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Resource

Systematic Analysis of Viral and Cellular MicroRNA Targets in Cells Latently Infected with Human γ-Herpesviruses by RISC Immunoprecipitation Assay

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SUMMARY

The mRNA targets of microRNAs (miRNAs) can be identified by immunoprecipitation of Argonaute (Ago) protein-containing RNA-induced silencing complexes (RISCs) followed by microarray analysis (RIP-Chip). Here we used Ago2-based RIP-Chip to identify transcripts targeted by Kaposi's sarcoma-associated herpesvirus (KSHV) miRNAs (n = 114), Epstein-Barr virus (EBV) miRNAs (n = 44), and cellular miRNAs (n = 2337) in six latently infected or stably transduced human B cell lines. Of the six KSHV miRNA targets chosen for validation, four showed regulation via their 3′UTR, while two showed regulation via binding sites within coding sequences. Two genes governing cellular transport processes (TOMM22 and IPO7) were confirmed to be targeted by EBV miRNAs. A significant number of viral miRNA targets were upregulated in infected cells, suggesting that viral miRNAs preferentially target cellular genes induced upon infection. Transcript half-life both of cellular and viral miRNA targets negatively correlated with recruitment to RISC complexes, indicating that RIP-Chip offers a quantitative estimate of miRNA function.

INTRODUCTION

Herpesviruses are large DNA viruses which after primary infection persist for life, leaving the infected individual at risk for reactivation and subsequent disease. They are divided into three subfamilies based on sequence homologies and unique biological features (α-, β-, and γ-herpesviruses). Humans are infected with two members of the γ-herpesvirus family, namely Kaposi's sarcoma-associated herpesvirus (KSHV) and Epstein-Barr virus (EBV). Both infect B cells and can induce proliferative diseases in humans (Barozzi et al., 2007). KSHV is involved in the development of several human tumors, including Kaposi's sarcoma, primary effusion lymphoma (PEL), and multicentric Castleman disease (reviewed in Dourmishev et al., 2003; Schulz, 2006). PEL tumor cells are thought to originate from postgerminal center B cells due to the presence of hypermutated immunoglobulin genes (Gaidano et al., 1997) and display an intermediate immunophenotype between immunoblasts and plasma cells (Carbone et al., 1996; Nador et al., 1996). The cell line body cavity-based lymphoma-1 (BCBL-1) was established from a malignant effusion (Renne et al., 1996) and serves as a model cell line for PEL. Four groups independently reported on 12 miRNAs expressed during latent infection in PEL cells (Cai et al., 2005; Grundhoff et al., 2006; Pfeffer et al., 2005; Samols et al., 2005), which are remarkably conserved among isolates from many different clinical sources (Marshall et al., 2007).

EBV has been linked to various human malignancies including Burkitt's and Hodgkin's lymphoma, NK/T cell and peripheral T cell lymphoma, posttransplant lymphoma, and nasopharyngeal and gastric carcinoma and is responsible for posttransplant lymphoproliferative disease (PTLD) (Delecluse et al., 2007). EBV readily transforms primary human B-lymphocytes which are the in vitro correlate of EBV-associated PTLD arising under severe immunosuppression (Carbone et al., 2008). EBV encodes for at least 25 miRNAs derived from three separate miRNA clusters (Cai et al., 2006; Grundhoff et al., 2006; Pfeffer et al., 2004; Zhu et al., 2009).

Since viral miRNAs were first described in 2004 (Pfeffer et al., 2004), only very few targets have been identified. For KSHV these include targets involved in angiogenesis, proliferation, immune evasion, or repression of apoptosis (Gottwein et al., 2007; Nachmani et al., 2009; Samols et al., 2007; Skalsky

et al., 2007; Ziegelbauer et al., 2009). In addition, EBV miRNAs promote cell survival (Choy et al., 2008) and target cellular chemokines (CXCL-11) (Xia et al., 2008). Similar to human cytomegalovirus miR-UL112-1, both kshv-miR-K12-7 and ebv-miR-BART2 target MICB, an activating stress-induced NK cell ligand involved in immune recognition of infected cells (Nachmani et al., 2009; Stern-Ginossar et al., 2007).

Recently, immunoprecipitation of RISCs followed by microarray analysis of the RISC-bound miRNA targets (RIP-Chip) was shown to permit the identification of hundreds of cellular miRNA targets with high specificity (Baroni et al., 2008; Beitzinger et al., 2007; Easow et al., 2007; Hendrickson et al., 2008; Karginov et al., 2007; Keene et al., 2006). Here we applied Argonaute 2 (Ago2)-RIP-Chip to identify both cellular and viral miRNA targets in six human B cell lines. We report on the identification of 2337 putative targets of cellular and 158 targets of viral miRNAs in human B cells. Thus, we provide a comprehensive atlas of cellular miRNA targets in human B cells and extend the list of cellular targets of all viral miRNA targets identified so far by > 5-fold.

RESULTS

Study Design

Cellular targets of human γ-herpesvirus miRNAs in B cells were identified by immunoprecipitation (IP) of RISCs followed by microarray analysis of the coimmunoprecipitated mRNAs (RIP-Chip). To identify KSHV miRNA targets, we used the KSHV-positive cell line BCBL-1 and the KSHV- and EBV-negative cell line DG75 transduced by a lentiviral vector to stably express the ten intronic KSHV miRNAs (DG75-10/12). DG75 transduced with eGFP (DG75-eGFP) served as control (Wang et al., 2004). To identify viral miRNA targets of EBV, the prototype B95.8 strain of EBV (BL41 B95.8) and its parental Burkitt's lymphoma cell line (BL41) were used. As B95.8 has lost more than half of the viral miRNAs (Cai et al., 2006), the Jijoye cell line featuring a nondeleted strain, where all known EBV miRNAs are present (Grundhoff et al., 2006), was included.

Identification of Cellular miRNA Targets in Human B Cells

We established a protocol for RISC IP in human B cells using the recently described monoclonal antibody to human Ago2 (α-hAgo2; 11A9) (Rudel et al., 2008). A monoclonal antibody against bromodesoxyuridine (BrdU) served as control. In all experiments the efficiency of the IP was analyzed by quantitative PCR. Usually, an ~1000-fold (670- to 3300-fold) enrichment of cellular Let7a levels was observed when comparing the Ago2-IP with the BrdU-IP samples. A consistent recovery rate of ~50% of Let7a was observed, indicating that Ago2-bound miRNAs comprise at least half of the cellular miRNA pool in human B cells (see Figure S1 available online). Enrichment of cyclin E1 mRNA (CCNE1), a cellular miRNA target of both hsa-miR-16 and hsa-miR-15a (Bandi et al., 2009; Liu et al., 2008), served as a positive control. It was typically enriched by ~8-fold.

Next, microarray analyses of two independent biological replicates for each cell line were performed using Affymetrix human Gene ST 1.0 arrays. For DG75-eGFP, DG75-10/12, and BCBL-1, both the α-Ago2-IP and the α-BrdU-IP samples were analyzed. For both BL41 and BL41 B95.8, the amounts of RNA obtained by the α-BrdU-IPs were too small (50–100 ng) for microarray analysis using the same experimental setting and microarray platform. As the control IP is thought to merely represent total RNA levels in the cell and total RNA has been successfully used as control for the Ago2-IP (Weinmann et al., 2009), we analyzed total RNA instead of the α-BrdU-IP sample for BL41, BL41 B95.8, and Jijoye. Enrichment of transcripts in the Ago2-IP was highly concordant for all six human B cell lines irrespective of whether total RNA or the α-BrdU-IP sample was used (Pearson's correlation coefficient between 0.34 and 0.96 for all combinations, $p < 2.2 \times 10^{-16}$). The complete set of data is provided in Table S1.

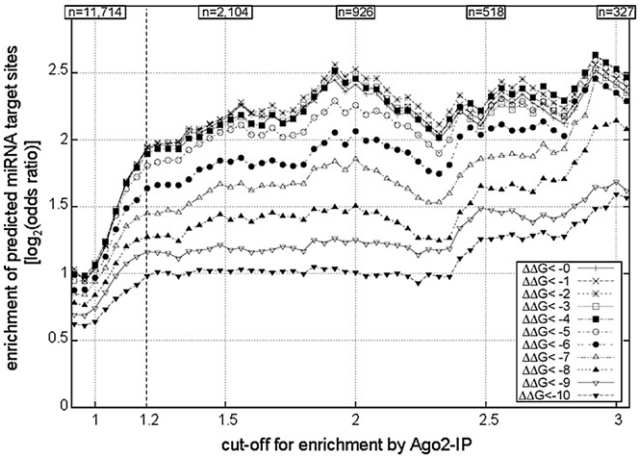

Figure 1. Overrepresentation of Predicted Binding Sites for Cellular miRNAs

Binding sites for these 44 cellular miRNAs expressed in human B cells (Landgraf et al., 2007) were predicted using PITA software and different cutoffs for free binding energies (ΔΔG) ranging from 0 to −10 kcal/mol. Odds ratios for predicted miRNA-binding sites are shown for all genes with a mean enrichment in the Ago2-IP versus control across all six cell lines greater than the indicated value. For each value, the number of transcripts with a mean enrichment greater than this cutoff is shown.

To test for overrepresentation of predicted miRNA-binding sites of cellular miRNAs among the enriched transcripts, we obtained 44 cellular miRNAs expressed in BL41, BL41 B95.8, and DG75 from the miRNA expression atlas (Landgraf et al., 2007) (Table S2A). We used the miRNA target prediction program PITA (Kertesz et al., 2007) and different ΔΔG cutoff values ranging from 0 to −10 kCal/mol to test for overrepresentation of favorable target sites of any of these miRNAs in target 3′UTRs (Figure 1). Overrepresentation was highly significant down to very low mean enrichments ($p < 10^{-58}$ for a mean enrichment of 1.2-fold). Based on statistical analysis, we identified 2337 transcripts significantly enriched across all six B cell lines ($p < 0.05$, one-sided paired t test) with a mean enrichment > 1.2-fold. Enrichment of their transcripts in the six cell lines is shown in Figure 2A, and a complete list is provided in Table S2B. For these genes, predicted binding sites were significantly overrepresented within 5′UTRs ($p = 8.59 \times 10^{-3}$), coding sequences ($p = 2.02 \times 10^{-27}$), and 3′UTRs ($p = 6.19 \times 10^{-63}$, Fisher's exact test and ΔΔG < −4 kCal/mol, see also Table S3A).