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Gamma-Herpesvirus-encoded miRNAs and their roles in viral biology and pathogenesis

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Abstract

To date, more than 200 viral miRNAs have been identified mostly from herpesviruses and this rapidly evolving field has recently been summarized in a number of excellent reviews (see [1, 2]). Unique to γ -herpesviruses, like Kaposi's sarcoma-associated herpesvirus and Epstein-Barr virus, is their ability to cause cancer. Here, we discuss γ -herpesvirus-encoded miRNAs and focus on recent findings which support the hypothesis that viral miRNAs directly contribute to pathogenesis and tumorigenesis. The observations that KSHV mimics a human tumorigenic miRNA (hsa-miR-155), which is induced in EBV-infected cells and required for the survival of EBV-immortalized cells, lead to a number of studies demonstrating that perturbing this pathway induces B cell proliferation *in vivo* and immortalization of human B cells *in vitro*. Secondly, the application of state of the art ribonomics methods to globally identify viral miRNA targets in virus-infected tumor cells provides a rich resource to the KSHV and EBV fields and largely expanded our understanding on how viral miRNAs contribute to viral biology.

Introduction

γ -herpesviruses encode miRNAs

miRNAs are short (21–23 nt), non-coding RNAs that bind to partially complementary sequences in the 3'UTR of target transcripts to inhibit their translation and/or induce their degradation. miRNAs have been identified in most eukaryotes, from single cell organisms like algae and amoebae to organisms all across the metazoa (miRBase). Metazoan miRNAs are crucial regulators of many biological processes including development, hematopoiesis, and stem cell differentiation to name a few and are aberrantly expressed in many human malignancies (for review [3]). In 2004 the first virally-encoded miRNAs were identified in Epstein-Barr virus (EBV)-infected Burkitt's lymphoma cells[4]. Since then more than 200 mature miRNAs have been identified in all herpesviruses analyzed so far, except for Varicella Zoster virus (for recent review see [1, 2]). While the initial study identified 5 EBV

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miRNAs in the B95-8 strain which contains a deletion, follow-up studies identified a total of 44 EBV miRNAs that are located within two clusters (Fig. 1) [5–7]. The EBV closely related Rhesus lymphocryptovirus (rLCV) encodes 36 miRNAs of which 18 have sequences conserved to EBV miRNAs [5, 8, 9]. Kaposi's sarcoma-associated herpesvirus (KSHV) contains 12 miRNA genes that are located within the latency-associated region, 10 in the intragenic region between v-FLIP and the Kaposin locus and two embedded within the K12 open reading frame (Fig. 1) [6, 10–12]. Rhesus Rhadinovirus, a γ -herpesvirus closely related to KSHV encodes 7 miRNA genes that are also located within the latency-associated region; however unlike EBV/rLCV, no sequence conservation has been observed between KSHV and RRV miRNAs [8, 9, 13]. Murine γ -herpesvirus type 68 (MHV68) contains 15 miRNA genes at the 5' end of the genome that are unusual since they are part of tRNA-like genes transcribed by RNA pol III and processed Drosha-independently by tRNaseZ [14–16]. A second example of non-canonical miRNA maturation was reported for Herpesvirus saimiri miRNAs that are cleaved from HSURs by the integrator complex [17]. However, most viral miRNAs, like their cellular counterparts, are processed from RNA Pol II transcripts as part of a 70–80 nts long RNA stem-loop, the primary (pri)-miRNA. The pri-miRNA is cleaved in the nucleus by the RNase III-like enzyme Drosha together with DGCR8 liberating the pre-miRNA, which is rapidly exported from the nucleus by Exportin 5/Ran-GTP, and cleaved by a cytoplasmic RNase III-like enzyme, Dicer, resulting in a 21–23 nt RNA duplex. The guide strand is then incorporated into the RNA-induced silencing complex, while the passenger strand is rapidly degraded (for review see [3, 18]). For many EBV and KSHV miRNAs both strands can be introduced into RISC and as a result the 12 KSHV miRNA hairpins give rise to a total of 25 miRNAs (one is edited). The major component of RISC is the Argonaute (Ago) protein. In mammalian cells there are four Ago proteins that are all incorporated into RISC, however, only Ago2 has endonuclease activity, thereby cleaving the target transcript. Ago contains two RNA-binding domains, the PAZ domain binding the miRNA 3' end and the PIWI domain interacting with the 5' end thereby guiding the miRNAs to their mRNA targets (reviewed by Yang et al. [19]). While the exact mechanism(s) of how miRNAs regulate gene expression are still under debate, miRNA targeting generally leads to translational inhibition which often but not always induces mRNA destabilization [20–22]. Mammalian miRNAs predominantly bind to the 3'UTR of mRNAs; however targeting of coding regions has also been observed, albeit less efficiently regulating target genes [20, 21, 23, 24]. MiRNA targeting is predominantly determined by the seed sequence, which comprises nts 2–8 at the miRNA 5' end that are fully complementary to the target [25]. Seed pairing is supplemented by additional base pairing at the 3' end of the miRNA. Moreover, exceptions exist where miRNAs lack perfect seed and instead show 3' compensatory base pairing [25]. To date no mechanistic differences have been reported for host or viral miRNA targeting and to understand miRNA function requires comprehensive target identification as well as identifying phenotypes associated with miRNA perturbation (loss- and gain- of function studies). Since γ -herpesviruses infect different cell types (i.e. lymphoid and endothelial for KSHV) tissue-specific miRNA expression levels will affect targeting. O'Hara reported differential KSHV and host miRNA expression profiles in KSHV-infected PEL cells, HUVEC cells and KS tumor cells [26]. Similarly, EBV miRNA profiles differ between different latency programs in B cells and nasopharyngeal carcinomas [5, 27].

Techniques and challenges to identify viral miRNA targets

Until recently, viral miRNA targets have mainly been identified by pair wise analysis approaches establishing that a gene initially predicted to be a target by a number of bioinformatic algorithms could be regulated by perturbation (over gain-of-function or loss-of-function) of a specific miRNA. These assays entail cloning and mutagenesis of 3'UTRs of potential targets down-stream of a reporter (luciferase or GFP) and/or monitoring potential targets by real-time RT-PCR in miRNA- or antimir-transfected cells (Table 1).

For viral miRNAs this approach has the added advantage of a built-in control by comparing infected to none-infected cells. While these approaches have identified a number of interesting targets discussed below in detail (Table 2), they mostly demonstrate whether a gene can be regulated by a particular miRNA. However, deciphering the complex regulatory networks of miRNAs and their contribution to viral biology requires to determine which host cellular and/or viral genes are regulated by viral miRNAs under a specific physiological condition as existing in EBV or KSHV latently infected B cells or tumor cells of lymphoid, epithelial, or endothelial origin. More genome-wide approaches such as transcriptome or proteome profiling, had limited success since these approaches determine expression differences but cannot distinguish direct from indirect or down-stream targeting [28–31].

Applying ribonomics approaches to generate tissue-specific target catalogues for KSHV and EBV miRNAs

Ribonomics approaches such as high-throughput sequencing of RNA isolated by cross-linking immunoprecipitation (HITS-CLIP) [32] and Photoactivatable-Ribonucleoside-Enhanced Cross-linking and immunoprecipitation (PAR-CLIP) [33] enable direct identification of miRNA targeted genes in EBV and KSHV infected cells of lymphoid origin [34–37]. Both techniques utilize UV cross-linking to fix RNA/protein interaction, followed by immunoprecipitation of Ago, which enriches miRNAs that are incorporated into RISC complexes and guided to their cognate targets. Compared to direct RNA immunoprecipitation as was also applied to KSHV and EBV infected PEL cells [38], these techniques prevent the risk of forming artificial post-lysis complexes by stabilizing specific RNA/protein interactions [39]. After Ago IP, enriched RNA/protein complexes are RNase treated, proteins are digested and the remaining miRNA and mRNA fragments after cDNA synthesis are analyzed by high throughput sequencing. HITS-CLIP uses direct cross-linking at 254 nm while for PAR-CLIP, cells are metabolically labeled with thiouridine (4SU), which is incorporated into newly synthesized RNA, and efficiently cross-linked at 365 nm and after reverse transcription leads to a T to C transition. This 4SU-induced PAR-CLIP footprint labels Ago/RNA interaction sites, which aids the bioinformatic analysis of large data sets [33]. HITS-CLIP can be efficiently applied to primary tissues since it does not require labeling prior to UV-cross-linking tissues [32]. Pros and cons of different ribonomics approaches to study RNA/Protein interaction as well as miRNA targets have recently been reviewed in great detail by Riley and Steitz [40]. Recently four laboratories reported HITS-CLIP or PAR-CLIP data sets for KSHV and EBV miRNAs: Riley et al. performed HITS-CLIP to study EBV miRNA targets in Jijoye cells, exhibiting type III latency and expressing all EBV miRNAs. More than 1600 potential EBV miRNA target were identified [36]. Skalsky et al. applied PAR-CLIP to a lymphoblastoid cell line (LCL) infected with an EBV laboratory strain, B95.8 lacking most of the BART miRNAs. This study reports a total of about 630 EBV miRNA targets [37]. The KSHV and EBV miRNA targetome was determined by Gottwein et al. in the KSHV/EBV co-infected primary effusion lymphoma (PEL) cell line BC-1 and the KSHV BC-3 line (PAR-CLIP, yielding more than 2000 putative targets genes) [34]. Finally, we identified more than 1600 putative targets for KSHV miRNAs in two KSHV-positive PEL cells BCBL-1 and BC-3 using HITS-CLIP [35]. These data sets, which represent a highly valuable resource, revealed in addition to the targets themselves, a number of emerging concepts that further our understanding on how viral miRNAs contribute to global gene expression in latently infected tumor cells.

More viral miRNA mimicry

Gottwein et al identified novel KSHV miRNAs with alternatively processed 5' ends, such as KSHV-miR-K10a_+1_5, which shares the seed sequence with an alternatively processed miR-142-3p_–1_5 (lacking one nt at the 5' end). It was demonstrated that miR-K10a_+1_5 indeed is a functional ortholog of miR-142-3p_–1_5 with a large set of common targets,

several of which were experimentally validated [34]. This is only the second functional ortholog of a human miRNA reported for KSHV in addition to miR-K12-11, which mimics the oncomir miR-155 [28, 30], a miRNA involved in lymphocyte activation [41]. MiR-142-3p variants are detected in KSHV PEL cells but not in uninfected and KSHV-infected endothelial cells TIME cells [42]. MiR-142-3p is a highly B cell-specific miRNA and simultaneous targeting of transcripts by miR-142-3p and miR-K10a may serve to ensure a miR-142-3p targeted pathway required for KSHV latency. In endothelial cells miR-K10a might mimic miR-142-3p function to create a more lymphoid cell-like environment [34, 43].

HITS-CLIP and PAR-CLIP revealed only few viral genes to be regulated by viral or host miRNAs

For KSHV, less than 2% of all reads were viral transcripts, which is not unexpected since all three studies analyzed latently infected cells where viral gene expression is highly restricted. In KSHV, both studies recovered target sites for miR-K10a, -K10b, miR-142-3p and the let7/miR-98 family in the 3'UTRs of LANA, vCyclin, v-FLIP, vIRF-3, and vIL-6, and within the ORFs of vCyclin and v-FLIP [34, 35]. The vIL-6 targeting was experimentally confirmed by luciferase reporter assay to be strongly down-regulated in the presence of miR-K10a [35]. Interestingly, vIL-6 is a mostly lytic gene involved in the inflammatory symptoms of KSHV-induced disorders such as MCD [44]. However, vIL-6 is also expressed at very low levels during latency in PEL cells [45].

For EBV targeting of EBNA2, LMP1 and BHRF1 was identified [36, 37]. LMP1 and BHRF1 clusters contained seed matches for the human miR-17/20/106 seed family and viral miRNAs (BART3, BART19-5p, BART5-5p, BART10-3p), which were all functionally validated. The miRNAs of the 17/20/106/93 seed family are part of three miRNA clusters, the oncogenic miR-17/92, the miR-106a-363 and miR-106b-25 cluster (reviewed by Olive et al. [46]). Both the miR-17/92 and 106/25 cluster are regulated by c-myc, which in the EBV-positive BL cells is the major driver of proliferation [47]. Hence, while the overall small number of viral targeting may suggest that latency-associated transcripts of γ -herpesviruses have been evolutionary purged from host miRNA seed matches over time, the opposite is true too, since the miR-17/20/106 target sites in LMP1 and BHRF1 3'UTRs are evolutionarily conserved between EBV and rhesus lymphocryptovirus (rLCV) [37], which separated more than 13 million years ago [5].

KSHV and EBV miRNAs target predominantly host transcripts within similar cellular pathways

HITS-CLIP as well as PAR-CLIP identified a large portion of previously validated viral miRNA targets (Table 2) and similar to human miRNAs, each viral miRNA targets between several dozen and a few hundred transcripts. This together with the high percentage of functionally confirmed targets in each study [34–37] strongly endorses HITS-CLIP and PAR-CLIP; however, since enriched clusters can contain multiple seed sequences, some targets may not be annotated correctly. Skalsky et al. utilized a recombinant EBV carrying a miRNA deletion and by this elegant approach determined that the false-positive rate in PAR-CLIP-identified targets was a modest 11% [37]. Surprisingly, while EBV and KSHV miRNAs show no seed sequence homology, Gottwein et al. report a high overlap between EBV and KSHV targets in BC-1 cells (>55%) [31, 34, 48, 49]. All four studies consistently reveal commonly targeted pathways, such as apoptosis, cell cycle control, intracellular transport, protein transport and localization, transcription regulation, proteolysis, and immune evasion. Prior to these studies only a few apoptotic genes were identified as KSHV [31, 48, 49] or EBV miRNA targets [50–52]. Similarly, few immune modulatory genes such as the NK ligand MICB [53, 54], IKBKE [55], LIP (CEBP/B) [56], IRAK1 and MYD88 [57] in KSHV, or CXCL11 [58] in EBV were previously identified. HITS-CLIP and PAR-CLIP

revealed dozens of apoptotic and immune modulatory genes targeted by KSHV and EBV miRNAs, thus not only confirming previous conclusions about viral miRNA functions but also underscoring the importance of these pathways. The fact that miRNAs with no sequence homology have evolved to target identical pathways strongly suggests that their downregulation is crucial for herpesvirus biology. In addition targets enriched for novel interesting cellular pathways, such as the glycolysis pathway in KSHV [35], the WNT pathway in EBV [8, 37], the MAPKKK cascade [34], and the PI3 kinase and Ras pathways [37] have been identified and future studies will determine their importance. We don't go deeper into details of specific new targets, but instead refer to the supplemental material describing both viral and cellular targetomes within all four studies [34–37].

Viral miRNAs co-target transcripts with human miRNAs

For KSHV 55–65% of viral miRNA targets had additional interaction sites for human miRNAs [35], in EBV 75–90% were co-targeted by human miRNAs [36, 37]. Interestingly, in Jijoye cells, 50% of the co-targeted transcripts are targets of the very abundant members of the miR-17/92 cluster. One factor that contributes to the high number of overlapping targets are seed homologies between human and viral miRNAs, such as miR-155/miR-K11, and miR-142-3p_–1_5/miR-K10a_+1_5 in KSHV or the miR-29/miR-BART1-3p and miR-18/miR-BART5-5p in EBV, which share full seed homology (nt 1–7, 2–7 or 2–8) [34, 43]. Additional miRNAs share a partial overlap (6-mer) that is offset by one nucleotide (e.g. miR-181/miR-K3, miR-15/miR-K6-5p, miR-27/miR-K11*, or miR-196/miR-K5* in KSHV (Haecker et al. unpublished observations). It has been well established that miRNAs with identical seed sequences (miR-155/miR-K11 and miR-142-3p/miR-K10a [28, 30] share some common targets but also have specific targets. The later concept has been elegantly analyzed by Riley et al who showed that nucleotides adjacent to the seed sequence had large effects on ortholog targeting [36]. Interestingly, seed homologies also exist among viral miRNAs. EBV miR-BHRF-1 and miR-BART4 or miR-BART1-3p and 3-3p share a seed that is off-set by only one nucleotide and the latter two indeed target the same 3'UTR in a luciferase reporter assay. Skalsky and coworkers suggest that this redundancy could help to ensure that important pathways are targeted during all stages of EBV latency which differ with respect to BART miRNA expression [37]. In somatic cells, miRNA-mediated post-transcriptional regulation mainly fine tunes transcript levels. Therefore, the effects of co-targeting cellular pathways under host miRNA control by viral miRNAs often expressed at high copy numbers could induce developmental expression patterns, as was reported for MAF in the transition from blood-endothelial to lymphoid endothelial cells [59]. Lastly while the overall viral miRNA levels in all four studies was 20 and 30%, in KSHV-infected BC-3 cells viral miRNAs comprised 70% of all RISC-associated miRNAs which was accompanied by an overall reduction of host miRNA targeting [35]. In BC-3 viral miRNAs may not be primarily enforcing cellular pathways under miRNA control but rather preventing host miRNAs access to the limited pool of RISC complexes [60]. Conceptually intriguing, this sequence-independent or non-canonical mode of viral miRNA-dependent regulation could result in a global up-regulation or de-repression of the host cellular transcriptome, which may be beneficial for large DNA viruses early after *de-novo* infection.

Biological functions of herpesviral miRNAs

Although recent works have identified many potential targets of KSHV and EBV miRNAs by PAR-CLIP and HITS-CLIP approaches, defining the role of a miRNA in infection and pathogenesis ultimately requires the demonstration of its biological function, particularly in the context of viral infection. Advances have been made recently to define the genuine biological functions of viral miRNAs though it remains limited in the context of viral infection. Previous studies have shown that several KSHV miRNAs regulate viral replication by directly or indirectly targeting the expression of viral genes [55, 61–65]. More

recent studies have identified a number of novel cellular targets of KSHV and EBV miRNAs and defined their cellular functions. Here, we briefly summarize these new findings based on their functional classes. It is important to point out that nearly all targets previously identified by these studies have been confirmed by the above mentioned ribonomics approach in the context of γ -herpesvirus infected tumor cells (Table 2) [34–37].

a) Regulation of cell growth and survival—For oncogenic viruses KSHV and EBV to cause malignant cellular transformation, it requires the deregulation of cell growth and survival pathways. Indeed, several KSHV and EBV miRNAs have been shown to promote cell growth and survival by overcoming cell cycle arrest and apoptosis.

KSHV miR-K1 evades cell cycle arrest by targeting a cyclin-dependent kinase inhibitor (CDKI) p21 (CIP1/WAF1) [66]. We have previously shown that miR-K1 targets I κ B α to activate the NF- κ B pathway, which presumably promotes cell survival [62]. Several KSHV miRNAs inhibit apoptosis. miR-K5, -K9, and -K10a/b target Bcl-2 associated factor (BCLAF1), a pro-apoptotic protein. miR-K10 facilitates cells escape from TNFSF12/TWEAK-induced apoptosis by repressing TNRSFR12A/TWEAKR [31]. miR-K1, -K3 and -K4-3p target caspase 3, a critical mediator of apoptosis [49]. KSHV miR-K11 targets SMAD5 to inhibit the TGF- β pathway [43, 67], which is also targeted by KSHV miRNA regulating THBS [29, 35]. We have also shown the miR-K10 inhibits the TGF- β pathway by targeting TGF- β type II receptor resulting in enhanced cell survival [42, 67]. Using a newly developed model of KSHV cellular transformation of primary cells, we have shown that a viral mutant with a cluster of 10 precursor miRNAs deleted (miR1–9, 10) failed to transform primary cells, and instead, caused cell cycle arrest and apoptosis [68]. Interestingly, expression of several miRNAs alone was sufficient to rescue the oncogenicity of the mutant virus, indicating that multiple miRNAs are likely to mediate KSHV cellular transformation [68].

In EBV, miR-BART5 represses the expression of the BH3-only protein PUMA, a pro-apoptotic protein that promote cytochrome C release from the mitochondria in response to apoptotic stimuli [50]. Another BH3-only protein, Bim, is targeted by multiple EBV cluster I miRNAs [51]. Another potential target of miR-BART16 is TOMM22, which is a part of a mitochondrial pore complex that serves as a receptor for the pro-apoptotic protein Bax [38]. Several KSHV and EBV miRNAs regulate oxidative stress. KSHV miR-K11 and EBV miR-BAR4, -BHRF1-2 target BACH-1 [28, 30, 37, 69]. Consequently, several BACH-1-regulated genes including hemeoxygenase 1 (HMOX1), the limiting enzyme in heme catabolism, and SLC7A11 (solute carrier family, xCT), a critical antioxidant that protects cells from reactive oxygen species (ROS), are upregulated. Thus, by targeting BACH-1, KSHV miR-K11 and EBV miR-BART4, -BHRF1-2 promote host cell survival, which is likely essential in the tumor oxidative stress environment.

b) Regulation of innate immunity and inflammation—In order to establish a life-long persistent infection in the host, herpesviruses evolve to evade host innate or adaptive immune responses. Several KSHV and EBV miRNAs have been shown to regulate host immune responses.

One important target that plays a critical role in evading natural killer (NK) cells is MICB, which was first reported for hCMV [54]. KSHV miR-K7 and EBV miR-Bart2-5p were also shown target MICB to inhibit NK cell recognition and activation albeit through different target sites [70], MICB was however not enriched by HITS-CLIP or PAR-CLIP [35] [37]. Downregulation of TWEAKR by miR-K10 not only blocks TWEAK-induced apoptosis but also inhibits the expression of proinflammatory cytokines IL-8 and MCP-1 [48]. Utilizing a humanized mouse model demonstrated that miR-K12-11, an ortholog of cellular oncomiR

miR-155, induces splenic B-cell expansion and potentially KSHV-associated lymphomagenesis by targeting C/EBP β [71], a transcriptional repressor of IL-6 and IL-10, which promote cell growth and angiogenesis and inhibit T-cell activation[56].

KSHV miR-K3 and miR-7 also induce basal secretion of IL-6 and IL-10 in macrophages by targeting LIP, a dominant-negative isoform of C/EBP β [71]. Several KSHV miRNAs inhibit innate immune responses. miR-K9 and miR-K5 target IRAK1 and MYD88, respectively, both of which mediate the TLR/IL-1R signaling cascade [72] while miR-K11 targets I-kappa-B kinase epsilon (IKK ϵ) to attenuate type I interferon signaling, a principal response mediating antiviral innate immunity [73]. By repressing interferon-inducible T cell chemokine, CXCL-11/I-TAC, EBV miR-BHRF1-3 inhibits the activation of host interferon response during EBV infection [58].

c) Functions of orthologs and variants—Unlike cellular miRNAs that tend to be conserved across mammalian species, most viral miRNAs are virus-specific. However, two viral miRNAs miR-K12-11 and miR-K10a share seed sequence homology with has-miR-155 and has-miR-142-3p, respectively. miR-155 is an oncomiR associated with development of several types of cancer [74]. By 3'UTR reporter assays and examination of protein levels following ectopic expression, the initial studies confirmed that miR-K11 and miR-155 regulate a common set of cellular targets [28, 30]. Subsequent studies confirmed the biological functions of miR-K11 in cells including inhibition of BACH-1, SMAD5 and C/EBP β as described above[56, 67, 69].

miR-K10 is another viral ortholog of miR-142-3p. Interestingly, recent RNA deep-sequencing reveals the presence of miRNA variants including miR-K10 and miR-142-3p variants [34]. A previous study has shown that pre-miR-K10 gene can be processed into two mature miRNAs, miR-K10a and miR-K10b, which differ in a single nucleotide[16]. Both miR-K10a and miR-K10b further exhibit 5' nucleotide variants, in that each mature miR is longer by a single nucleotide at the 5' end, generating two other miR variants, miR-K10a_+1_5 and miR-K10b_+1_5. Cellular miR-142-3p also has a similar variant, miR-142-3p_-1_5, that shares the same seed sequence with miR-K10a_+1_5 [34, 75]. Because of the sequence alteration in the seed sequence, albeit minor, the target pool of these miRNAs is expected to increase, resulting in acquisition of additional functions. Importantly, miR-K10 variants are expressed in KSHV-infected PEL cells and endothelial cells, indicating their functional relevance [26]. Interestingly, miR-142-3p variants are expressed only in PEL cells but not in uninfected and KSHV-infected endothelial cells suggesting some levels of cell type specificity. We have demonstrated that both miR-K10 and miR-142-3p variants inhibit the TGF- β pathway by target TGF- β type II receptor [42]. As stated above, results from the PAR-CLIP study identified KSHV vCyclin/LANA and vIL-6 as the targets of miR-K10 and miR-143-3p [34] [35]. Thus, these miRNAs might also function to fine-tune the expression of KSHV genes during latency. As a result of deep-sequencing, more miRNA variants are likely to be revealed, which should further extend the functional repertoire of γ -herpesvirus miRNAs.

Conclusion and outlook

Remarkable advancements have been made in our understanding of the functions and mechanisms of action of herpesviral miRNAs in recent years. Genome-wide PAR-CLIP and HITS-CLIP analyses have led to the identification of many targets of KSHV and EBV miRNAs. The results have established the landscape regarding the complex roles of viral miRNAs and are likely to guide future functional studies. Of particular interest is the investigation of functional redundancy of viral miRNAs revealed in several studies. In addition to targeting the same gene, a set of miRNAs could also target different genes of the

same pathway or related pathways, which can lead to similar functional outcomes. While PAR-CLIP and HITS-CLIP analyses have resulted in the identification of enriched miRNA pathways, further application of systems biology should place the miRNAs and their targets in appropriate functional networks and hierarchical positions. Nevertheless, final delineation of the contribution of viral miRNAs to viral biology has to be in the context of viral infection. Recent development of model systems as well as the application of existing model systems combined with reverse genetics approaches should facilitate the functional analysis of viral miRNAs during viral infection [76–79]. A major limitation of studying human γ -herpesvirus pathogenesis is the lack of easily tractable animal models [80, 81]. Within this context, the realization that many KSHV and EBV miRNAs, although not sequence related, target very similar pathways (i.e. apoptosis and immune evasion) may allow the characterization of miRNA phenotypes in related γ -herpesviruses such as MHV-68 for which animal models are available [82, 83]. Finally, the advancement of humanized mice such as the NOD/SCID IL2R γ ^{–/–} mice and KS xenograft models should facilitate the eventual development of KSHV and EBV infection mouse models [71, 84]. Such models will ultimately be necessary to evaluate virally-encoded miRNAs as potential therapeutic cancer targets as currently pursued in the context of HCV-related hepatocellular carcinoma [85].

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Highlights

1. HITS-CLIP and PAR-CLIP provide putative target lists for KSHV and EBV miRNAs
2. Both KSHV and EBV mimic host cellular miRNAs in lymphoid and endothelial cells
3. EBV and KSHV miRNAs regulate b cell proliferation and contribute to tumorigenesis
4. Although with different sequences γ -herpesvirus miRNAs target common cellular pathways

Figure 1. Schematic representation of miRNAs found in γ -herpesviruses

Genomes are represented for EBV, LCV, RRV, KSHV and MHV-68 with black arrows for ORFs, black triangles for tRNA genes, and black bars or rectangles for repeat sequences. MiRNA locations are indicated with orange arrows. Genomes are not drawn to scale. Abbreviations: U_S, unique short; U_L, unique long; LAT, latency associated transcript.

Table 1

Target identification techniques applied to γ -herpesvirus-encoded miRNAs

Perturbations			Measurements			Prediction algorithms	
Gain-of-function	Loss-of-function		Expression		Biochemical binding	Factors*	Programs
			mRNA	protein			
Transfection	Genetic knockout					Seed pairing	MirZ; mirWIP; PicTar; PITA; RNA22; TargetScan
Retroviral transduction	Silencing constructs (Antagonists, sponges, decoys)	Gene-specific	qPCR	Western blot	Luciferase reporter assay	Overall pairing	miRanda; RNA22
						Pairing stability	MirWIP; PITA; RNA22; RNAhybrid
						Conservation	MirZ; RNA22; TargetScan
						Accessibility	mirWIP; PIT A; TargetScan
		Genome-wide	Microarray RNA-seq	SILAC	CLIP-seq; HITS-CLIP, PAR-CLIP	Site number	MiRanda; mirZ; PicTar; PITA; mirWIP; TargetScan

mirZ, <http://www.biomedcentral.com/1471-2105/8/69>;
mirWIP, <http://www.nature.com/nmeth/journal/v5/n9/full/nmeth.1247.html>;
PicTar, <http://www.sciencedirect.com/science/article/pii/S0960982206010591>;
PITA, <http://www.nature.com/ng/journal/v39/n10/abs/ng2135.html>;
RNA22, <http://www.sciencedirect.com/science/article/pii/S0092867406010993>;
TargetScan, <http://genome.cshlp.org/content/19/1/92.full>;
miRanda, http://nar.oxfordjournals.org/content/36/suppl_1/D154.full;
RNAhybrid, <http://rnajournal.cshlp.org/content/10/10/1507.full>;

Table 2

Cellular targets of Herpesvirus miRNAs (References have to be done last)

Cellular target	Virus/sub-family	miRNA	(Proposed) functional consequences	Reference(s)
BACH1	KSHV/ γ	miR-K11	Pro-proliferative, increased viability under oxidative stress	[28] [30] [69]
BCLAF1	KSHV/ γ		Inhibit caspase activity, facilitate lytic reactivation	[31]
CASP3	KSHV/ γ	miR-K1, -K3, -K4-3p	Inhibition of apoptosis	[49]
CDKN1A/p21	KSHV/ γ	miR-K1	release of cell cycle arrest	[28]
C/EBPbeta, C/EBPbeta p20	KSHV/ γ	miR-K11 miR-K3, -K7,	De-repression of IL6/IL10 secretion; Modulation of macrophage cytokine response	[71] [56]
IKBKE	KSHV/ γ	miR-K11	Suppression of antiviral immunity via IFN signaling	[55] [28]
IRAK1	KSHV/ γ	miR-K9	Decreased activity of TLR/IL1R signaling cascade	[57]
MAF1	KSHV/ γ	miR-K1, -K6-5p, -K11	Induce endothelial cell reprogramming	[59]
MICB	KSHV/ γ	miR-K7	Immune evasion	[70]
MYD88	KSHV/ γ	miR-K5	Decreased activity of TLR/IL1R signaling cascade	[57]
NFIB	KSHV/ γ	miR-K3	Promote latency	[64]
NFKBIA	KSHV/ γ	miR-K1	Promote latency	[62]
RBL2	KSHV/ γ	miR-K4-5p	De-repression of DNA methyl transferases (DNMT1, 3a, 3b)	[65]
SMAD5	KSHV/ γ	miR-K11	Resistance to growth inhibitory effects	[67]
TGFBRII	KSHV/ γ	miR-K10a, -K10b	Resistance to growth inhibitory effects	[42]
THBS1	KSHV/ γ	miR-K1, -K3-3p, -K6-3p, -K11	Pro-angiogenic	[29]
TNFRSF10B/TWEAKR	KSHV/ γ	miR-K10a	Reduced induction of inflammatory response and apoptosis	[48]
CXCL-11	EBV/ γ	miR-BHRF1-3	Immune modulation	[58]
MICB	EBV/ γ	miR-BART2-5p	Immune evasion	[70]
PUMA	EBV/ γ	miR-BART5	Anti-apoptotic	[50]

Listed are targets that have been functionally confirmed at least by luciferase reporter repression upon ectopic miRNA expression, and de-repression of the reporter upon target site mutation; KSHV = Kaposi's sarcoma-associated herpesvirus, EBV = Epstein-Barr virus.