The miRNA-targetome of KSHV and EBV in human B-cells

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The miRNA-targetome of KSHV and EBV in human B cells

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Micro-RNAs (miRNAs) are small non-coding RNA molecules which provide a subtle layer of regulation to thousands of cellular genes. The identification of virally encoded miRNAs added another layer of complexity to the dense interaction between viruses and their natural hosts. While it has been shown that viral miRNAs can regulate both cellular and viral gene expression, target identification has been a difficult and cumbersome task. The immunoprecipitation of Argonaute (Ago)-protein containing RNA-induced silencing complexes (RISC) followed by microarray analysis (RIP-Chip) allows the identification of miRNA-targetomes at whole transcriptome level. We applied Ago2-based RIP-Chip to identify cellular transcripts targeted by Kaposi's sarcoma-associated herpesvirus (KSHV, n = 114), Epstein-Barr virus (EBV, n = 44) and cellular miRNAs (n = 2,337) in six latently infected or stably transduced human B-cell lines. While RIP-Chip yields a plethora of high-confidence miRNA targets and provides a quantitative estimate of miRNA function, additional biochemical methods like HITS-CLIP or PAR-CLIP and bioinformatic analysis are required to identify individual miRNA binding sites. Together, these methods will be useful to unravel the network of regulation exerted by both viral and cellular miRNAs, thereby providing the basis for functional studies on miRNA-mediated regulation of gene expression in herpesvirus infections.

Key words: miRNA, viral, kaposi's sarcoma associated herpesvirus, epstein-barr-virus, RIP-chip, microarray

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The Herpesvirus Family

Herpesviruses are large double stranded DNA viruses which have evolved with their animal and human hosts over millions and millions of years. More than 120 different herpesviruses have so far been identified in mammals, birds, reptiles and even invertebrates (e.g., oysters). They can be divided into three subfamilies (α-, β- and γ-herpesviruses) based on sequence homologies and biological features.[1] A unique feature of all herpesviruses is their ability to establish a life-long infection. Thus, once infected the virus is maintained for life. The virus persists in the nucleus of infected cells as an extrachromosomal episome with a stable copy-number in a state termed 'latency'. During this phase only a few viral proteins are expressed, which mainly contribute to maintenance of latency and immune evasion.[2] Triggered by specific endogenous or exogenous factors, like stress, immune suppression, UV-light or hormones, the virus can reactivate into the lytic stage of infection. This results in recurrent production and release of infectious virus particles finally resulting in death of the infected cell.

To date, eight human herpesviruses (HHV-1 to HHV-8) have been discovered. Two of them, KSHV (Kaposi's sarcoma associated Herpesvirus, HHV-8) and EBV (Epstein-Barr virus, HHV-4), are γ-herpesviruses that have oncogenic potential and are able to infect B-cells. In immunocompromised individuals, infection with EBV or KSHV can result in lymphoproliferative disorders.[3] KSHV is involved in the development of several human tumors, including Kaposi's sarcoma, primary effusion lymphoma and multicentric Castleman's disease.[4,5] In most infected cells the virus resides in the latent form, and during this phase of

infection only four viral proteins (of at least 89) are expressed via a set of differentially spliced transcripts from a common locus called "latency associated region" (LAR), including Kaposin, an oncogenic protein, v-FLIP, a viral homologue of the cellular FLICE-inhibitory protein, v-Cyclin, a viral homologue of cellular D-type-cyclins and LANA (Latency associated nuclear antigen).[6]

Herpesviruses and microRNAs

As not all of the drastic changes that a cell or a tissue undergoes upon latent KSHV infection can be explained by the expression of these four proteins, it was an interesting discovery made by three independent groups in 2005 in parallel, that a set of 12 conserved pre-miRNAs is expressed from the KSHV LAR-locus during virus latency.[7-9]

Micro-RNAs (miRNAs) are endogenous, 21–23 nucleotide long, non-coding RNA molecules that regulate protein-coding gene expression in plants and animals via the RNA silencing machinery. Human cells express at least approximately 700 miRNAs which are involved in the regulation of virtually every biological pathway. It was intriguing to speculate that viral miRNAs regulate both cellular and viral gene expression to support the establishment and maintenance of latency, as well as lytic reactivation. Interestingly, it was soon identified by two different groups in parallel that KSHV encodes an orthologue (or in our opinion rather an analogue) of cellular miR-155,[10,11] an evolutionary conserved miRNA upregulated in several types of cancer. This resulted in the identification of 16 cellular transcripts targeted by both kshv-K12-11 and hsa-miR-155. While these targets are known to play important roles in B-cell function, innate immunity, apoptosis and cell cycle control, the biological effects of their regulation by kshv-miR-K12-11 remain to be elucidated. Interestingly, it was subsequently discovered that EBV infection resulted in a NFκB-dependent induction of hsa-miR-155 in human B-cells,[12] indicating that miRNA-mediated downregulation of hsa-miR-155 targets seems to be in favor of these two human γ-herpesviruses. In general, viral miRNAs show only very little sequence and positional conservation which probably reflects the great flexibility of these viral modulators. The finding that both KSHV and EBV regulate hsa-miR-155 targets may indicate that this is not necessarily the same for the targets of their miRNAs. Interestingly, the Mandelboim lab identified the activating natural killer cell ligand MICB, which is induced in cells upon infection resulting in NK-cell killing of the infected cell, to be targeted by both KSHV and EBV as well as human cytomegalovirus,[13,14] thereby highlighting the role of this protein in antiviral defense against herpesviruses in general. Interestingly, none of these viruses targeted the closely related NK-cell activating ligand MICA, while both MICA and MICB are also heavily regulated by cellular miRNAs.

Targets of miRNAs From Herpesviruses

In the last three years, a series of papers were published in which single cellular targets of KSHV miRNAs were presented. For example, in 2007 the Renne lab identified THBS1, a major regulator of cell adhesion, migration and angiogenesis, to be targeted by KSHV-encoded miRNAs which may contribute to the pathogenesis of Kaposi sarcoma.[15] Other targets included the transcription factors CEB/P and MAF, an inhibitor of NFκB activation (IKBA), RBL2, an inhibitor of DNA-methylases, whose regulation by KSHV miRNAs had an effect on epigenetic regulation of viral latency, and the cyclin dependent kinase (CDK)-inhibitor p21.[16-20] In addition, also one viral target was identified, the transcriptional activator RTA (KSHV ORF50), which is the master regulator of lytic reactivation in KSHV. In summary, until recently approximately 25 cellular and viral targets of KSHV miRNAs had been identified.

Little is still known about the function of viral miRNAs in the context of EBV infection. EBV is a very successful pathogen that infects >90% of all adult humans worldwide. It has been linked to various human malignancies including Burkitt's, Hodgkin's, NK/T-cell, peripheral T-cell and post-transplant lymphomas, nasopharyngeal and gastric carcinomas, as well as posttransplant lymphoproliferative disease (PTLD). EBV latent gene expression in various EBV-associated malignancies and EBV-derived cell lines has led to the identification of three different and distinct latency programs. These latency programs are the result of differential promoter activity and are influenced by host cell factors. EBV encodes at least 25 miRNAs, which are located in two primary transcripts, the BHRF1 transcript which also encodes the BHRF1 ORF, and the BamHI A rightward transcripts (BARTs). The BARTs contain two clusters of miRNAs, which have been termed Cluster 1 and Cluster 2. Among different cell lines, the miRNAs of EBV are expressed at dramatically differing levels. Interestingly, the prototype B95.8 strain of EBV has lost more than half of the viral miRNAs during cell culture passages indicating that expression of these viral miRNAs may be of disadvantage for the virus in cell culture.[21] Until recently, only three cellular targets for EBV encoded miRNAs including the chemokine CXCL-11, the p53-regulated modulator of apoptosis (PUMA) and the immune ligand MICB, were known.[13,22,23] In addition there are several viral genes known to be regulated by EBV-encoded miRNAs. These are, for example, the viral Polymerase BALF5 and the latent membrane protein LMP2A.[24,25]

All attempts to identify targets for γ-herpesviral miRNAs either relied only on bioinformatic predictions, or employed microarray analysis on total RNA to identify viral miRNA targets, based on the very modest effect of miRNAs on the RNA stability of their targets. While these approaches have led to successful identification of a few cellular and viral targets for these viral miRNAs, the experimental work is cumbersome and full of difficulties. Bioinformatic approaches are hampered by the fact that miRNAs require only limited complementarity to their binding sites, as even a complete SEED complementarity of seven nucleotides is not always essential. Similar, the microarray expression profiling analysis on total RNA also has major limitations: First, transcript levels of miRNA targets are not necessarily affected if regulation occurs predominantly by translational repression.

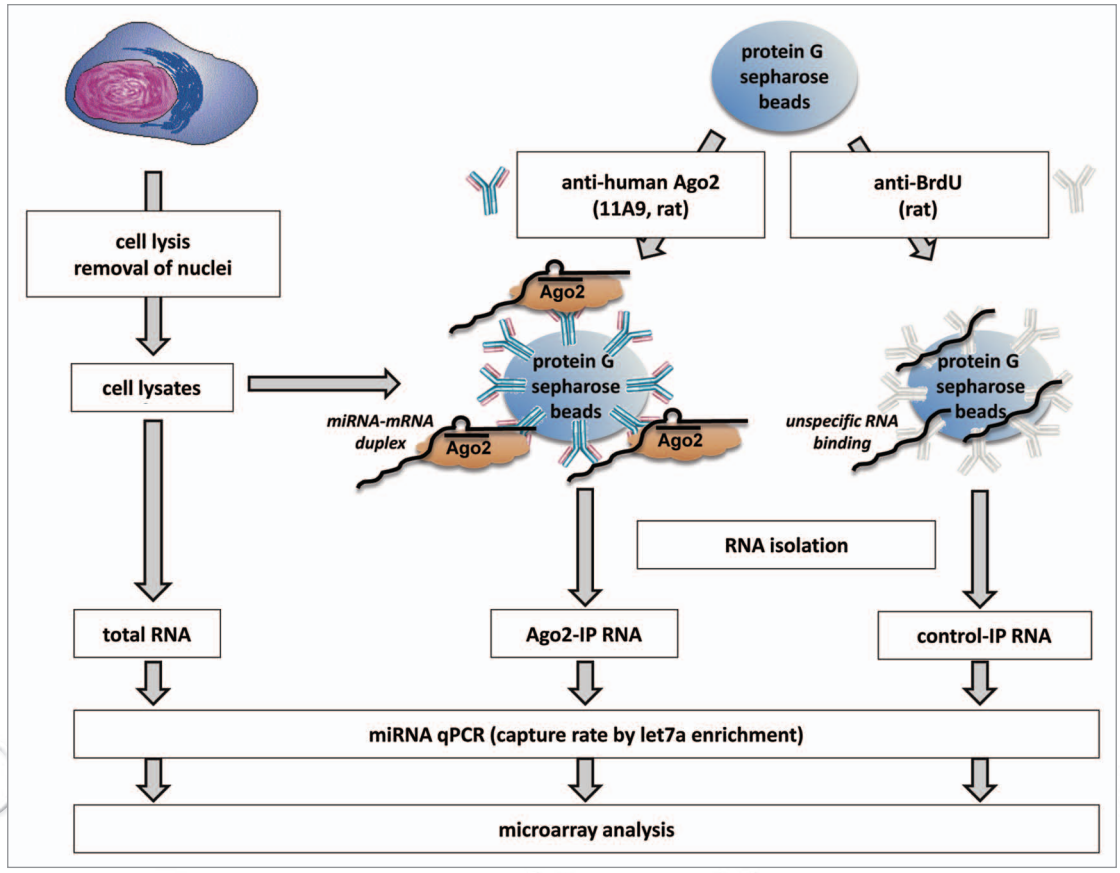

Figure 1. RIP-Chip workflow. Protein-G sepharose beads are coated with a monoclonal antibody to Ago2 or a control antibody (anti-BrdU). Cells are lysed, cleared by centrifugation and lysates stored at -80°C until immunoprecipitation (IP). Following the IP, sepharose beads are lysed and RNA molecules are recovered using Qiazol reagent. Most importantly, the efficiency of every IP is controlled by quantitative PCR for an abundant cellular miRNA (e.g., let7a or miR-16). In addition, the capture efficiency of the IP is determined by comparing miRNA levels between the Ago2-IP and total RNA (=input). In human cells, we generally observed a capture efficiency of ~50% and an enrichment of let7a of ~1,000-fold.

Second, since the observed effects are usually only very small and mostly do not exceed 2-fold many bona fide targets are missed. Finally, secondary targets are difficult to distinguish as a multitude of transcripts may be affected due to secondary effects. As even sophisticated bioinformatic approaches have not been able to solve these problems in a satisfactory way, the identification of viral miRNA targets was based on a trial-and-error strategy and required a high number of genes to be screened, and therefore was inadmissibly labor-intensive.

RISC-immunoprecIpitation Allows the Identification of miRNA Targets

RIP-Chip analysis, the immunoprecipitation of RNA-induced silencing complexes (RISC) with an Argonaute-specific monoclonal antibody followed by RNA-extraction and subsequent quantification of mRNAs on microarrays, has recently been utilized to identify mRNAs that are associated with the RNA-silencing machinery and therefore are targets of cellular miRNAs.[26-31] First approaches used cell lines which expressed c-myc-tagged Ago2, and used a Myc-specific antibody to pull down miRNA/targets complexes. Recently, the Meister lab developed a highly specific monoclonal antibody to human Argoaute2 (α-hAgo2; 11A9). We now used this monoclonal antibody and established an Ago2-based RIP-Chip protocol to identify transcripts targeted by KSHV, EBV and cellular miRNAs in six latently infected or stably transduced human B-cell lines (**Fig. 1**).[32] This resulted in the identification of a large number of high-confidence targets for KSHV (n = 114) and EBV (n = 44) and cellular miRNAs (n = 2,337). In short, RISC-immunoprecipitation with the AGO2-specific antibody resulted in a ~1,000-fold enrichment of cellular miRNAs (e.g., let7a or miR-16) compared to an unspecific control antibody. About 50% of cellular miRNAs could be recovered indicating that at least half of all cellular miRNAs in human B-cells are incorporated into AGO2-containing RISC-complexes. Two known targets of cellular miRNAs (CCNE1 and MICA) were typically enriched by ~8-fold as determined by RT-PCR. We then used microarray analysis (Affymetrix Gene ST 1.0 arrays) to identify both cellular and viral miRNA targets. Enrichment of transcripts in the Ago2-IP vs. control (BrdU-IP or total RNA) ranged up to ~40-fold with up to ~1,400 transcripts showing >2-fold enrichment. We analyzed the enrichment profiles of 11,714 protein coding genes for

the presence of binding sites for 44 known B-cell specific miRNAs. Interestingly, transcripts, enriched as little as 1.2-fold, already showed highly significant overrepresentation of predicted miRNA target site using either PITA or RNAhybrid. Binding sites of cellular miRNAs were particularly overrepresented in transcript 3'-UTRs ($p = 6.2 \times 10^{-63}$) and coding sequences ($p = 2.010^{-27}$) but not in 5'-UTRs ($p = 8.9 \times 10^{-3}$). Therefore, we considered 2.337 transcripts significantly enriched >1.2-fold across all six cell lines to be targets of cellular miRNAs. Targets of KSHV and EBV miRNAs were defined by their property to be enriched to a substantially greater extent (>3-fold) in the infected cell lines (BCBL-1 and Jijoye) compared to two control cell lines. This resulted in 114 targets of KSHV and 44 of EBV miRNAs, and the analysis revealed that there was a significant enrichment of binding sites for viral miRNAs on these transcripts. The substantially greater number of KSHV miRNA targets simply reflects the slightly greater mean enrichment in BCBL-1 than in Jijoye which we did not compensate for by applying additional normalization steps. This approach yielded high confidence targets of viral miRNAs. First, 18/19 (95%) of the viral miRNA targets were confirmed by quantitative PCR. We then randomly picked 6 putative KSHV miRNA targets and performed dual luciferase assays. Four targets revealed regulation via their 3'-UTR, two via binding sites located within coding sequences. Interestingly, we predominantly identified binding sites for the most prominent KSHV miRNAs showing expression of >1,000 copies/cell as determined by quantitative PCR. In addition, we validated two genes known to govern cellular transport pathways, namely *importin 7* (IPO7) and trans*porter of the mitochondrial out membrane 22* (TOM22) as EBV miRNA targets. While regulation of TOM22 may help EBV to prevent BAX-induced apoptosis downregulation of IPO7 may be involved in modulating cytokine signaling in EBV infected cells. The recent finding that EBV may modulate gene expression also in uninfected cells via secretion of viral miRNAs within exosomes is an interesting concept to be tested in further studies.[33]

miRNAs and the Half-Life of Transcripts

It has been a long standing debate to what extent miRNAs regulate the stability of their targets. So far all available data rely on measurements of changes in total RNA thereby reflecting the changes in RNA stability.[34,35] Also these studies only showed a very minor effect of cellular miRNAs on transcript levels hardly exceeding 2-fold, a main concept commonly pursued to identify viral miRNA targets still is to screen for potential effects on total RNA levels by microarray analysis. We applied a novel approach we recently developed, now termed 4sU-tagging, to directly measure RNA half-lives and not their impact on total RNA levels. This approach is based on metabolic tagging of nascent RNA (e.g., for 60 min) by adding 4-thiouridine (4sU) to the cell culture medium followed by isolation of total RNA, thiol-specific biotinylation of 4sU-tagged nascent RNA and separation of total cellular RNA into nascent and pre-existing untagged RNA using streptavidin-coated magnetic beads.[36,37] RNA half-lives can then be determined based on nascent/total, nascent/untagged or untagged/total RNA ratios. Thus, we determined RNA half-lives for >10,000 transcripts in 4 of the 6 human B-cell lines to investigate the effect of both cellular and viral miRNA on RNA stability. We found a small, however highly significant negative correlation in between median RNA half-life and enrichment in the Ago2-IP ($p < 10^{-16}$), reflecting recruitment of transcript to Ago2-RISC-complexes. For KSHV miRNA targets, this was exclusively seen in the KSHV positive cell line BCBL-1 indicating that both viral and cellular miRNAs have a small, however significant impact on target RNA stability resulting in a mean reduction in RNA half-life of ~10–15%. Although this does not exclude that individual transcripts are destabilized in a more pronounced way it indicates that the effect of miRNAs on target RNA stability is generally too small to be efficiently utilized to able to confirm about 50% of the predicted binding sites in our KSHV and EBV miRNA targets. Recent advances in UV-cross-linking technologies (HITS-CLIP and PAR-CLIP) followed by next-generation sequencing (CLIP-Seq) now provide access to miRNA binding sites at whole transcriptome level.[38,39] As these methods are most likely not quantitative due to cloning and sequencing biases, a combination of RIP-Chip confidently identify their targets. On the other hand, these data also demonstrate that RIP-Chip analysis offers a quantitative estimate of miRNA function. Therefore, the extent of enrichment observed in the RISC-IP may serve to identify the most strongly and relevantly regulated targets.

In contrast to other methods, RIP-Chip analysis is based on positive selection of miRNA targets. It is important to note, however, that a lack of enrichment does not exclude a transcript to be targeted by miRNAs. This may be for several reasons. First, some genes may simply be expressed below the detection limit of the arrays and may thus escape detection. In addition, targets showing as little as 1.2-fold enrichment still contained a significant above-average numbers of predicted miRNA binding sites. Therefore, already very little enrichment is compatible with miRNA-mediated regulation. We restricted our analysis to the most strongly enriched targets, i.e., >3-fold. Therefore, we are quite certain that many additional targets can be found in our set of data when less stringent criteria are employed. While only three of the published KSHV miRNA targets fulfilled our criteria as miRNA targets (PIK3CA, SLA and NFKBIA), we observed an enrichment of >1.5-fold for approximately 60% of the published KSHV and EBV miRNA targets. For example, MICB was significantly stronger enriched in the Ago2-IPs of both KSHV- and EBV-infected cells as identified by microarray analysis and confirmed by quantitative PCR.

Conclusions

While RIP-Chip analyses are able to identify high-confidence miRNA targets and provide limited information about their biological relevance, the determination of the actual binding sites still remains a cumbersome task. Using the RNAhybrid algorithm, we were only able to confirm about 50% of the predicted binding sites in our KSHV and EBV miRNA targets.

Recent advances in UV-cross-linking technologies (HITS-CLIP and PAR-CLIP) followed by next-generation sequencing (CLIP-Seq) now provide access to miRNA binding sites at whole transcriptome level.[6,15] As these methods are most likely not quantitative due to cloning and sequencing biases, a combination of RIP-Chip and CLIP-seq appears to be the way of choice to continue, which will soon result in the identification of hundreds of binding sites for both cellular and viral miRNAs. While this will dramatically improve our understanding in the biology of miRNA-mediated regulation of gene expression, the biological consequences and the relevance of their regulation for example at different stages of the virus life cycle remain to be elucidated.

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