cells. This finetuning is regulated by **ebv-miR-BART1-5p, -BART6** and **-BART17-5p**, located in BART cluster 1 (fig. 5-1) [39]**.**

These miRNAs are an imperfect match to the 3' UTR of the mRNA coding for LMP1, resulting in the repressing of LMP1 expression, regulating the LMP1 mediated activation of NFκB and apoptosis resistance [39]. The modulation of LMP1 by miRNAs indicates a role for viral miRNAs in regulating viral pathogenesis [31].

4.3 LMP2a

Lung *et al* have shown that LMP2a is a downstream target of **ebv-miR-BART22** (fig. 5-1). LMP2a is an EBV encoded protein with a relative high immunogenicity for cytotoxic T lymphocytes and regulation of LMP2a promotes tumor cell survival by evading the immune system. LMP2a binds and sequesters tyrosine kinases and inhibits thus B cell receptor signaling, preventing unwanted antigen triggered activation of EBV positive B cells which causes them to enter the lytic cycle [5]. MiR-BART22 is abundantly detected in NPCs, a type of tumor restricted to South-China. South-Chinese EBV strains have a polymorphism that promotes biogenesis of miR-BART22 [2].

4.4 RTA

Lu *et al* proposed that the miRNA cluster can serve as a genetic element that helps maintaining the latent status of the viral chromosome. Viral miRNAs repress expression of immediate early gene transcription by a mechanism that contains translational suppression of RTA (replication and transcription activator) and the maintenance of DNA methylation of the ORF50 promoter as well as other regions of the viral and cellular genome. KSHV miRs help maintaining latency by downregulating the lytic activator protein RTA/ORF50 and by increasing the global levels of DNA methyltransferases (DNMTs) and CpG methylation. **MiR-K12-5** represses ORF50 mRNA levels and it is ORF50 3' UTR dependent, although it is not directly targeting it. MiR-K12-4-5p targets the cellular repressor protein Rbl2 which is thought to play a role in transcription regulation of DNMTs. The microRNAs can upregulate DMNTs activity and increase DNA methylation on both viral and cellular chromosomal sites [72].

Kshv-miR-K9* can directly influence lytic regulation by modulating the expression of RTA. Expression of RTA triggers reactivation from latency. At the same time however, a knock-out model of RTA shows that induction of lytic replication is no longer possible. Administering synthetic miR-K9* leads to a 30% reduction of RTA levels [45]. Kshv-miR-K5 achieves the opposite: it makes cells more sensitive to lytic reactivation [29].

MiRNAs are all about the fine-tuning of gene expression. They do not cause major changes but are involved in subtle regulation. A shift in the sensitivity for reactivation provides the virus with the opportunity to adapt to changing environmental circumstances, which might require a different reactivation frequency. Depletion of miR-K9* leads to a 2-3 fold increase of lytic reactivation. This serves as some kind of safety mechanism to prevent accidental induction of lytic replication by transcriptional noise (the presence of low levels of proteins without induction) to control RTA. It seems illogical that latent miRNA represses a latent gene. RTA is in charge of reactivation when it gets down to it, but miR-K9* can fine tune this mechanism [45].

5. Cellular targets of viral miRNA

5.1 PUMA

Ebv-miR-BART5 targets PUMA (p53 upregulated modulator of apoptosis, also known as BBC3 or Bcl-2 binding component 3). There are four isoforms of PUMA (α, β, γ and δ) which share the same 3' UTR, however, only isoform α and β have pro-apoptotic activity.

PUMA has been identified as a possible miRNA target by bio-informatics analysis and functional screening. It is a pro-apoptotic protein belonging to the BH3-only group of the Bcl2 family. PUMA is an immediate downstream target of p53, but is also able to induce p53-independent apoptosis as reaction on a wide variety of stimuli. P53 and PUMA are the master regulators of apoptosis and cellular growth. The p53 gene itself is a transcript for cellular miRNAs too. It is likely that both p53 and PUMA are regulated by several miRNAs.

It is thought that apoptosis is prevented in NPC by EBV-miRNA mediated inhibition of PUMA (fig. 5-1). Evidence supporting this theory is the fact that PUMA has a 3' UTR target site for miR-BART5. Furthermore, the expression of miR-BART5 in NPC cells is negatively correlated with PUMA and manipulation of ebv-miR-BART5 (overexpression or knockdown) has the opposite effect. This leads to the conclusion that ebv-miR-BART5 has an anti-apoptotic effect in both NPC as EBV-GC cells. This link is not surprising as p53 in NPC is seldom mutated but activated, probably via LMP1, so p53 activation has to be inhibited to preserve EBV infection. Underexpression of PUMA was only observed in ~60% of the BART positive samples, so it might be possible that there are several redundant mechanisms to regulate PUMA. This could be studied using overexpression and loss of function experiments. In EBV infected cells inhibition of miR-BART5 leads to an increase of PUMA-mediated apoptosis [30].

5.2 CXCL-11

Xia *et al* have identified the CXCL-11/I-TAC mRNA as a target of **ebv-miR-BHRF1-3.** CXCL-11/I-TAC is an IFN inducible T cell attracting chemokine. The miRNA sequence is 100% identical to its target sequence in the 3' UTR of the CXCL-11/I-TAC mRNA and the miR thus serves as a potential effector in the modulation of the cytokine network. Negative modulation of CXCL-11/I-TAC can function as an immunomodulatory mechanism in tumor cells [31].There is an inverse correlation between levels of BHRF1-3 and CXCL-11, which has an immunomodulatory function in lymphomas. Ebv-miR-BHRF1-3 is found to be mainly expressed in type III latency cell lines. Primary BLs and PELs have a higher BART expression but no BHRF1-3 microRNAs [43].

5.3 MICB

Both **kshv-miR-K12-7** and **ebv-miR-BART2** target major histocompatibility complex class I-related chain B (MICB), an activating stress induced NK ligand that plays a role in immune recognition of infected cells. MICB is upregulated by oncogenic stress and viral infection and is a ligand for NK and CD8 positive cells. Downregulation of MICB leads to a reduction of NK mediated killing of infected cells. The mRNA encoding MICB is targeted by several herpesvirus encoded miRNAs without a clear sequence homology and might be an example of convergent evolution of herpesvirus miRNAs [26, 73].

5.4 MAF

Hansen *et al* proposed that KSHV miRs play a role in cellular differentiation and that they may mediate transcriptional reprogramming of endothelial cells. They have studied this via microarrays in LEC cells. They found that the leucine zipper transcription factor MAF (musculoaponeurotic fibrosarcoma oncogenes homologue) is targeted by KSHV miRNAs [74]. MAFs play a role in tissue specification and terminal differentiation of a wide range of cell types [75]. KSHV induces transcriptional reprogramming in LECs [76]. Hansen *et al* show that different KSHV miRs and specifically **miR-K12-11** and **K12-6** cooperate to target the LEV specific transcription factor MAF to modulate the fate of LEC during infection. They conclude that viral miRNAs play a role in viral induced reprogramming. MAF has miRNA target sites in its 3' UTR for both **miR-K12-11** and **K12-6,** and for both miRs there is more than one predicted site. MAF 3' UTR reporter assays have shown that both miRs indeed are capable of silencing MAF. This is consistent with previous statements that different miRNAs can target the same mRNA. MAF is downregulated early in infection and stays that way throughout infection. Blocking the mentioned miRNAs abolishes their effect on MAF. MiRNA target sites that lie within 40 base pairs of each other can act cooperatively during silencing, further spaced out target sites act independent and can have an additive effect [14, 15]. The latter is the case with MAF [74].

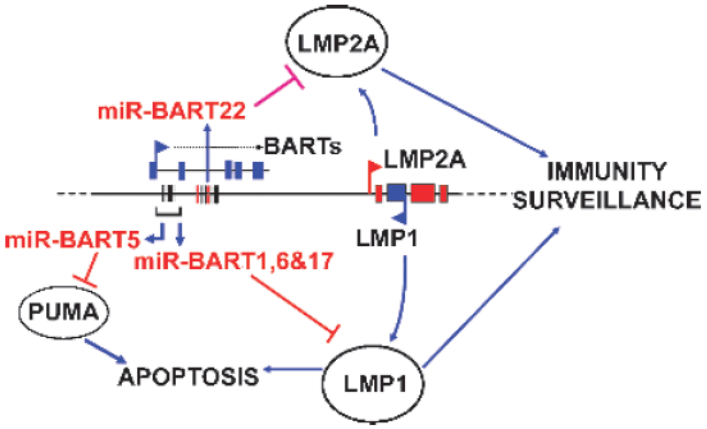


Figure 5-1 Cellular and viral targets of ebv-BART-miRNAs related to host cell survival. miR-BART5 can inhibit apoptosis by modulating the expression of PUMA. miR-BART22 regulates the expression of viral LMP2a and miR-BART1, 6 & 17 can modulate LMP1 expression [2]

5.5 IkBα

KSHV miR regulates NFkB inhibitor IkBα

The group of Lei *et al* have created mutant KSHV viruses that lack a cluster of 14 miRNAs. This results in higher levels of RTA and MCP compared to wild

type infection. Additionally, there is more of the early viral lytic protein ORF59 and the infected cells produce more progeny virions after lytic induction. This suggests that the miRNA cluster inhibits the expression of viral lytic genes in latent cells and also in cells that are induced to viral lytic replication. Zhu *et al* have predicted several putative targeting sites for **Kshv-miR-K6** in the 3' UTR of RTA and ZTA via computational analysis [77]. However, Lei *et al* could not confirm this. The NFκB activity in the ΔmiRNA infected cells was lower compared to wt and there was also less nuclear p65 staining as well as p50 and cRel. The conclusion is that there is less NFκB activity in ΔmiRNA infected cells. Overexpression of the miRNA cluster in both wild type as ΔmiRNA infected cells yields a higher NFκB activity.

Inhibition of the NFκB pathway facilitates viral replication and can be mediated by miRNAs. The suspicion that NFκB was mediated by miRNA targeting of IκBα was confirmed with the 3' UTR reporter assay and **kshv-miR-K1** was identified as effector (fig. 5-2). The IκBα 3' UTR has two putative binding sites for miR-K1 and deletion of both sequences abolishes the effect of miR-K1. Deletion of just one binding site has no effect. Expression of miR-K1 enhances NFκB expression in a dose-dependent manner. Deletion of the miRNA cluster enhances lytic replication and expression of just miR-K1 can reverse this (judged by the MCP and RTA levels) [1].

EBV LMP1 and KSHV vFLIP activate the NFκB pathway to promote cell growth and survival and to stimulate viral latency [78, 79]. The NFκB pathway is thus used to inhibit viral replication and to promote cell survival [1].

5.6 BACH-1 & c-Maf

KSHV-miRNAs modulate xCT via BACH-1 and c-Maf

xCT is an inducible subunit of the amino acid membrane transporter system x_c^- that mediates amino acid exchange. Cystine transporter xCT has a protective role during oxidative stress en protects against cell death. xCT expression is upregulated during hypoxia en inflammation [80]. KSHV miRNAs are thought to target the negative transcription regulators of ARE (antioxidant response element) that regulates xCT expression. xCT expression is regulated by competitive binding of positive and negative transcription factors to ARE in the xCT promoter. A positive regulator binding to the cis-acting ARE is nuclear factor erythroid 2-related factor-2 (Nrf-2) [81] and examples of negative regulators include BACH-1 and c-Maf. These negative regulators bind to ARE and decrease binding of Nrf-2 by competitive binding and this depresses ARE mediated gene expression [82]. BACH1 is a transcriptional repressor involved in regulating oxidative stress. It binds to NF-E2 sites on DNA and cooperates with MAF proteins to repress the transcription of heme oxygenase 1 (HO-1). MicroRNA targeting of BACH-1 should thus lead to an increase of HO-1 activity, which stimulates survival of *de novo* infected cells [83]. xCT is recently found to be able to act as a fusion-entry receptor for KSHV as well [84]. Qin *et al* found that **kshv-miR-K12-11** upregulates the expression of xCT in macrophages and endothelial cells. This is achieved partly by suppression of the negative regulator of gene transcription BACH-1. KSHV miRNAs are also found to induce the macrophage secretion of reactive nitrogen species (RNS) what protects these cells from RNS-induced cell death by upregulation of xCT. Reducing the NOS activity and RNS secretion of macrophages lowers their permissiveness for KSHV. More advanced KS tumors have a higher expression of xCT than early stage tumor cells. Thus Qin *et al* propose that miRNAs facilitate KS pathogenesis by cooperative mechanisms that regulate xCT en RNS secretion. This would facilitate *de novo* infection and increase the survival of infected cells in the tumor microenvironment.

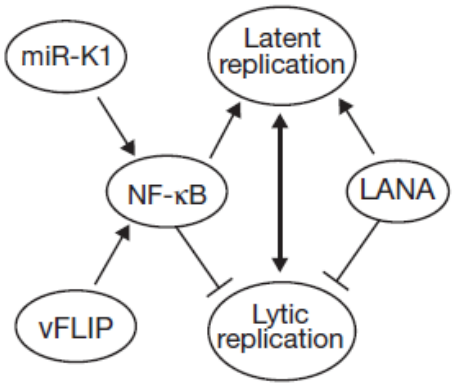


Figure 5-2 Model of KSHV latency and replication regulated by latent genes and miR-K1[1]

It has been suggested before that BACH-1 and c-Maf are regulated by KHSV-miRs [28, 85, 86]. MiR-K12-11 is an ortholog of cellular miR-155 which targets and reduces the expression of BACH-1 [28]. MiR-155 reduces the expression of c-Maf in T-cells [86] and KSHV miRs are found to downregulate c-Maf expression in epithelial cells [85]. On this basis Qin *et al* proposed that K12-11 cooperates with other miRNAs to regulate xCT expression. They found that K12-11 downregulates BACH-1 and induces xCT expression in both macrophages and endothelial cells. Screening the genes that are involved in xCT expression and RNS secretion regulation revealed several binding sites for kshv-K12-1, K12-9 and K12-11. In the 3' UTR of BACH-1 binding sites for K12-4 could be identified but not for K12-1 or K12-9. The virus is involved in the autocrine upregulation of its own receptor, but what the biological implications are is not clear yet [68].

5.7 THBS1

Thrombospondin (THBS1) has been identified as a potential KSHV-miR target via gene expression profiling of cells engineered to stably express 10 KSHV pre-miRNAs by the group of Renne. Trombospondin regulates angiogenesis and cell growth by activating transforming growth factor β (TGF-β). There is a reduced THBS1 activity in KS tumors [27].
KSHV miRNAs downregulate the expression of multi-cellular glycoprotein THBS1 which has anti-angiogenic and antiproliferative activity and stimulates immune evasion [87]. THSB1 is targeted by several KSHV miRNAs, in particular **kshv-miR-K12–1, miR-K12-3-3p, miR-K12-6-3p,** and **miR-K12–11**. The down regulation of THBS1 leads to reduced levels of TGF-β [27].

5.8 TOMM22 & IPO7

Some new miRNA targets have been found after immunoprecipitation of RISCs followed by microarray analysis of the RISC bound miRNA targets. This technique has identified hundreds of new cellular miRNA targets [88]. The method is based on positive selection of viral miRNA targets. TOMM22 and IPO7 are also identified using this technique. TOMM22 and IPO7 are targets of **ebv-miR-BART16** and **ebv-mir-BART3**, respectively. These two proteins are involved in protein import from the cytosol into the mitochondria (TOMM22) [89] or the nuclei (IPO7) [90]. Antisense knockdown of TOMM22 inhibits the association of pro-apoptotic BAX with the mitochondria and prevents BAX-induced apoptosis [91]. IPO7 has been linked to the innate immunity. Antisense knockdown of IPO7 in macrophages produces an inhibited production of pro-inflammatory cytokine IL-6 after LPS stimulation [92]. Yang *et al* showed also that FBX09 is needed for the efficient production of IL-6 in certain macrophage types and FBX09 was also on the target list of Dölken *et al*. EBV miRNA seems to modulate cellular trafficking and protein localization to prevent activation of the innate immune system and apoptosis [37]. By reducing the IκBα levels the NFκB pathway gets activated, which mediates vFLIP inhibition of KSHV lytic replication [93].

5.9 BCLAF1

The Blc-2 associated transcription factor (BCLAF1) gene encodes a transcriptional repressor that can interact with several members of the Bcl-2 protein family. Overexpression of BCLAF1 promotes apoptosis by preventing the transcription of Bcl-2; this can be suppressed by co-expression of Bcl-2 proteins. Ziegelbauer *et al* focused on identifying cellular targets of **kshv-miR-K5**. Their study showed that the seed sequence of miR-K5 has a perfectly homologous target sequence in BCLAF1. Transfection of miR-K5 leads to downregulation of BCLAF1 levels. Furthermore they cloned the gene of interest 3' UTR downstream of a luciferase reporter gene and cotransfected it with its target or random miRNA. If the miRNA is matched to the target the luciferase signal will be downregulated. They mutated the seed sequence to confirm their findings. If the same mutation was introduced in the miRNA the effect was restored. Downregulation of BCLAF1 is associated with a reduced sensitivity to apoptosis and BCLAF1 action also impairs lytic replication [29].

5.10 Homology cellular miRNA

A fraction of the viral microRNAs exert their effect by mimicking the hosts own microRNAs.
For example, e**bv-miR-BART5** is an intron of the BART transcript and shares seed sequence homology with the cellular miR-18a and -18b which are part of the miR-17-92 cluster that is often upregulated in B cell lymphomas [28, 94]. *In silico* analysis showed that **Ebv-miR-BART1-3p** has seed similarity with miR-29abc, a cellular microRNA that plays a role in apoptosis, however, this has not been proved experimentally yet [95].

miR-155

KSHV, EBV and other viruses influence the cellular levels of miR-155. Increased levels of miR-155 are found in several tumor types. Host miR-155 can be processed from sequences present in BIC RNA, a spliced and polyadenylated but non-protein-coding RNA that accumulates in human B cell lymphomas. An elevated level of miR-155 might directly or indirectly reduce the synthesis of a protein with tumor suppressor or pro-apoptotic function. **Kshv-miR-K12-11** is an ortholog of cellular miR-155 which targets and reduces the expression of BACH-1 [28]. MiR-155 reduces the expression of c-Maf in T-cells [86] and KSHV miRs are found to downregulate c-Maf expression in epithelial cells, stimulating the survival in *de novo* infected cells [85].
Marek's disease virus serotype 1 (MDV-1) encodes a miR-155 ortholog as well [96] and EBV induces the expression of miR-155 in latency III B cells [97]. Reticulo-endotheliosis virus strain T induces cellular miR-155 too, which promotes cell survival [98]. This might point out a selective advantage for the viruses to increase miR-155 expression.

Viral effects on cellular miRNA

The expression of cellular miRNAs is influenced strongly by viral infections. This includes both host antiviral defenses and viral factors that influence the host micromilieu. For example, EBV induces the expression of both miR-155 and miR-146a in infected B cells [97]. miR-146a is also upregulated upon bacterial infection and is suggested to play a role in the negative feedback loop to limit innate immune responses [99]. During latency III EBV LMP1 activates the promoter of 146a what leads to a 146a induced decline of IFN-responsive genes [97]. LMP1 also induces the expression of cellular miR-29b which leads to downregulation of T cell leukemia gene 1 (TCL1), a gene with roles in cell survival and proliferation [100]. LMP1 in its turn is being targeted by EBV miRNAs, so EBV can influence both viral and host miRNA to modulate antiviral IFN signaling to increase the persistence of infected cells. It is also possible that viruses disturb the normal host miRNA synthesis by overexpression their own viral miRNAs.

6. Conclusion

MicroRNAs are short (18-25 nt), non-coding stretches of RNA that can selectively modulate posttranslational gene expression. Recently it has been shown that viruses utilize miRNAs as well. They can both manipulate their host's miRs and encode their own. Gamma-herpesviruses encode the most miRNAs: EBV has 25 pre-miRs, rLCV 32 and KSHV 12. The best characterized cellular targets of viral miRNAs are those of KSHV encoded miRNAs [4].

The discovery of viral miRNAs provides us with new insights on transformation and immune evasion [31]. Knocking down parts of the miRNA processing machinery like Dicer leads to an increased viral infection [101].

Viral miRNA can target both viral and cellular transcripts, the first are easier to identify due to its smaller genome size. Viruses can use miRNA to create an optimized environment for successful infection and replication. Furthermore they are involved in cellular reprogramming to control the latent-lytic switch and they can modulate the host immune response. MiRNAs function dose-dependent, 2-fold differences already have significant biological consequences. Viral microRNAs are not very conserved, this lack of sequence homology can attributed to the higher rate of mutations and faster evolution in viruses when compared to eukaryotes [24].

Viral targets include the EBV DNA polymerase BALF5 to prevent accidental induction of viral replication and the viral proteins LMP1 and -2a to promote cell survival and evasion of the immune system. Cellular miRNA targets include MICB, which plays a role in immune recognition of infected cells and IκBα, the brake on the NFκB pathway. Homology to cellular microRNAs is an additional method that viruses exploit to modulate their environment.

Other regulators of cell survival and growth are targeted by viral miRNA as well. For example, cellular protein BCLAF1 , which plays a role in apoptosis, is targeted by kshv-miR-K5 in both B cells and endothelial cells [29]. siRNA inhibition of BCLAF1 leads to an increase of lytic replication.

Viruses are able to mimic cellular miRNAs or affect their expression in order to control existing regulatory pathways. The other way around, cellular miRNAs are able to directly influence viral replication as well, some miRNAs are even thought to directly target mammalian RNA virus genomes. Future research would be about identifying mRNA targets of viral miRNAs and finding out how these viral miRNAs are involved in the latent-lytic switch and immune suppression and what the contribution is of these viral miRNA to viral pathogenesis. The latter is interesting to find out if these viral miRNAs could be targeted for antiviral therapy.

Concluding, we know that viral miRNAs play a role in establishing latent infection and driving carcinogenesis in epithelial cells (KSHV), but both cellular and additional viral targets remain to be found, identified and characterized [30].

7. Abbreviations

ARE	antioxidant response element
BART	BamH1-A rightward transcript
BHRF1	BamH1 rightward reading frame 1
BL	Burkitt's lymphoma
DGCR8	DiGeorge critical region 8
DNMTs	DNA methyltransferases
EBNA	EBV nuclear antigen
EBV	Epstein-Barr virus
EBVaGC	EBV-associated gastric carcinoma
hCMV	human Cytomegalovirus
HL/HD	Hodgkins lymphoma/ disease
IM	infectious mononucleosis
KSHV	Kaposi sarcoma-associated herpesvirus
LANA	latency associated nuclear antigen
LMP	latent membrane proteins
MAF	musculoaponeurotic fibrosarcoma oncogenes homologue
MH68	mouse herpesvirus 68
miRNA	microRNA
MICB	major histocompatibility complex class I-related chain B
NPC	nasopharyngeal carcinoma
RISC	RNA induced silencing complex
RNS	reactive nitrogen species
rLCV,	rhesus lymphocryptovirus
RTA	replication and transcription activator
SV40	simian virus 40
TRBP	Tar RNA binding protein

8. Literature

1. Lei, X., et al., *Regulation of NF-kappaB inhibitor IkappaBalpha and viral replication by a KSHV microRNA.* Nat Cell Biol. **12**(2): p. 193-9.
2. Lung, R.W., et al., *Modulation of LMP2A expression by a newly identified Epstein-Barr virus-encoded microRNA miR-BART22.* Neoplasia, 2009. **11**(11): p. 1174-84.
3. Kilger, E., et al., *Epstein-Barr virus-mediated B-cell proliferation is dependent upon latent membrane protein 1, which simulates an activated CD40 receptor.* EMBO J, 1998. **17**(6): p. 1700-9.
4. Skalsky, R.L. and B.R. Cullen, *Viruses, microRNAs, and Host Interactions.* Annu Rev Microbiol.
5. Caldwell, R.G., et al., *Epstein-Barr virus LMP2A drives B cell development and survival in the absence of normal B cell receptor signals.* Immunity, 1998. **9**(3): p. 405-11.
6. Kuppers, R., *B cells under influence: transformation of B cells by Epstein-Barr virus.* Nat Rev Immunol, 2003. **3**(10): p. 801-12.
7. Cullen, B.R., *Viruses and microRNAs.* Nat Genet, 2006. **38 Suppl**: p. S25-30.
8. Griffiths-Jones, S., et al., *miRBase: microRNA sequences, targets and gene nomenclature.* Nucleic Acids Res, 2006. **34**(Database issue): p. D140-4.
9. Friedman, J.M., P.A. Jones, and G. Liang, *The tumor suppressor microRNA-101 becomes an epigenetic player by targeting the polycomb group protein EZH2 in cancer.* Cell Cycle, 2009. **8**(15): p. 2313-4.
10. Pfeffer, S., et al., *Identification of virus-encoded microRNAs.* Science, 2004. **304**(5671): p. 734-6.
11. Bartel, D.P., *MicroRNAs: target recognition and regulatory functions.* Cell, 2009. **136**(2): p. 215-33.
12. Liu, C.G., et al., *An oligonucleotide microchip for genome-wide microRNA profiling in human and mouse tissues.* Proc Natl Acad Sci U S A, 2004. **101**(26): p. 9740-4.
13. Umbach, J.L., et al., *MicroRNAs expressed by herpes simplex virus 1 during latent infection regulate viral mRNAs.* Nature, 2008. **454**(7205): p. 780-3.
14. Grimson, A., et al., *MicroRNA targeting specificity in mammals: determinants beyond seed pairing.* Mol Cell, 2007. **27**(1): p. 91-105.
15. Baek, D., et al., *The impact of microRNAs on protein output.* Nature, 2008. **455**(7209): p. 64-71.
16. Obernosterer, G., et al., *Post-transcriptional regulation of microRNA expression.* RNA, 2006. **12**(7): p. 1161-7.
17. Luciano, D.J., et al., *RNA editing of a miRNA precursor.* RNA, 2004. **10**(8): p. 1174-7.
18. Kawahara, Y., et al., *Redirection of silencing targets by adenosine-to-inosine editing of miRNAs.* Science, 2007. **315**(5815): p. 1137-40.
19. Das, A.K. and G.G. Carmichael, *ADAR editing wobbles the microRNA world.* ACS Chem Biol, 2007. **2**(4): p. 217-20.
20. Franco-Zorrilla, J.M., et al., *Target mimicry provides a new mechanism for regulation of microRNA activity.* Nat Genet, 2007. **39**(8): p. 1033-7.
21. Krutzfeldt, J., et al., *Silencing of microRNAs in vivo with 'antagomirs'.* Nature, 2005. **438**(7068): p. 685-9.
22. Care, A., et al., *MicroRNA-133 controls cardiac hypertrophy.* Nat Med, 2007. **13**(5): p. 613-8.
23. Pfeffer, S., et al., *Identification of microRNAs of the herpesvirus family.* Nat Methods, 2005. **2**(4): p. 269-76.
24. Ghosh, Z., B. Mallick, and J. Chakrabarti, *Cellular versus viral microRNAs in host-virus interaction.* Nucleic Acids Res, 2009. **37**(4): p. 1035-48.
25. Gottwein, E., et al., *A viral microRNA functions as an orthologue of cellular miR-155.* Nature, 2007. **450**(7172): p. 1096-9.

26. Nachmani, D., et al., *Diverse herpesvirus microRNAs target the stress-induced immune ligand MICB to escape recognition by natural killer cells.* Cell Host Microbe, 2009. **5**(4): p. 376-85.
27. Samols, M.A., et al., *Identification of cellular genes targeted by KSHV-encoded microRNAs.* PLoS Pathog, 2007. **3**(5): p. e65.
28. Skalsky, R.L., et al., *Kaposi's sarcoma-associated herpesvirus encodes an ortholog of miR-155.* J Virol, 2007. **81**(23): p. 12836-45.
29. Ziegelbauer, J.M., C.S. Sullivan, and D. Ganem, *Tandem array-based expression screens identify host mRNA targets of virus-encoded microRNAs.* Nat Genet, 2009. **41**(1): p. 130-4.
30. Choy, E.Y., et al., *An Epstein-Barr virus-encoded microRNA targets PUMA to promote host cell survival.* J Exp Med, 2008. **205**(11): p. 2551-60.
31. Xia, T., et al., *EBV microRNAs in primary lymphomas and targeting of CXCL-11 by ebv-mir-BHRF1-3.* Cancer Res, 2008. **68**(5): p. 1436-42.
32. Zhu, J.Y., et al., *Identification of novel Epstein-Barr virus microRNA genes from nasopharyngeal carcinomas.* J Virol, 2009. **83**(7): p. 3333-41.
33. Gottwein, E., X. Cai, and B.R. Cullen, *A novel assay for viral microRNA function identifies a single nucleotide polymorphism that affects Drosha processing.* J Virol, 2006. **80**(11): p. 5321-6.
34. Elbashir, S.M., W. Lendeckel, and T. Tuschl, *RNA interference is mediated by 21- and 22-nucleotide RNAs.* Genes Dev, 2001. **15**(2): p. 188-200.
35. Grad, Y., et al., *Computational and experimental identification of C. elegans microRNAs.* Mol Cell, 2003. **11**(5): p. 1253-63.
36. Grundhoff, A., C.S. Sullivan, and D. Ganem, *A combined computational and microarray-based approach identifies novel microRNAs encoded by human gamma-herpesviruses.* RNA, 2006. **12**(5): p. 733-50.
37. Dolken, L., et al., *Systematic analysis of viral and cellular microRNA targets in cells latently infected with human gamma-herpesviruses by RISC immunoprecipitation assay.* Cell Host Microbe. **7**(4): p. 324-34.
38. Epstein, M.A., B.G. Achong, and Y.M. Barr, *Virus Particles in Cultured Lymphoblasts from Burkitt's Lymphoma.* Lancet, 1964. **1**(7335): p. 702-3.
39. Lo, A.K., et al., *Modulation of LMP1 protein expression by EBV-encoded microRNAs.* Proc Natl Acad Sci U S A, 2007. **104**(41): p. 16164-9.
40. Klein, E., L.L. Kis, and G. Klein, *Epstein-Barr virus infection in humans: from harmless to life endangering virus-lymphocyte interactions.* Oncogene, 2007. **26**(9): p. 1297-305.
41. Sixbey, J.W., et al., *Epstein-Barr virus replication in oropharyngeal epithelial cells.* N Engl J Med, 1984. **310**(19): p. 1225-30.
42. Niedobitek, G., H. Herbst, and H. Stein, *Epstein-Barr virus/complement receptor and epithelial cells.* Lancet, 1989. **2**(8654): p. 110.
43. Xing, L. and E. Kieff, *Epstein-Barr virus BHRF1 micro- and stable RNAs during latency III and after induction of replication.* J Virol, 2007. **81**(18): p. 9967-75.
44. Khan, G., et al., *Is EBV persistence in vivo a model for B cell homeostasis?* Immunity, 1996. **5**(2): p. 173-9.
45. Bellare, P. and D. Ganem, *Regulation of KSHV lytic switch protein expression by a virus-encoded microRNA: an evolutionary adaptation that fine-tunes lytic reactivation.* Cell Host Microbe, 2009. **6**(6): p. 570-5.
46. Thorley-Lawson, D.A., *Epstein-Barr virus: exploiting the immune system.* Nat Rev Immunol, 2001. **1**(1): p. 75-82.
47. Schaefer, B.C., J.L. Strominger, and S.H. Speck, *Redefining the Epstein-Barr virus-encoded nuclear antigen EBNA-1 gene promoter and transcription initiation site in group I Burkitt lymphoma cell lines.* Proc Natl Acad Sci U S A, 1995. **92**(23): p. 10565-9.

48. Schaefer, B.C., J.L. Strominger, and S.H. Speck, *The Epstein-Barr virus BamHI F promoter is an early lytic promoter: lack of correlation with EBNA 1 gene transcription in group 1 Burkitt's lymphoma cell lines.* J Virol, 1995. **69**(8): p. 5039-47.
49. Levitskaya, J., et al., *Inhibition of antigen processing by the internal repeat region of the Epstein-Barr virus nuclear antigen-1.* Nature, 1995. **375**(6533): p. 685-8.
50. Fahraeus, R., et al., *Expression of Epstein-Barr virus-encoded proteins in nasopharyngeal carcinoma.* Int J Cancer, 1988. **42**(3): p. 329-38.
51. Takada, K., K. Yamamoto, and T. Osato, *Analysis of the transformation of human lymphocytes by Epstein-Barr virus. II. Abortive response of leukemic cells to the transforming virus.* Intervirology, 1980. **13**(4): p. 223-31.
52. Voinnet, O., *Induction and suppression of RNA silencing: insights from viral infections.* Nat Rev Genet, 2005. **6**(3): p. 206-20.
53. Cai, X., et al., *Epstein-Barr virus microRNAs are evolutionarily conserved and differentially expressed.* PLoS Pathog, 2006. **2**(3): p. e23.
54. Cosmopoulos, K., et al., *Comprehensive profiling of Epstein-Barr virus microRNAs in nasopharyngeal carcinoma.* J Virol, 2009. **83**(5): p. 2357-67.
55. Cai, X. and B.R. Cullen, *Transcriptional origin of Kaposi's sarcoma-associated herpesvirus microRNAs.* J Virol, 2006. **80**(5): p. 2234-42.
56. Barth, S., et al., *Epstein-Barr virus-encoded microRNA miR-BART2 down-regulates the viral DNA polymerase BALF5.* Nucleic Acids Res, 2008. **36**(2): p. 666-75.
57. Zhang, L. and J.S. Pagano, *Interferon regulatory factor 7: a key cellular mediator of LMP-1 in EBV latency and transformation.* Semin Cancer Biol, 2001. **11**(6): p. 445-53.
58. Pratt, Z.L., et al., *The microRNAs of Epstein-Barr Virus are expressed at dramatically differing levels among cell lines.* Virology, 2009. **386**(2): p. 387-97.
59. Walz, N., et al., *A global analysis of evolutionary conservation among known and predicted gammaherpesvirus microRNAs.* J Virol. **84**(2): p. 716-28.
60. Sun, R., et al., *A viral gene that activates lytic cycle expression of Kaposi's sarcoma-associated herpesvirus.* Proc Natl Acad Sci U S A, 1998. **95**(18): p. 10866-71.
61. Haque, M., et al., *Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) contains hypoxia response elements: relevance to lytic induction by hypoxia.* J Virol, 2003. **77**(12): p. 6761-8.
62. Cesarman, E., et al., *Kaposi's sarcoma-associated herpesvirus-like DNA sequences in AIDS-related body-cavity-based lymphomas.* N Engl J Med, 1995. **332**(18): p. 1186-91.
63. Decker, L.L., et al., *The Kaposi sarcoma-associated herpesvirus (KSHV) is present as an intact latent genome in KS tissue but replicates in the peripheral blood mononuclear cells of KS patients.* J Exp Med, 1996. **184**(1): p. 283-8.
64. Ballestas, M.E., P.A. Chatis, and K.M. Kaye, *Efficient persistence of extrachromosomal KSHV DNA mediated by latency-associated nuclear antigen.* Science, 1999. **284**(5414): p. 641-4.
65. Samols, M.A., et al., *Cloning and identification of a microRNA cluster within the latency-associated region of Kaposi's sarcoma-associated herpesvirus.* J Virol, 2005. **79**(14): p. 9301-5.
66. Muralidhar, S., et al., *Identification of kaposin (open reading frame K12) as a human herpesvirus 8 (Kaposi's sarcoma-associated herpesvirus) transforming gene.* J Virol, 1998. **72**(6): p. 4980-8.
67. Cai, X., et al., *Kaposi's sarcoma-associated herpesvirus expresses an array of viral microRNAs in latently infected cells.* Proc Natl Acad Sci U S A, 2005. **102**(15): p. 5570-5.
68. Qin, Z., et al., *Upregulation of xCT by KSHV-encoded microRNAs facilitates KSHV dissemination and persistence in an environment of oxidative stress.* PLoS Pathog. **6**(1): p. e1000742.
69. O'Hara, A.J., et al., *Pre-micro RNA signatures delineate stages of endothelial cell transformation in Kaposi sarcoma.* PLoS Pathog, 2009. **5**(4): p. e1000389.

70. Xu, Y., A. Rodriguez-Huete, and G.S. Pari, *Evaluation of the lytic origins of replication of Kaposi's sarcoma-associated virus/human herpesvirus 8 in the context of the viral genome.* J Virol, 2006. **80**(19): p. 9905-9.
71. Umbach, J.L. and B.R. Cullen, *In-depth analysis of Kaposi's sarcoma-associated herpesvirus microRNA expression provides insights into the mammalian microRNA-processing machinery.* J Virol. **84**(2): p. 695-703.
72. Lu, F., et al., *Epigenetic regulation of Kaposi's sarcoma-associated herpesvirus latency by virus-encoded microRNAs that target Rta and the cellular Rbl2-DNMT pathway.* J Virol. **84**(6): p. 2697-706.
73. Stern-Ginossar, N., et al., *Host immune system gene targeting by a viral miRNA.* Science, 2007. **317**(5836): p. 376-81.
74. Hansen, A., et al., *KSHV-encoded miRNAs target MAF to induce endothelial cell reprogramming.* Genes Dev. **24**(2): p. 195-205.
75. Eychene, A., N. Rocques, and C. Pouponnot, *A new MAFia in cancer.* Nat Rev Cancer, 2008. **8**(9): p. 683-93.
76. Wang, L., et al., *The Kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8) K1 protein induces expression of angiogenic and invasion factors.* Cancer Res, 2004. **64**(8): p. 2774-81.
77. Zhu, F.X., T. Cusano, and Y. Yuan, *Identification of the immediate-early transcripts of Kaposi's sarcoma-associated herpesvirus.* J Virol, 1999. **73**(7): p. 5556-67.
78. Chaudhary, P.M., et al., *Modulation of the NF-kappa B pathway by virally encoded death effector domains-containing proteins.* Oncogene, 1999. **18**(42): p. 5738-46.
79. Matta, H. and P.M. Chaudhary, *Activation of alternative NF-kappa B pathway by human herpes virus 8-encoded Fas-associated death domain-like IL-1 beta-converting enzyme inhibitory protein (vFLIP).* Proc Natl Acad Sci U S A, 2004. **101**(25): p. 9399-404.
80. Lo, M., Y.Z. Wang, and P.W. Gout, *The x(c)- cystine/glutamate antiporter: a potential target for therapy of cancer and other diseases.* J Cell Physiol, 2008. **215**(3): p. 593-602.
81. Ishii, T., et al., *Transcription factor Nrf2 coordinately regulates a group of oxidative stress-inducible genes in macrophages.* J Biol Chem, 2000. **275**(21): p. 16023-9.
82. Dhakshinamoorthy, S., et al., *Bach1 competes with Nrf2 leading to negative regulation of the antioxidant response element (ARE)-mediated NAD(P)H:quinone oxidoreductase 1 gene expression and induction in response to antioxidants.* J Biol Chem, 2005. **280**(17): p. 16891-900.
83. McAllister, S.C., et al., *Kaposi sarcoma-associated herpesvirus (KSHV) induces heme oxygenase-1 expression and activity in KSHV-infected endothelial cells.* Blood, 2004. **103**(9): p. 3465-73.
84. Kaleeba, J.A. and E.A. Berger, *Kaposi's sarcoma-associated herpesvirus fusion-entry receptor: cystine transporter xCT.* Science, 2006. **311**(5769): p. 1921-4.
85. Hong, Y.K., et al., *Lymphatic reprogramming of blood vascular endothelium by Kaposi sarcoma-associated herpesvirus.* Nat Genet, 2004. **36**(7): p. 683-5.
86. Rodriguez, A., et al., *Requirement of bic/microRNA-155 for normal immune function.* Science, 2007. **316**(5824): p. 608-11.
87. McClure, L.V. and C.S. Sullivan, *Kaposi's sarcoma herpes virus taps into a host microRNA regulatory network.* Cell Host Microbe, 2008. **3**(1): p. 1-3.
88. Baroni, T.E., et al., *Advances in RIP-chip analysis : RNA-binding protein immunoprecipitation-microarray profiling.* Methods Mol Biol, 2008. **419**: p. 93-108.
89. Saeki, K., et al., *Identification of mammalian TOM22 as a subunit of the preprotein translocase of the mitochondrial outer membrane.* J Biol Chem, 2000. **275**(41): p. 31996-2002.
90. Gorlich, D., et al., *A novel class of RanGTP binding proteins.* J Cell Biol, 1997. **138**(1): p. 65-80.

91. Bellot, G., et al., *TOM22, a core component of the mitochondria outer membrane protein translocation pore, is a mitochondrial receptor for the proapoptotic protein Bax.* Cell Death Differ, 2007. **14**(4): p. 785-94.
92. Yang, I.V., et al., *Identification of novel genes that mediate innate immunity using inbred mice.* Genetics, 2009. **183**(4): p. 1535-44.
93. Ye, F.C., et al., *Kaposi's sarcoma-associated herpesvirus latent gene vFLIP inhibits viral lytic replication through NF-kappaB-mediated suppression of the AP-1 pathway: a novel mechanism of virus control of latency.* J Virol, 2008. **82**(9): p. 4235-49.
94. Gottwein, E. and B.R. Cullen, *Viral and cellular microRNAs as determinants of viral pathogenesis and immunity.* Cell Host Microbe, 2008. **3**(6): p. 375-87.
95. Waidner, L.A., et al., *MicroRNAs of Gallid and Meleagrid herpesviruses show generally conserved genomic locations and are virus-specific.* Virology, 2009. **388**(1): p. 128-36.
96. Burnside, J., et al., *Marek's disease virus encodes MicroRNAs that map to meq and the latency-associated transcript.* J Virol, 2006. **80**(17): p. 8778-86.
97. Cameron, J.E., et al., *Epstein-Barr virus latent membrane protein 1 induces cellular MicroRNA miR-146a, a modulator of lymphocyte signaling pathways.* J Virol, 2008. **82**(4): p. 1946-58.
98. Bolisetty, M.T., et al., *Reticuloendotheliosis virus strain T induces miR-155, which targets JARID2 and promotes cell survival.* J Virol, 2009. **83**(23): p. 12009-17.
99. Xiao, C. and K. Rajewsky, *MicroRNA control in the immune system: basic principles.* Cell, 2009. **136**(1): p. 26-36.
100. Anastasiadou, E., et al., *Epstein-Barr virus encoded LMP1 downregulates TCL1 oncogene through miR-29b.* Oncogene. **29**(9): p. 1316-28.
101. Chable-Bessia, C., et al., *Suppression of HIV-1 replication by microRNA effectors.* Retrovirology, 2009. **6**: p. 26.