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Rapid Communication

Merkel cell polyomavirus encodes a microRNA with the ability to autoregulate viral gene expression

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ABSTRACT

microRNAs (miRNAs) are post-transcriptional regulators of gene expression that play a role in viral infection. We have developed a method to identify viral-encoded miRNAs from viruses in which abundant amounts of infected material is limiting. We show that Merkel Cell Polyomavirus (MCV), a recently identified human virus associated with cancer, encodes a miRNA. This miRNA is expressed from the late strand, lies antisense to the early transcripts and negatively regulates expression of chimeric reporters containing a portion of the early transcripts. Interestingly, different viral isolates have sequence polymorphisms in the pre-miRNA region that result in amino acids substitutions but fully preserve the processing and activity of the miRNAs.

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Introduction

microRNAs (miRNAs) are post-transcriptional regulators of gene expression that play a role in numerous and diverse cellular processes including viral infection (reviewed in Gottwein and Cullen, 2008; Grey et al., 2008; Nair and Zavolan, 2006; Pfeffer, 2008; Samols and Renne, 2006; Sarnow et al., 2006; Sullivan, 2008; Sullivan and Ganem, 2005). Over 120 viral miRNAs have been described from divergent families of DNA viruses. A functional understanding of the majority of viral miRNAs remains incomplete or unknown, however recent studies clearly demonstrate that some viral miRNAs will regulate host gene expression, viral gene expression, or both. Viral miRNAs that regulate host transcripts with relevance to tumorigenesis and immune evasion have been described. In addition, diverse viruses, including an ascovirus (Hussain, Taft, and Asgari, 2008), and several members of the Herpesviridae and Polyomaviridae have been shown to encode miRNAs that autoregulate viral gene expression (Gottwein and Cullen, 2008; Pfeffer, 2008). It has been hypothesized that viral miRNA-mediated autoregulation of gene expression contributes to regulation of the virus lifecycle including the maintenance of latency and activation of lytic replication (Umbach et al., 2008) — the proper balance of which is key to immune evasion strategies. Therefore, proper functioning of autoregulatory miRNAs likely profoundly affects the fitness of some viruses during natural infection *in vivo*. Thus, combined with the recent successes in rationally-designed anti-miRNA inhibitors (Elmen et al., 2008; Krutzfeldt et al., 2005), these observations suggest viral miRNAs might make worthy anti-viral drug targets. Members of the Polyomaviridae include the model laboratory tumor viruses murine polyomavirus (MuPyV) and Simian Virus 40 (SV40), and the human pathogens BK virus (BKV) and JC virus (JCV). We have previously shown that multiple members of the Polyomaviridae, including SV40, JCV, BKV, and MuPyV encode miRNAs that autoregulate early gene expression at late times of infection (Seo et al., 2008; Sullivan et al., 2005, 2006; Sullivan et al., submitted for publication). Recently, three new human polyomaviruses have been described, including KI virus (KIV), WU virus (WUV) and Merkel Cell Polyomavirus (MCV) (Allander et al., 2007; Feng et al., 2008; Gaynor et al., 2007). All three were discovered using new molecular-based technologies. It seems clear from these and other related technologies that we are in for an avalanche of new virus discoveries. Many of these viruses are being discovered faster than *in vitro* culture systems can be developed. Here we present a method for identifying viral-encoded miRNAs that does not require an experimental infectious system. We show that MCV, a likely etiologic agent of human cancer, encodes a miRNA on the late strand that (similar to other reported Polyomaviral miRNAs) has the potential to autoregulate early gene expression. These results further emphasize an important role for miRNAs in Polyomaviral biology and suggest a possible target for future therapeutic strategies.

Results

We set out to develop an experimental strategy to identify viral-encoded miRNAs that does not require abundant amounts of infected material. We chose to examine whether MCV encoded a miRNA for the

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following reasons: first, MCV was only recently discovered and so far no experimental infectious systems have been reported. Second, MCV is linked to human cancer and is likely a *bona fide* human pathogen, suggesting a possible need to develop anti-viral therapeutic targets. Third, because we have previously shown that divergent members of the polyomavirus family (including the SV40-like polyomaviruses and MuPyV) encode miRNAs to autoregulate early gene expression, it seemed probable that other members of the family, including MCV, would also encode miRNAs of similar function.

Our strategy is to computationally predict likely candidate pre-microRNAs (pre-miRNAs) and then to clone them, including flanking sequence, into a heterologous expression vector under control of the cytomegalovirus major early promoter (CMV). These plasmids are then transiently transfected and screened for *bona fide* miRNA expression using several criteria including: Northern blot analysis, cloning and sequencing, and activity in chimeric target reporter assays. We ran the sequence of both published MCV isolates in the viral miRNA prediction algorithm Vmir (Grundhoff et al., 2006; Sullivan and Grundhoff, 2007; Sullivan et al., 2005). A relatively stringent arbitrary cutoff score of 200 was used and we focused on transcripts of the late orientation where all other Polyomaviral miRNAs have so far been identified. Only two candidates met these criteria (Fig. 1A). One candidate, MR62 was found near the origin of replication and was predicted for both the MCC339 and MCC350 isolate genomes. However, this candidate was discarded because this region of the genome produced false positive candidates from several other members of the Polyomaviridae (CSS, unpublished observations). The other candidate, MR17, was the top scoring Vmir prediction for MCC339. However, due to three nucleotide substitutions in the pre-miRNA hairpin region (Fig. 1B), MR17 was ranked only the sixth best candidate for MCC350. We nonetheless pursued candidate MR17 (although it shared no sequence identity with any other known pre-miRNAs of viral or host origin), because it did however, map to a region of the genome we have previously shown encodes a pre-miRNA in MuPyV (Sullivan et al., submitted for publication).

A portion of the genome including candidate MR17 and flanking regions was cloned from either the MCC339 or MCC350 isolates into an expression vector. The resulting plasmids were then transfected into 293T cells. Total RNA was harvested and Northern blot analysis was conducted with the probes described in Fig. 2A. The 5p probe detected 2 bands from RNA harvested from cells transfected with either the MCC339 or MCC350 expression vector. The slower migrating band is approximately 65 nucleotides (consistent with it being a pre-miRNA), and the faster migrating band is approximately 22 nucleotides—exactly where a *bona fide* miRNA should migrate. The 3p probe detected the slower ~65 nucleotide band and low amounts of a ~22 nucleotide band. Importantly, no specific bands were detected from RNA harvested from cells transfected with a negative control vector in which a 300 nucleotide portion of MCC350 (containing the pre-miRNA and flanking regions) was deleted. In addition, a control probe directed against the terminal "loop" portion of the predicted pre-miRNA hairpin, which should not be stabilized by the downstream miRNA processing and effector machinery, only recognized the ~65 nucleotide band. These results rule out non-specific RNA degradation as a source of the 22 nucleotide band detected with the 5p probe. Combined, these results strongly suggest that multiple isolates of MCV encode a pre-miRNA in the late orientation, of which both the 5p and 3p arms of the hairpin are processed into miRNAs. In accordance with the established miRBase precedence (Griffiths-Jones, 2006), we have named these miRNAs "MCV-mir-M1 5p" and "MCV-mir-M1 3p".

To map the derivative miRNAs with more precision we generated a small RNA library from cells expressing the MCC350 miRNAs. Small RNAs were gel purified and ligated to linkers, and reverse transcription was performed to generate a cDNA library. PCR was then conducted with primers specific to each arm of the Vmir predicted pre-miRNA hairpin. Using this strategy we were able to individually map the 3′ and 5′ ends of both the 3p and 5p miRNAs. Notably, the miRNA sequences we mapped from the MCC350 isolate are completely conserved with the MCC339 isolate. (Fig. 1B).

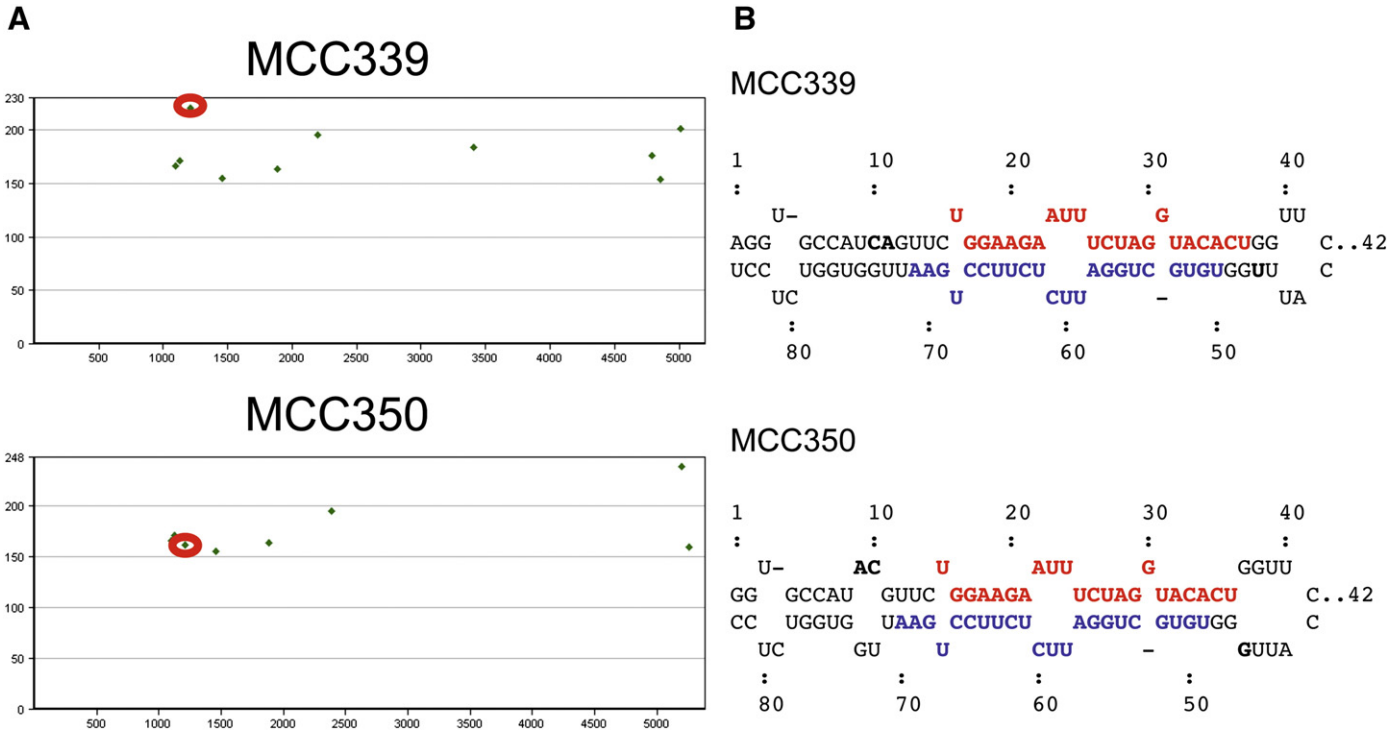

Fig. 1. Vmir predictions for MCV pre-miRNAs. (A) Candidate pre-miRNAs are indicated for both the MCC 339 and MCC 350 isolates (diamonds). The candidate that scored positive in both MCC339 and MCC350 (referred to as MR17 in the text) is marked with a red circle. (B) The secondary structure predictions for MCV precursors are shown. The polymorphic sites between MCC 350 and MCC 339 are shown (bold). The fine-mapped sequences of the 5p (red) and 3p miRNAs (blue) are indicated.

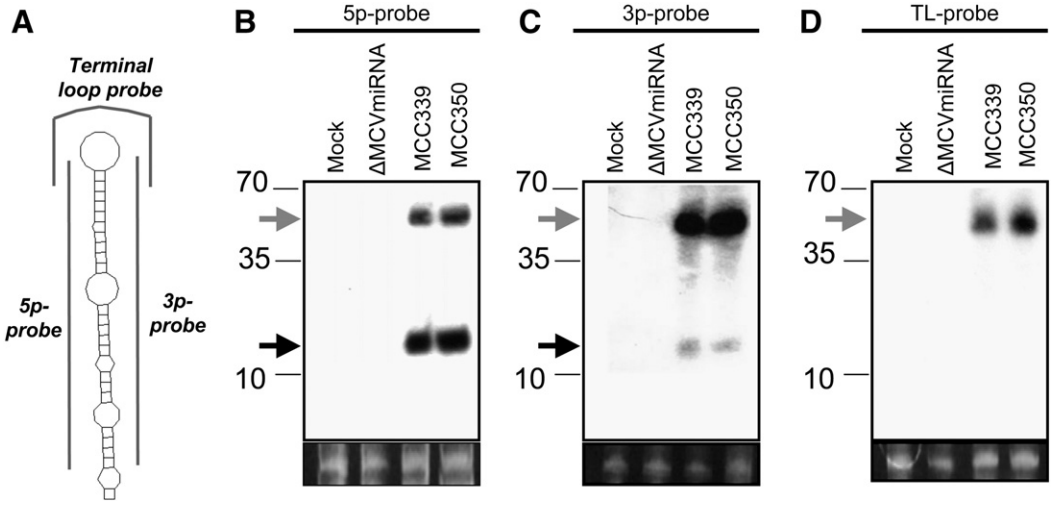

Fig. 2. The Merkel Cell Polyomavirus isolates MCC 339 and MCV 350 encodes miRNAs. (A) Diagram of probes used. Each sample was assayed with a probe that detects the 5p or 3p arm or terminal loop portion of the pre-miRNA. (B–D) Northern blot analysis for the MCV miRNAs. RNA harvested from cells transfected with the mock, negative control Δ MCV miRNA, the MCC339 or MCC350 miRNA expression vectors was analyzed with the indicated probes. Ethidium bromide staining of the low molecular weight RNA is shown as a loading control. Bands corresponding to the 5p and 3p miRNAs (black arrow) or the pre-miRNA (gray arrow) are indicated.

We next sought to determine if MCV-mir-M1 was a functionally active miRNA. miRNAs are initially transcribed as long precursor molecules that contain a characteristic ~80–100 nucleotide hairpin secondary structure. This hairpin is recognized and excised by the nuclear microprocessor multi-protein complex resulting in an ~53–80 nucleotide precursor miRNA (pre-miRNA) (reviewed in Gottwein and Cullen, 2008). The pre-miRNA is further processed by the cytoplasmic, RNAse III-like endonuclease Dicer into a transient double-stranded ~22 nucleotide intermediate. Eventually, a portion of a single "arm" of the pre-miRNA hairpin is incorporated in a stable manner into the multi-protein RNA Induced Silencing Complex (RISC). RISC-bound miRNAs can then bind to target mRNAs with perfect or imperfect complementarity, typically resulting in cleavage or translational repression of the targeted mRNA. We have previously shown that multiple members of the Polyomaviridae encode miRNAs that are active within RISC to direct the cleavage of the complementary early strand RNAs late during infection (Seo et al., 2008; Sullivan, 2008; Sullivan et al., 2005, 2006; Sullivan et al., submitted for publication). Given that MCV-mir-M1 is encoded on the late strand, antisense to the early transcripts (Figs. 3A, B), we predicted that it would direct the cleavage of the early transcripts. To test this hypothesis, we engineered a chimeric luciferase reporter construct, in which a 300 nucleotide portion of the early transcripts (including the MCV-mir-M1 complementary and flanking regions) was cloned into the 3′ UTR. Co-transfection of this reporter with either the MCC339 or MCC350 MCV-mir-M1 expression plasmids results in a dramatic reduction in luciferase activity (Fig. 3C, left panel). Co-transfections of vector alone, an irrelevant miRNA (JCV), or a vector encoding MCC350 with the miRNA region deleted had no effect on this reporter (Fig. 3C, left panel) arguing that the effect we observe is specific to the MCV miRNAs. Importantly, no effect of the MCV miRNAs was detected when they were co-transfected with a control reporter plasmid that lacked complementary sites to MCV-mir-M1 (Fig. 3C, right panel). From these results, two inferences can be made. First, MCV-mir-M1 is fully active within RISC, lending further support as to the authenticity of this miRNA. Second, MCV-mir-M1 functions to direct cleavage of the early RNAs during MCV infection.

Discussion

The post-genomic era has ushered in several new virus discovery techniques that will likely provide a windfall of new pathogens in the near future. Many of these viruses will not have a readily available experimental system making developing and testing anti-viral drugs a challenge. We propose that one useful strategy will be to identify virus-encoded miRNAs and utilize existing successful antisense anti-miRNA drug strategies (Elmen et al., 2008; Krutzfeldt et al., 2005) to develop potential therapeutics. A first step to realizing this goal is to develop methods to identify miRNAs encoded by such pathogens. In this work, we describe such a method and prove its utility on the recently described polyomavirus MCV. We show that two different strains of MCV encode the miRNA despite having a difference of 3 nucleotide substitutions in the pre-miRNA hairpin. Interestingly, these substitutions result in 2 non-conservative amino acid substitutions in the Large T antigen protein that is encoded on the early strand (Fig. 3B). However, these changes do not significantly alter the predicted pre-miRNA hairpin secondary structures on the late strand (Fig. 1B) and the pre-miRNAs from both the MCC339 and MCC350 isolates have similar processing efficiencies (Fig. 2). Furthermore, the derivative miRNAs from both the MCC350 and MCC339 isolates have similar activities in directing the cleavage of the early transcript reporter (Fig. 3C). These results suggest evolutionary pressure exists for MCV to maintain expression of the miRNAs.

We used stringent criteria to conclude that MCV-mir-M1 is a *bona fide* miRNA. The miRNA we identified is detectable via Northern blot analysis (Fig. 2) and is active at directing the inhibition of reporter gene expression, most likely through a RISC-mediated mechanism (Fig. 3C). Furthermore, fine mapping studies of the MCV-mir-M1 and its cognate*3P sequence (the strand that is less efficiently incorporated into RISC) results in a predicted duplex secondary structure with 2–3 nucleotide 3′ overhangs — a hallmark of both microprocessor and Dicer processing. Combined, these results prove MCV-mir-M1 is a *bona fide* miRNA. A notable caveat to our approach is the possibility that we have missed other possible *bona fide* miRNAs encoded by MCV. Indeed, Vmir predicted four candidates that scored higher than MR17 in the MCC350 genome (Fig. 1A). While Vmir is a useful starting point for identifying novel miRNAs in viral genomes, a high Vmir score alone is relatively uninformative due to the possibility of false positives. Therefore, ruling out the existence of additional MCV miRNAs is not possible until exhaustive, saturated small RNA cloning is performed on RNA obtained from all stages of the virus lifecycle (e.g. persistent and lytic infection). Obviously, these studies await the development of laboratory models of MCV infection.

What is the function of the MCV miRNA? Two lines of reasoning strongly suggest that MCV-mir-M1 functions to autoregulate early

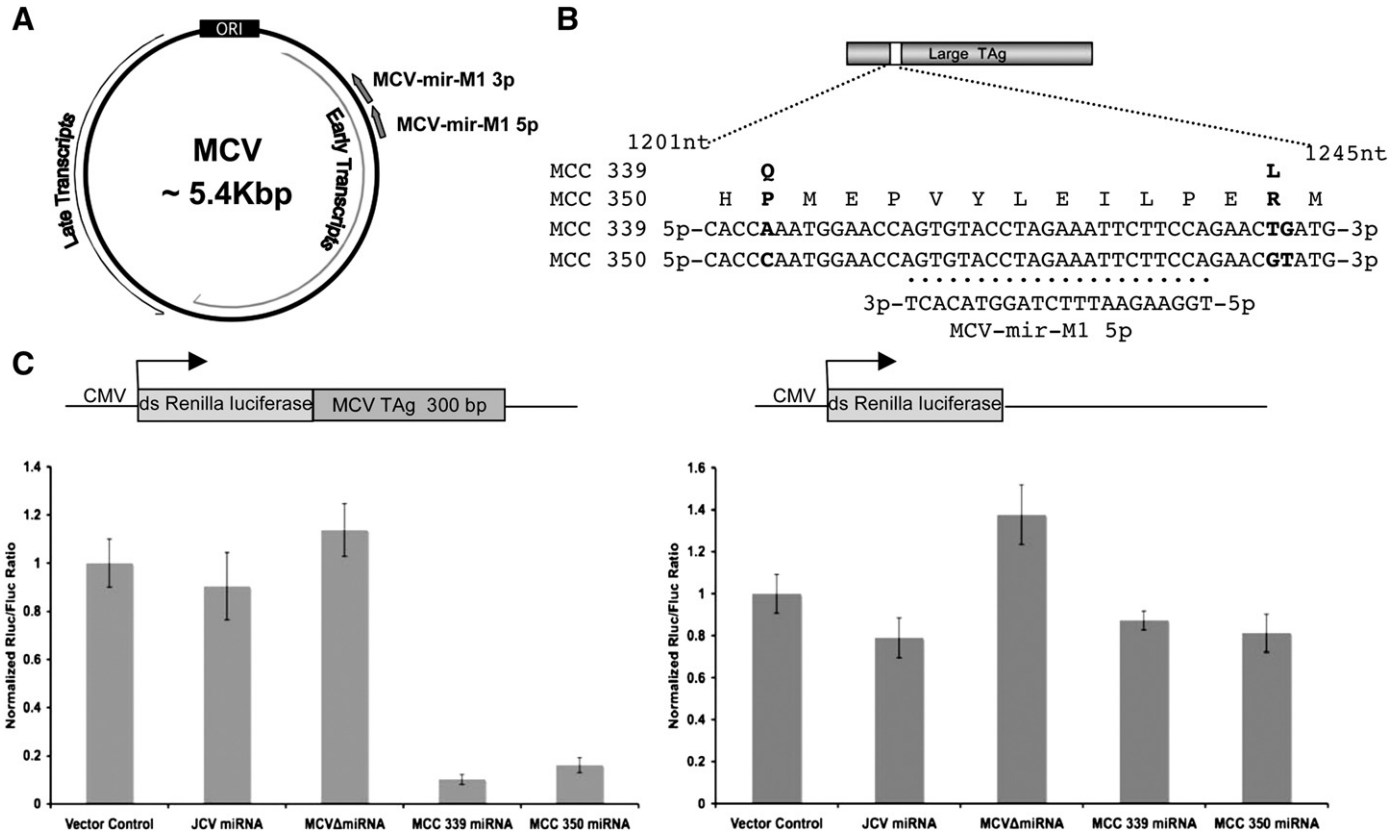

Fig. 3. The MCV miRNAs downregulate early transcripts. (A) Map of MCV genomic organization with the location of the 5p and 3p miRNA indicated with gray arrows. (B) Alignment of the MCV early transcripts (5′–3′) with the complementary 5p miRNA indicated (3′–5′). Shown are the derived amino acids of MCV Large T Antigen that correspond to this region. (C) Diagrams of the MCV miRNA reporter construct (top, left) and control reporter (top, right) are shown. The vectors consist of a *Renilla* luciferase reporter with or without a single copy of a 300 nucleotide region of the Large T antigen early transcript (complementary to the MCV miRNA) cloned into the 3′UTR. Cells were transfected with either reporter plus a plasmid expressing vector alone, the MCV339 miRNA, the MCV350 miRNA, the JCV miRNA, or Δ MCV miRNA (a negative control MCV plasmid in which the pre-miRNA region has been deleted). Firefly luciferase was used as a transfection control and *Renilla* luciferase levels are plotted normalized relative to firefly luciferase levels.

viral gene expression at late times postinfection. First, every polyomavirus we have examined in detail, including the SV40-like polyomaviruses (SV40, JCV and BKV) and MuPyV encode miRNAs at late times of infection that direct the cleavage of early RNA acting to autoregulate early protein levels at late times during infection (Seo et al., 2008; Sullivan et al., 2005, 2006; Sullivan et al., submitted for publication). The genomic location, antisense and complementary to the early RNAs, supports a similar activity for MCV-mir-M1. Second, we have demonstrated that a chimeric reporter containing a portion of the early RNAs is specifically downregulated by expression of MCV-mir-M1. Importantly, these results do not rule out a potential additional function for MCV-mir-M1 in regulating a cellular target. However, since MCV-mir-M1 shares no sequence identity with any of the other known Polyomaviral miRNAs, it seems highly unlikely that these miRNAs could bind to and regulate the same cellular target. Thus, our results strongly suggest that a major function of the MCV miRNA, similar to the other Polyomaviral miRNAs, will be to autoregulate early gene expression at late times of infection.

Finally, the fact that the MCV miRNA is likely only expressed during lytic infection (when the late genes are expressed), limits the potential utility of it as an anti-viral drug target. To date, MCV is associated with tumorigenesis in cases where the viral genome has integrated into the host genome in a stable fashion and is likely only competent to express early gene products. It is currently unclear if any of these tumors are undergoing active lytic infection. Nonetheless, blocking the MCV miRNA function will do little to inhibit the tumorigenic activity of T antigens that are expressed from the tumor genome. However, as the MCV field is brand new, it remains possible that lytic infection may indeed one day be linked to pathogenesis, and in that case, MCV-mir-M1 is a plausible therapeutic target. Irrespective, our study provides a proof of principle that viral miRNAs can rapidly be identified in newly emerging pathogens.

Materials and methods

Computational prediction of MCV miRNA precursors and miRNA Northern blot analysis

Vmir (Grundhoff et al., 2006; Sullivan and Grundhoff, 2007; Sullivan et al., 2005) was used to predict candidate pre-miRNAs in the MCV genome (the accession numbers are; MCC350: EU_375803, MCC339:EU_375804). The candidates were cloned into pcDNA3.1-puro, transiently transfected into human embryonic kidney cells (HEK) 293T. Total RNA was harvested using an in house Trizol-like reagent (2 M Guanidinium Thiocyanate, 20 mM Citrate buffer (PH 4.5), 5 Mm EDTA, 0.25% Sarkosyl, 48% saturated phenol (PH 4.5), 2.1% isoamyl alcohol, 0.5% beta-mercaptoethanol, 0.1% 8-Hydroxyquioline, 0.0025% Coomassie blue) and Northern blot analysis was conducted as previously described (Seo et al., 2008) using the following probes:

MCV5′ probe: GGAACCAGTGTACCTAGAAATTCTTCCAGAACGTA
MCV 3′ probe: AGACCACCAATTCAGGAAGAGAATCCAGCACACCCA
MCV loop: CACACCCAATGGAACCAGTGTAC

Small RNA library generations and fine mapping of MCV miRNA

For fine mapping of the MCV miRNA, a library was generated as previously described (Seo et al., 2008). Briefly, small RNA (10–40

nucleotides) was harvested from 293T cells transfected with the MCC350 miRNA expression plasmid. The small RNA was ligated and TA cloned into PCR2.1. miRNA specific primers were used in a PCR reaction to map both the 3′ and 5′ ends of the MCV miRNAs. The primers used were:

5′ linker forward primer: GTTGATCAGAGCCCAGGG;
3′ linker backward primer: ATTGATGGTGCCTACAG;
MCV 5p forward primer: AGTGTACCTAGAAATTCC
MCV 5p backward primer: GTTCTGGAAGAATTTCTA
MCV 3p forward primer: ATTCAGGAAGAGAATCCA
MCV3p backward primer: GTGTGCTGGATTCTCTTC

DNA constructs, transfection and luciferase assays

All DNA constructs were confirmed by sequence analysis. pCDNA3.1MCVmiRNA (MCC350), that expresses the MCV miRNAs, was generated by subcloning into the Hind III/Xba I sites of pCDNA3.1puro expression vector (Sullivan and Ganem, 2005). A plasmid containing the entire MCV genome (kindly provided by Drs. Chang and Moore, University of Pittsburgh) served as template to subclone the region of the MCV genome containing the MCV pre-miRNA as well as ~1 Kb of flanking regions. The resulting plasmid was named "pcDNA3.1puroMCV350miRNA". To construct the MCV339 miRNA expression vector, we conducted PCR mutagenesis using pcDNA3.1puroMCV350miRNA as the template. As a negative control, we engineered a deleted version of pcDNA3.1puroMCV350miRNA in which 300 nucleotides of sequence, encompassing the pre-miRNA and surrounding regions (nts 1017–1318 of the MCC350) was deleted. The luciferase reporter assays were performed as previously described (Seo et al., 2008) in HEK 293 cells. The reporter contains a single copy of a 300 nucleotide sequence (1051–1350 nt positions from one of the MCC genomes MCC350) corresponding to the complementary and flanking regions of the early transcripts. As a transfection control, *Renilla* luciferase levels were normalized to a co-transfected firefly luciferase reporter as previously described (Seo et al., 2008).

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