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HITS-CLIP and PAR-CLIP advance viral miRNA targetome analysis

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Abstract

MiRNAs regulate gene expression by binding predominantly to the 3'UTR of target transcripts to prevent their translation and/or induce target degradation. In addition to the more than 1200 human miRNAs, human DNA tumorviruses such as Kaposi's sarcoma-associated herpesvirus (KSHV) and Epstein-Barr virus (EBV) encode miRNAs. Target predictions indicate that each miRNA targets hundreds of transcripts, many of which are regulated by multiple miRNAs. Thus, target identification is a big challenge for the field. Most methods used to date investigate single miRNA-target interactions and are not able to analyze complex miRNA-target networks. To overcome these challenges, cross-linking and immunoprecipitation (CLIP), a recently developed method to study direct RNA-protein interactions in living cells, was successfully applied to miRNA target analysis. It utilizes Argonaute (Ago)-immunoprecipitation to isolate native Ago-miRNA-mRNA complexes. In four recent publications two variants of the CLIP method (HITS-CLIP and PAR-CLIP) were utilized to determine the targetomes of human and viral miRNAs in cells infected with the gamma-herpesviruses KSHV and EBV that are associated with a number of human cancers. Here, we briefly introduce herpesvirus-encoded miRNAs and then focus on how CLIP technology has largely impacted our understanding of viral miRNAs in viral biology and pathogenesis.

I. MiRNA-mediated Regulation of Gene Expression

The first miRNA, *lin-4*, was discovered in 1993 in *C. elegans* as a small RNA regulating the expression of the *lin-14* gene¹. MiRNA-mediated regulation of gene expression was at first believed to be a nematode-specific phenomenon. This changed in 2000 with the discovery of *let-7*², which was soon after found to be conserved across species³, and triggered the search for more miRNA-encoding genes. Since then the number of miRNAs registered at miRBase^{4, 5} has risen to more than 18,000 entries (release 18, Nov. 2011). MiRNAs are short (21-23 nt), non-coding RNAs that bind to partially complementary sequences mostly

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in the 3'UTR of target transcripts to inhibit their translation and/or induce their degradation. The majority of miRNAs are processed from larger, capped and poly-adenylated RNA pol II transcripts as part of a 70-80 nt RNA stem-loop, the primary (pri)-miRNA. However, some gamma-herpesviral and a recently reported retroviral miRNA are processed from pol III transcripts^{6, 7}. The pri-miRNA is cleaved in the nucleus by Drosha/DGCR8. This liberates a shorter stem-loop, the pre-miRNA, which is then exported into the cytoplasm by Exportin/Ran-GTP, where it is cleaved by Dicer, resulting in a 21-23 nt RNA duplex. Usually only one strand, the guide strand, is incorporated into the RNA-induced silencing complex (RISC) to accomplish the silencing function, while the other strand (passenger strand) is degraded. In contrast to siRNAs, miRNAs in most cases are only partially complementary to their targets. MiRNA target specificity is predominantly determined by the seed sequence, which comprises 7-8 nt at the miRNA 5' end that are fully complementary to the target⁸⁻¹⁰. Seed pairing can be supplemented by base pairing interactions at the 3' end of the miRNA¹⁰. Moreover, Shin et al. showed a new class of miRNAs lacking perfect seed and 3' end pairing and binding instead with 11-12 central nucleotides¹¹, while Chi et al. describe miRNAs binding with a G-bulge in the mRNA opposite to miRNA position five and six¹².

A 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 to cleave the target transcript¹³. Ago proteins contain two RNA-binding domains, the PAZ domain binding the 3' end of the mature miRNA and the PIWI domain interacting with the 5' end. Together they position the miRNA for target interaction (reviewed by Yang et al.¹⁴). In the case of full-length complementarity between miRNA and mRNA, miRNAs have a siRNA-like function leading to direct cleavage of the mRNA by Ago2. This mode of action dominates in plants¹⁵, but is very rare in animals. Here, miRNAs predominantly function by inhibiting protein translation (reviewed by Fabian & Sonenberg¹⁶).

It is still a matter of debate, however, what the mechanism of translational repression is. Several studies involving micro-array experiments showed a decrease in transcript levels in response to miRNA targeting. This occurs via deadenylation of the targeted mRNA, which leads to decapping, initiating standard mRNA turnover processes¹⁷⁻¹⁹. However, the effects on mRNA levels are usually modest, feeding the idea that repression must be based on translational inhibition, which is supported by a few studies showing that RISC binding interferes with translation initiation (reviewed by Fabian et al.²⁰). These are challenged, on the other hand, by recent high-throughput approaches indicating that for the majority of targets with decreased protein levels also the mRNA levels are reduced²¹⁻²³.

Animal miRNAs predominantly bind to the 3'UTR of mRNAs. Increasing evidence shows that miRNAs can also function in CDS (e.g.²⁴⁻²⁷). The latter target sites, however, although functional, usually are less effective than 3'UTR target sites^{21, 22, 28, 29}. Interestingly, target sites for the same miRNA in the 3'UTR and CDS of a transcript can have synergistic effects²⁹. Although miRNA-induced changes in transcript levels are mostly moderate (1.5-2 fold), a recent very elegant paper showed that small changes in transcript levels can lead to large changes in protein levels if a certain threshold is crossed³⁰.

II. Herpesviruses and Viral Mirnas

MiRNAs have been identified in many eukaryotes, from single cell organisms like algae and amoebae to organisms all across the metazoa (miRBase), and in 2004 the first virally-encoded miRNAs were also discovered in the herpesvirus Epstein-Barr virus (EBV)³¹. Since then more than 200 mature miRNAs have been identified in all herpesviruses analyzed so far, except for Varicella Zoster virus (for review see^{32, 33}). Herpesviruses are double-stranded DNA viruses, which are categorized into three subfamilies: *Alpha*-, *Beta*-, and *Gammaherpesvirinae*. To date, three human alpha-, three beta-, and two gammaherpesviruses are known. Typical for herpesviruses is their ability to establish lifelong latent infections in the host. For this the virus needs to remain hidden from the host immune surveillance, which is achieved by very limited viral gene expression together with the downregulation of or interference with the host innate immune defense mechanisms³⁴. During latency the viral genome persists as a circular episome that in dividing cells is replicated by the host cell replication machinery upon cell division. Certain triggers such as inflammation can cause the reactivation of the virus into the lytic cycle, which leads to replication of the viral DNA and production of progeny virus. For two human gammaherpesviruses latent infection can be associated with tumorigenesis: Kaposi's Sarcoma-associated herpesvirus (KSHV) is the etiological agent of Kaposi's sarcoma (KS), primary effusion lymphoma (PEL), and a subset of multicentric Castleman's disease (MCD), while EBV causes Burkitt's lymphomas (BL), non-Hodgkin's lymphoma, and nasopharyngeal carcinoma (NPC) (reviewed by Wen et al.³⁵). In contrast, the pathogenesis of human alpha- and beta-herpesviruses is due to the lytic life cycle, which is characterized by uncontrolled viral replication. The subsequent immune responses result in tissue destruction, such as in the case of the encephalitis caused by herpes simplex virus 1 (HSV-1).

In many herpesviruses (e.g. EBV, KSHV, Rhesus rhadinovirus (RRV), HSV, and Marek's disease virus (MDV)) the viral miRNAs are expressed as part of the latency program³², implying a function in the maintenance of latency. This could indeed be confirmed for HSV-1 and -2, where several miRNAs are expressed as part of the latency-associated transcript (LAT), a long non-coding RNA that is transcribed antisense to lytic genes. Based on their antisense position several miRNAs were predicted to target immediate early lytic genes, which could be experimentally confirmed for ICP0 (HSV-1 and -2) and ICP34.5 (HSV-2)^{36, 37}. Umbach et al. identified an additional miRNA in HSV-1 located in a different transcript downstream of LAT. This miRNA was experimentally confirmed to decrease protein levels of ICP4³⁶, the major viral transactivator required for reactivation and productive infection. Also in KSHV it could be shown that two viral miRNAs target the major lytic transactivator, RTA^{38, 39}, while several EBV miRNAs target the viral oncoprotein LMP1, which is responsible for transformation⁴⁰.

Most viral miRNA targets identified thus far, however, are cellular transcripts. Although until recently the number of experimentally confirmed targets was very low, they nevertheless clearly indicated a role of viral miRNAs in cellular pathways critical for the herpesvirus life cycle. Major themes are the targeting of pro-apoptotic genes such as PUMA (EBV⁴¹), SMAD2 (MDV⁴²), THBS1 (KSHV⁴³), TWEAKR (KSHV⁴⁴), and CASP3

(KSHV⁴⁵) and genes involved in immune evasion, such as CXCL16 (Mouse cytomegalovirus, mCMV⁴⁶), CXCL11 (EBV⁴⁷), and MICB (Table 1). Interestingly, despite the rather limited sequence conservation between herpesviral miRNAs, they display astonishing functional conservation. MiRNAs from three different viruses, Human cytomegalovirus (hCMV), EBV, and KSHV, target MICB^{48, 49}, which is involved in the antiviral natural killer (NK) cell response. Other viral miRNA targets are involved in angiogenesis, cell cycle control, and endothelial cell differentiation. A comprehensive list of validated targets and their presumable functions is summarized in Table 1.

III. Tools For MiRNA Target Identification

Viral miRNAs provide a unique opportunity to examine miRNA function: they are not expressed in non-infected cells, thereby allowing to compare the cellular transcriptome and proteome in presence and absence of the miRNAs. Viral miRNAs are, with few exceptions, not sequence-related to the metazoan miRNAs. Thus, a target with a functional seed match for a viral miRNA can in most cases unequivocally be assigned to this miRNA. Finally, viruses encode only a limited number of miRNA genes (typically between 2 and 40), which simplifies the transcriptome-wide target analysis compared to the more than 1,200 human miRNAs⁵. Most of the targets described above have been identified by either one or a combination of mainly three strategies: bioinformatics target prediction, gene-specific experimental target verification, and expression profiling.

A. Bioinformatics Target Prediction

Numerous target prediction algorithms have been developed in the last 10 years^{9, 50-57}. Target prediction is complicated by the incomplete complementarity between a miRNA and its target and the still poorly understood rules of target recognition. Therefore, most algorithms are based on target binding via the seed sequence (nt 2-8) of the miRNA, the so far best characterized target recognition mechanism. Some programs additionally take supporting base pairing interactions, e.g. at the miRNA 3' end, the overall thermodynamic stability of the miRNA/mRNA duplex, and evolutionary conservation into account. However, there are pronounced differences in the prediction results obtained by the different programs, reflecting in part the use of different sources for the 3'UTR sequences (TargetsCan⁹, Miranda⁵⁴), but also the high false positive rate (with estimates going up to 70%^{21, 22, 58-60}) due to the high statistical probability with which a 7-mer sequence occurs in the genome. Considering only evolutionary conserved seed matches strongly enhances performance^{21, 22}, but is not applicable for viral miRNA target prediction due to the lack of conservation even among closely related viruses. Additionally, current predictions mostly don't incorporate new findings such as alternative targeting strategies, or the existence of functional target sites beyond those in the 3'UTR, i.e. in protein encoding regions and even 5'UTRs, which leads to false-negative predictions. Thus bioinformatics target prediction provides a useful start, especially if nothing is known about potential targets of a miRNA, but always needs experimental follow-up.

B. Target Identification by Luciferase Reporter Assay, Quantitative RT-PCR, and Western Blot

The most commonly used approaches to experimentally validate a specific miRNA-target interaction are luciferase reporter assay, quantitative RT-PCR, and western blot. While the latter two measure downstream effects of miRNA expression without being able to distinguish between direct and indirect effects, the luciferase reporter assay reports direct miRNA-target interactions. They are based on cloning a 3'UTR with a potential miRNA target site downstream of a luciferase reporter gene. The reporter vector is transiently transfected into cells together with a miRNA expression vector or synthetic miRNA mimics, and the luciferase levels are determined in presence and absence of the miRNA. In case of a direct target effect, mutation of the seed match site in the 3'UTR should abolish the reporter repression through the miRNA. These data can be further backed up by western blot to visualize the effect on the corresponding protein product. However, all three assays study at best very few targets at a time, which by far doesn't capture the capacity of a single miRNA to regulate a complex network of targets.

C. Target Identification by Transcriptome or Proteome Profiling

A more global experimental approach is gene expression profiling, which uses overexpression of a miRNA in a cell line and monitoring of changes in transcript levels by micro-array or high-throughput sequencing. Down-regulated genes often are enriched for seed matches of the overexpressed miRNA^{28, 61}, which is an indication for direct targeting. Although evidence is accumulating that miRNA-mediated repression is mostly due to decreased transcript levels²¹⁻²³, some targets seem to be predominantly repressed at the translational level⁶² and therefore missed by micro-array analysis. Recently, two groups performed high-throughput proteomics^{21, 22}, and indeed found targets that showed no change on the mRNA level but decreased protein levels. However, the majority of their results showed that miRNA-mediated repression occurs via modest mRNA destabilization. A drawback of both approaches (transcriptome and proteome profiling) is that they observe downregulation of targets within a background of secondary effects, making it difficult to sort out true, direct targets. Although seed match enrichment can point in the right direction, theoretically every target identified by either of the two methods needs to be confirmed by luciferase reporter assays to show the direct miRNA-mRNA interaction.

A caveat of all the methods discussed above is the exogenous expression of miRNAs, which often results in expression above physiological levels, and frequently is introduced into a non-natural transcriptome background, thus potentially reporting non-physiological interactions or even artifacts due to overloading RISC with either overexpressed miRNAs or high concentrations of mimics. A reverse approach is the silencing of a miRNA by transfection of an antisense oligonucleotide (e.g. antagomirs⁶³). This avoids miRNA overexpression effects. On the downside, however, this approach is only as specific as the antisense oligo, which can be a limiting factor especially when targeting a specific member of a miRNA family, resulting in off-target effects. An elegant tandem-array approach was utilized by Ziegelbauer et al., who combined overexpression and inhibition of miRNAs to stringently identify targets that were repressed in the presence of the mimic and de-repressed in the presence of the antagomir⁶⁴.

IV. Identification of MiRNA Regulatory Networks by Cross-linking and Immunoprecipitation (CLIP)

A. The HITS-CLIP and PAR-CLIP Techniques

Two recently developed methods, high-throughput sequencing of RNA isolated by cross-linking immunoprecipitation (HITS-CLIP⁶⁵) and Photoactivatable-Ribonucleoside-Enhanced Cross-linking and Immunoprecipitation (PAR-CLIP⁶⁶), can – not completely but to a reasonable extent – address several of the issues discussed above. Both are based on UV cross-linking of RNA-protein complexes in living cells, followed by capturing of RISC complexes via Ago-immunoprecipitation. In brief, after cross-linking, (partial) RNase digest and immunoprecipitation, RISC complexes are further purified by SDS-PAGE. RNAs representing both miRNAs and their bound targets are extracted from the complexes of the correct migration size (i.e. desired length of cross-linked and clipped mRNA) and converted into small RNA libraries for high-throughput sequencing by adapter ligation and RT-PCR (Figure 1). Sequencing reads originating from Ago interactions with clipped mRNAs form narrow clusters of overlapping reads on transcripts after alignment to the reference genome. The clusters are usually enriched for seed matches of the most highly expressed miRNAs in the studied cell type⁶⁵⁻⁶⁷, especially within 20-30 nt upstream and downstream of the cluster peak position. This observation allowed to define the Ago footprint region to about 40 to 60 bases in length⁶⁵.

The experimental setup of CLIP studies has major advantages over conventional Ago-IP protocols such as RIP-Chip⁴⁶): (i) the cross-linking allows more stringent purification conditions, thus ensuring very limited carry-over of contaminations; and (ii) most importantly, while also in RIP-Chip-enriched transcripts can be screened for miRNA seed matches, they have to be searched over the entire transcript length, while in CLIP experiments the location of the seed-match is narrowed to the site of the Ago/miRNA/mRNA interaction, which adds specificity. Consequently, a high percentage of the HITS-CLIP or PAR-CLIP-predicted viral miRNA targets are confirmed in validation experiments⁶⁸⁻⁷⁰.

The major difference between HITS-CLIP and PAR-CLIP is the cross-linking technique. HITS-CLIP uses 254 nm UV light to cross-link RNA-protein complexes. As any of the 4 nucleotides is cross-linkable to amino acids (aa), there is a high chance for the presence of a favorably positioned nt-aa pair in each RNA binding protein (RBP)-target interaction. HITS-CLIP can thus be performed with any cell line or primary tissue without requiring pre-treatment. In contrast, in PAR-CLIP cells are first supplemented with the nucleoside analog 4-Thiouridine (4SU) that is incorporated into nascent mRNAs. 4SU cross-links are induced at 365 nm. Although cross-linking of 4SU is more efficient than that of the unmodified bases, it depends on the modified nucleotide being in a cross-linkable position (a 4SU must be in close distance to one of the reactive aa in the RNA binding pocket of Ago). Moreover, it can only be performed in cells lines, not in primary tissue samples. An important feature of PAR-CLIP, however, is a T to C transition of the cross-link site, since the cross-linked 4SU favors a U-G instead of a U-A base pair during reverse transcription⁶⁶ (Figure 1), thus clearly labeling the Ago/RNA interaction site. Recent work from the Darnell lab

demonstrated that 254 nm UV cross-linking as used in HITS-CLIP leads to single base deletions and transitions at the cross-link site during reverse transcription (although at a lower percentage than the T to C transitions in PAR-CLIP). While they are more complex to map during the bioinformatics analysis due to mutations of all four nucleotides occurring with different efficiencies, these cross-linking induced mutation sites (CIMS) also precisely map the location of the protein/RNA interactions⁷¹.

So far it is not well understood how the HITS-CLIP and PAR-CLIP experimental platforms compare. Certainly, there are a number of factors that contribute to differences in libraries, such as the cross-linking method, the nucleotide-specificity of the RNase used for clipping (RNase T1 vs. RNase A) and the extent of RNase digest⁷², as well as the choice of linkers (ligation bias⁷³). On the bioinformatics side, the algorithms and parameters chosen for alignment to the reference genome, algorithms and cut-off criteria used for cluster calling, and definition of seed matches influence which targets will be present in the final target lists. To date, only one study directly compared HITS-CLIP and PAR-CLIP data sets and determined a correlation of $R^2 > 0.4$ to 0.65 between the two platforms⁷². In this study, all libraries were analyzed with the CLIPZ database⁷⁴, a software pipeline specifically designed to analyze PAR-CLIP and HITS-CLIP data. Thus the observed differences between PAR-CLIP and HITS-CLIP datasets in this study are solely due to experimental differences/variation suggesting that cross-linking technique and RNase treatment differentially enrich for certain miRNA targets.

B. Viral MiRNA Regulatory Networks in EBV- and KSHV-Infected B Cells

Three laboratories including ours recently applied HITS-CLIP or PAR-CLIP to study the function of KSHV and EBV miRNAs: Riley et al. used HITS-CLIP to study EBV miRNA targets in Jijoye cells originally isolated from a patient tumor, exhibiting type III latency gene expression and expressing all 44 EBV miRNAs⁶⁷. They found more than 1600 potential EBV miRNA targets. 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 targets⁷⁰. The KSHV and EBV miRNA targetome was studied by Gottwein et al. in the KSHV/EBV co-infected primary effusion lymphoma (PEL) cell line BC-1 and the KSHV targetome in BC-3 (PAR-CLIP, more than 2000 putative targets)⁶⁹. Finally, our laboratory identified more than 1600 putative targets of KSHV miRNAs in the KSHV-infected PEL cells BCBL-1 and BC-3 using HITS-CLIP⁶⁸.

The three studies investigating EBV miRNAs report 7-25% of all sequenced miRNAs to originate from EBV. Similarly, KSHV miRNAs contribute about 20-30% of all miRNA reads in BCBL-1 and BC-1. In BC-3, however, KSHV miRNAs astonishingly comprised up to 70% of the sequencing reads. Deep sequencing data revealed miRNAs with alternatively processed 5' ends, such as KSHV-miR-K10a_+1_5 (additional nt at the 5' end) reported by Gottwein et al. Interestingly, this new version of miR-K10a shares the seed sequence with an alternatively processed miR-142-3p_-1_5 (lacking one nt at the 5' end). It could be shown 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 validated⁶⁹; in fact only

15% of the putative miR-K10a_+1_5 targets seem to be miR-K10a-specific. This is only the second functional ortholog of a human miRNA reported for KSHV in addition to miR-K11, which mimics the oncomir miR-155^{69, 70, 75, 76}, a miRNA involved in lymphocyte activation⁷⁷⁻⁷⁹. MiR-142-3p is a highly B cell-specific miRNA. Simultaneous targeting of transcripts by miR-142-3p and miR-K10a may serve to ensure the downregulation of certain miR-142-3p targets that are important for maintenance of KSHV latency. In non-B cells miR-K10a might mimic miR-142-3p function to create a more B cell-like environment⁶⁹. Interestingly, EBV has developed a slightly distinct strategy: instead of expressing a human miRNA ortholog it induces miR-155 expression in type III latency cells to contribute to EBV-mediated signaling⁸⁰⁻⁸².

C. HITS-CLIP and PAR-CLIP Revealed Only Few Viral Genes to be Regulated by Viral or Host MiRNAs

The majority of the mRNA sequencing reads align to the host genome, and only a very small number (< 2%) to the viral genomes, which is not unexpected since all studies investigate 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, and v-FLIP, the ORFs of vCyclin and v-FLIP, and in the 3'UTRs of vIRF-3 and vIL-6. The latter was experimentally confirmed by luciferase reporter assay to be strongly down-regulated in the presence of miR-K10a⁶⁸. vIL-6 is a mostly lytic gene involved in the inflammatory symptoms of KSHV-induced disorders such as MCD^{83, 84}. However, vIL-6 has also pro-proliferative effects⁸⁵ and was recently found to be expressed at very low levels during latency⁸⁶. Possibly, targeting of vIL-6 by miR-K10a helps to keep vIL-6 levels low enough to profit from moderate growth support during latency while at the same time avoiding inflammatory responses.

In EBV, both studies consistently report targeting of EBNA2, LMP1 and BHRF1^{67, 70}. While EBNA2 displays an atypical pattern of wide clusters all over the transcript, there are very distinct clusters in the 3'UTRs of LMP1 and BHRF1 with 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. Neither study, however, could confirm the previously reported miR-BART1-5p target sites in the LMP1 3'UTR⁴⁰. Remarkably, instead of evolving to avoid targeting of the viral genome by human miRNAs, the miR-17/20/106 target sites in LMP1 and BHRF1 3'UTRs are even evolutionarily conserved between EBV and rhesus lymphocryptovirus (rLCV)⁷⁰, which separated more than 13 million years ago⁸⁷, indicating the importance of these target sites, although their role in viral biology is currently unknown^{67, 70}. 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.⁸⁸). Both the miR-17/92 and 106/25 cluster are activated by the transcription factor c-myc^{89, 90}, which in the EBV-positive BL cells is the major driver of proliferation⁹¹⁻⁹³. MiR-17/92 activation via c-myc moreover leads to enhanced angiogenesis and inhibition of apoptosis. On the contrary, high expression of LMP1 can induce apoptosis⁹⁴. Thus, targeting of LMP1 might serve to fine-tune a balance between proliferation and cell death during latency^{40, 67}.

D. The Majority of Viral MiRNA Targets are Human Transcripts

HITS-CLIP as well as PAR-CLIP identified a large portion of previously validated viral miRNA targets. This together with the high percentage (>70%) of potential targets that could be functionally confirmed in each study⁶⁸⁻⁷⁰ reflects the confidence of the reported target sets produced by HITS-CLIP and PAR-CLIP. By using genetically engineered miRNA knockout viruses to compare potential miRNA interaction sites obtained in the presence or absence of a certain viral miRNA, Skalsky et al. moreover determined that the false-positive rate in PAR-CLIP-identified targets was just 11%⁷⁰. The biggest target set for KSHV and EBV miRNAs was previously obtained by RIP-Chip, but it reported only 114 targets for KSHV and 44 for EBV⁴⁶. Combined, the four CLIP studies demonstrate that, similar to human miRNAs, each viral miRNA targets between several dozen and a few hundred transcripts.

1.KSHV and EBV MiRNAs Target Similar Cellular Pathways—Interestingly, 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%)⁶⁹. Consistently, there are many common themes in the pathways targeted by viral miRNAs in all four studies, such as apoptosis, cell cycle control, intracellular transport, protein transport and localization, transcription regulation, proteolysis, and immune evasion. So far only a few apoptotic genes were identified as KSHV^{44, 45, 64} or EBV miRNA targets^{41, 95, 96}. Similarly, our knowledge of targeted immune modulatory genes such as the NK ligand MICB^{48, 49}, IKBKE⁹⁷, LIP (CEBP/B)⁹⁸, IRAK1 and MYD88⁹⁹ in KSHV, or CXCL11⁴⁷ in EBV was very limited. The recent CLIP studies now suggest that dozens of apoptotic⁶⁷⁻⁷⁰ and immune modulatory^{68, 70} genes are targeted by KSHV and EBV miRNAs, thus not only confirming previous conclusions about viral miRNA functions but also underscoring the importance of these pathways. Interestingly, Skalsky et al. also found that many of the genes identified in LCLs with target sites for EBV miRNAs contain additional predicted target sites for KSHV or hCMV miRNAs⁷⁰. The fact that miRNAs with no sequence homology have evolved to target identical pathways (e.g. immune evasion⁷⁰) indicates that their downregulation is crucial for herpesvirus biology.

Moreover, the suggested miRNA targetomes of the four studies are strongly enriched for novel interesting cellular pathways, such as the glycolysis pathway in KSHV⁶⁸, the WNT pathway in EBV^{67, 69}, the MAPKKK cascade⁶⁹, and the PI3 kinase and Ras pathways⁷⁰. Follow-up studies will show how miRNA regulation of these targets contributes to viral biology and/or pathogenesis. We don't go deeper into details of specific new targets in this review, but instead point the interested reader to the extensive supplemental material about the identified targetomes provided by all four studies⁶⁷⁻⁷⁰.

2.Viral MiRNAs Co-target Transcripts with Human MiRNAs—For KSHV as well as for EBV a significant overlap between human and viral miRNA targets was reported. While in KSHV 55-65% of viral miRNA targets had additional interaction sites for human miRNAs⁶⁸, in EBV 75-90% were co-targeted by human miRNAs^{67, 70}. Interestingly, in Jijoye cells, 50% of the co-targeted transcripts are targets of the very abundant members of the miR-17/92 cluster⁶⁷. 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). Others 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). The concept that viral and human miRNAs with identical seeds indeed share common targets has been experimentally validated in several cases for miR-155/miR-K11 and miR-142-3p/miR-K10a^{68, 69, 100-103}). The study by Riley et al. elegantly extends these observations, by analyzing how the nucleotides around the seed sequence can influence targeting by these orthologs. It shows that miR-BART5-5p targets LMP1 but miR-18a does not despite an identical seed sequence (nt 2-7). In this case the nucleotides directly adjacent to both sides of the seed seem to be the decisive factor. They cause the target alignment to be shifted by one nucleotide between the two miRNAs and LMP1 (nt 1-7 for miR-18a, and 2-8 for Bart-5-5p), resulting in a weaker predicted duplex for miR-18a⁶⁷. Interestingly, seed homologies exist also among EBV miRNAs, e.g. miR-BHRF-1 and miR-BART4 or miR-BART1-3p and 3-3p share a seed that is off-set by one nucleotide and the latter two indeed target the same 3'UTR in a luciferase reporter assay⁷⁰. Skalsky and coworkers suggest that this could help to ensure that important pathways are targeted at all times since EBV miRNA expression depends on latency stage and cell type (lymphoid versus epithelial).

The strong target overlap between human and viral miRNA targets, however, is only partially due to seed homologies. The majority of identified transcripts have distinct target sites for the co-targeting miRNAs in their 3'UTR. The functionality of multiple target sites (viral/viral or viral/human) within one 3'UTR was not only confirmed for several transcripts⁶⁷⁻⁷⁰, but it was also shown that these interactions can be cumulative^{43, 67, 68}. The concept of co-targeting of a 3'UTR by multiple miRNAs certainly is not new, but these four CLIP studies for the first time indicates that it occurs at a large extent between viral and human miRNAs in infected cells. Co-targeting could reflect a hijacking of existing human pathways by viral miRNAs, especially in cases where viral miRNAs mimic their human counterparts⁷⁰. Since in somatic cells miRNA-mediated post-transcriptional regulation fine tunes transcript levels, the effects of viral miRNAs (often expressed at high copy numbers) targeting the same genes could have significant consequences for the host cell by tipping gene expression in the direction that is advantageous for the virus. We call it active interference here. In an inverse scenario (passive interference), the high levels of viral miRNAs in infected cells (comprising up to 70% of the total RNA count) could have a different effect: instead of ensuring targeting of certain pathways by viral miRNAs, their large numbers could tip the competition for the existing limited pool of RISC complexes¹⁰⁴, thereby excluding many human miRNAs from access to RISC^{67, 68}. Conceptually intriguing, this sequence-independent passive mode of viral miRNA-dependent regulation could result in a global up-regulation of host cellular genes.

In summary, the recent CLIP studies not only provide a much broader picture of the viral miRNA targetomes but also point to novel cellular pathways that may play important roles in viral biology. Moreover, these comprehensive target databases are a valuable resource for the miRNA research community that allows to search for specific miRNA-target interactions

and targeted pathways of interest (not only of viral but also human miRNAs) for the subsequent design of functional follow-up studies.

D. Opportunities and Challenges of HITS-CLIP and PAR-CLIP Studies

As with all methods that create large datasets, the complex data analysis is the bottleneck for all researchers taking on HITS-CLIP or PAR-CLIP studies. The drylab (bioinformatics) often cannot keep up with the wet lab, and many laboratories don't have the infrastructure and/or expertise nearby that would allow them to create their own analysis pipeline. To our knowledge, currently only two analysis tools are publically accessible, CLIPZ⁷⁴, which can be used for HITS-CLIP and PAR-CLIP datasets, and PARalyzer¹⁰⁵, developed specifically for PAR-CLIP. The limited availability of free analysis tools and resulting use of individual solutions by many different research groups makes the comparison of published target lists difficult. A true comparison would require the reanalysis of the original datasets with the same pipeline using identical parameters.

Nevertheless, Ago CLIP has opened an important door in miRNA targetome analysis. CLIP is the first method that truly allows the simultaneous analysis of hundreds of direct miRNA-mRNA interactions instead of single miRNA-target pairs, and thus is the best tool to provide information about the large and complex miRNA regulation networks. Moreover, instead of overexpression studies that are often conducted in unrelated cell lines, the targets of a miRNA can now be studied at physiological levels and in its physiological, cell type-specific environment. HITS-CLIP (but not PAR-CLIP) in addition can be applied to primary tissues to analyze organ or tumor-specific targetomes and the consequences of miRNA deregulation in various diseases including cancers. Finally, miRNA knockout viruses in combination with CLIP can be used to dissect the functions of single miRNAs within the complex network of other viral and human miRNAs, which will likely help to better understand the mechanisms of miRNA-mediated gene regulation.

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Figure 1. Comparison of HITS-CLIP and PAR-CLIP experimental procedures

RNA-protein cross-linking occurs in living cells. HITS-CLIP uses cross-linking at 254 nm, while in PAR-CLIP cells are fed with 4-Thiouridine (4SU), which is incorporated into nascent RNA and cross-links at 365 nm. Cross-linked cells are lysed and lysates are treated with RNase to trim the mRNA. In HITS-CLIP mRNAs are partially digested with RNase A, which cleaves after pyrimidines, leaving mRNAs with lengths between 50 and >100 bp. PAR-CLIP performs an extensive RNA digest with RNase T1, which cleaves after guanine nucleotides, resulting in very short mRNA fragments of 20-30 nt. After Ago-immunoprecipitation, linker ligation and radioactive labeling of the RNA, cross-linked Ago-miRNA-mRNA complexes are further purified by SDS-PAGE. HITS-CLIP yields two major bands, the Ago-miRNA complexes migrating at ~100 kDa, and the Ago-mRNA and double cross-linked Ago-miRNA-mRNA complexes migrating at 120-130 kDa (corresponding to varying mRNA lengths between 50- and 80 bp). PAR-CLIP produces only one band at 100-110 kDa containing both RNA species. Complexes are cut out and RNAs are extracted and ligated to the 5' linker. Reverse transcription and PCR amplification creates the final libraries for Illumina sequencing. In PAR-CLIP, the cross-linked 4SU base pairs with Guanosine instead of Adenosine during reverse transcription, resulting in a T to C transition in the final PCR product that allows identification of the crosslink site. N = any

nucleotide; 4SU = 4-Thiouridine; G = Guanosine; Y = Pyrimidine; XL = symbolizes a cross-link position

Table 1
Cellular targets of Herpesvirus miRNAs

Cellular target	Virus/sub-family	miRNA	(Proposed) functional consequences	Reference(s)
BACH1	KSHV/ γ	miR-K11	Pro-proliferative, increased viability under oxidative stress	101 102 106
BCLAF1	KSHV/ γ		Inhibit caspase activity, facilitate lytic reactivation	64
CASP3	KSHV/ γ	miR-K1, -K3, -K4-3p	Inhibition of apoptosis	45
CDKN1A/p21	KSHV/ γ	miR-K1	release of cell cycle arrest	101
C/EBPbeta, C/EBPbeta p20	KSHV/ γ	miR-K11 miR-K3, -K7,	De-repression of IL6/IL10 secretion; Modulation of macrophage cytokine response	103 98
IKBKE	KSHV/ γ	miR-K11	Suppression of antiviral immunity via IFN signaling	97 101
IRAK1	KSHV/ γ	miR-K9	Decreased activity of TLR/IL1R signaling cascade	99
MAF1	KSHV/ γ	miR-K1, -K6-5p, -K11	Induce endothelial cell reprogramming	107
MICB	KSHV/ γ	miR-K7	Immune evasion	49
MYD88	KSHV/ γ	miR-K5	Decreased activity of TLR/IL1R signaling cascade	99
NFIB	KSHV/ γ	miR-K3	Promote latency	108
NFKBIA	KSHV/ γ	miR-K1	Promote latency	109
RBL2	KSHV/ γ	miR-K4-5p	De-repression of DNA methyl transferases (DNMT1, 3a, 3b)	39
SMAD5	KSHV/ γ	miR-K11	Resistance to growth inhibitory effects	100
TGFBR2	KSHV/ γ	miR-K10a, -K10b	Resistance to growth inhibitory effects	110
THBS1	KSHV/ γ	miR-K1, -K3-3p, -K6-3p, -K11	Pro-angiogenic	43
TNFRSF10B/TWEAKR	KSHV/ γ	miR-K10a	Reduced induction of inflammatory response and apoptosis	44
CXCL-11	EBV/ γ	miR-BHRF1-3	Immune modulation	47
Dicer	EBV/ γ	miR-BART6	Global reduction of miRNA synthesis	111
MICB	EBV/ γ	miR-BART2-5p	Immune evasion	49
PUMA	EBV/ γ	miR-BART5	Anti-apoptotic	41
CCNE2	hCMV/ β	miR-US25-1	Block cell cycle to allow DNA replication, desensitize for apoptotic signals	112
MICB	hCMV/ β	miR-UL112	Immune evasion	48
CXCL16	mCMV/ β	miR-M23-2	Immune evasion	46
C/EBPbeta	MDV1/ α	miR-M4-5p		113
c-Myb	MDV1/ α	miR-M4-5p	Hematopoiesis	113
GPM6B	MDV1/ α	miR-M4-5p		113
MAP3K7IP2	MDV1/ α	miR-M4-5p	Repression of NFkappaB induction via IL-1	113
PU.1	MDV1/ α	miR-M4-5p		113 114
RREB1	MDV1/ α	miR-M4-5p	Lymphocyte differentiation	113
SMAD2	MDV1/ α	miR-M3	Cell survival	42

Listed are all 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, hCMV = Human cytomegalovirus, mCMV = Mouse cytomegalovirus, MDV1 = Marek's disease virus