MCV-miR-M1 targets the host-cell immune response resulting in the attenuation of neutrophil chemotaxis.

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Abstract

Virus-encoded miRNAs are emerging as key regulators of persistent infection and host-cell immune evasion. Merkel cell polyomavirus (MCPyV), the predominant aetiological agent of Merkel cell carcinoma (MCC), encodes a single miRNA, MCV-miR-M1, which targets the oncogenic MCPyV large T antigen (LT). MCV-miR-M1 has previously been shown to play an important role in establishment of long-term infection, however, the underlying mechanism is not fully understood. A key unanswered question is whether, in addition to auto-regulating LT, MCV-miR-M1 also targets cellular transcripts to orchestrate an environment conducive for persistent infection. To address this, we adopted an RNA-Seq-based approach to identify cellular targets of MCV-miR-M1. Intriguingly, bioinformatics analysis of transcripts that are differentially expressed in cells expressing MCV-miR-M1 revealed several genes implicated in immune evasion. Subsequent target validation led to the identification of the innate immunity protein, SP100, as a direct target of MCV-miR-M1. Moreover, MCV-miR-M1-mediated modulation of SP100 was associated with a significant decrease in CXCL8 secretion, resulting in the attenuation of neutrophil chemotaxis towards Merkel cells harbouring synthetic MCPyV. Based on these observations we propose that MCV-miR-M1 targets key immune response regulators to help facilitate persistent infection, which is a pre-requisite for cellular transformation in MCC.

Introduction

Merkel cell polyomavirus (MCPyV) is the aetiological agent in MCPyV-positive Merkel cell carcinoma (MCC), a rare but aggressive skin cancer that typically occurs in immunocompromised individuals (recently reviewed by (Grundhoff and Fischer, 2015, Liu et al., 2016a)). Following the discovery of MCPyV in 2008 (Feng et al., 2008), research has principally focused on the role of the large and small tumour antigens (LT and sT, respectively). To this end, the last decade has seen a dearth of studies demonstrating how LT and sT usurp or perturb an array of host cell mechanisms to promote virus replication, create a conducive environment for persistent infection and promote transformation and MCC metastasis (Abdul-Sada et al., 2017, Arora et al., 2012, Cheng et al., 2013, Griffiths et al., 2013a, Houben et al., 2012, Knight et al., 2015, Kwun et al., 2013, Kwun et al., 2017, Liu et al., 2011, Shuda et al., 2008, Verhaegen et al., 2017). MCPyV-mediated attenuation of the innate immune response following infection and during virus replication is thought to enable a quasi-latent MCPyV infection in Merkel cells, an event presumed to be a pre-requisite for the development of MCC. Several elegant studies have demonstrated that LT and sT function to diminish the immunological footprint of MCPyV by targeting host innate immune components, including TLR9 (Shahzad et al., 2013) and the NF-κB essential modulator, NEMO (Abdul-Sada et al., 2017, Griffiths et al., 2013a). This approach is representative of convergent and divergent mechanisms that have evolved in most, if not all, DNA viruses. For example, similar immune evasion strategies have been reported in the pathogenic human polyomaviruses, JC and BK (Bauman et al., 2011) and in human papillomavirus (Tummers et al., 2015). The master exponents of host-immune evasion are herpesviruses, which utilise a plethora of approaches to manipulate the host cell immune response, including; destabilisation of host cell mRNA (Rowe et al., 2007), proteasome-mediated degradation of host antiviral proteins (Wiertz et al., 1996) and derailment of host cell innate immunity (Sun et al., 2015). As is the case for MCPyV, early

studies on herpesvirus-mediated host immune evasion centred on virus-encoded proteins, however, pioneering work in HCMV (Stern-Ginossar et al., 2007) followed by studies in EBV (Xia et al., 2008) and KSHV (Nachmani et al., 2009), rapidly established a pivotal role for virus-encoded miRNAs in the derailment of the host cell immune response.

From the perspective of a virus, non-coding RNAs (ncRNA) are extremely attractive regulatory molecules; they take up very little genomic real estate, a single ncRNA can impact hundreds of cellular targets and, crucially, they are non-immunogenic. In recent years, there has been a significant shift in our appreciation of the prevalence and importance of virus miRNAs in the replication and associated pathologies of DNA viruses. Human herpesviruses from all three subfamilies (α-, β- and γ-) have been reported to dampen immune response via the expression of numerous miRNAs that target different facets of the host-immune response network (Giffin and Damania, 2014, Zuo et al., 2017). This strategy is not limited to the large DNA viruses, the pathogenic polyomaviruses BK and JC encode identical miRNAs that target and translationally represses the NK-receptor ligand, ULBP3, an event that is required to establish a persistent infection in host cells (Bauman et al., 2011). MCPyV encodes a single primary miRNA, that is processed to produce MCV-miR-M1-5p and MCV-miR-M1-3p (Lee et al., 2011, Seo et al., 2009). MCV-miR-M1 is expressed from the antisense strand of the LT ORF and thus exhibits perfect sequence complementarity to a region in exon two of the MCPyV LT mRNA transcript. Indeed, MCV-miR-M1 has been shown to attenuate the expression of LT via dual-luciferase reporter assay (Seo et al., 2009), a function it shares with orthologous miRNAs encoded by the other human polyomaviruses (Imperiale, 2014).

MCV-miR-M1-mediated attenuation of LT expression is believed to dampen the host cell immune response during polyomavirus replication, a hypothesis that is supported by studies on

SV40 polyomavirus, where susceptibility to cytotoxic T-cells was significantly increased in SV40 miRNA-mutant infected cells compared with cells infected with wild type SV40 (Sullivan et al., 2005). Strikingly, a similar observation was recently reported for MCPyV, where a miRNA-deficient synthetic MCPyV loses its ability to establish long term infection (Theiss et al., 2015). While these observations suggest an essential role for polyomavirus miRNAs in host immune evasion and persistent infection, it is yet to be determined if polyomavirus miRNAs function solely by downregulating LT. An intriguing possibility is that, MCV-miR-M1, like the BK/JC miRNA, also manipulates the expression of cellular immune system transcripts to facilitate an environment in the host-cell that is conducive for long-term infection.

To date, the only proposed cellular targets for MCV-miR-M1 were derived via *in silico* analysis of the MCV-miR-M1-5p seed sequence (Lee et al., 2011). However, a meticulous miRNA-seq-based study of MCV-miR-M1 expression has since called into doubt the validity of these targets, due to a discrepancy in the seed sequence used in their identification (Theiss et al., 2015). Herein, we describe an unbiased RNA-seq-based analysis of MCV-miR-M1-5p and MCV-miR-M1-3p cellular targets. These data demonstrate that MCV-miR-M1 alters the expression of numerous cellular transcripts. Specifically, direct targeting of the intrinsic antiviral protein, SP100, during MCPyV replication leads to a reduction in the secretion of CXCL8 and a significant decrease in neutrophil chemotaxis towards host-cells harbouring replicative MCPyV. Together, these data describe a mechanism for MCV-miR-M1-mediated subversion of the host cell immune response that is likely to contribute to the reported role of MCV-miR-M1 in establishing long-term MCPyV infection in skin.

Results

MCV-miR-M1 downregulates the expression of numerous cellular immune transcripts.

To determine the effect of MCV-miR-M1 expression on cellular transcript levels independently of other MCPyV transcripts and proteins, we developed inducible stable cell line, transient transfection and mimic-based *in vitro* expression systems for MCV-miR-M1. The comparative expression of MCV-miR-M1 in these different systems was assessed via stem-loop qRT-PCR (Figure 1a) and their functional activity against their cognate target in LT determined using dual-luciferase assay (DLA) (Figure 1b). As can be seen in Figure 1b, MCV-miR-M1 mimics exhibited increased activity against the MCV-miR-M1 cognate recognition sequence compared with transient transfection of an MCV-miR-M1 expression construct. This observation, in combination with the opportunity to dissect 5p and 3p cellular targets (an important consideration given the fact that the MCV-miR-M1 orthologue in JC/BK polyomavirus derails host-cell innate immune response via the 3p arm (Bauman et al., 2011)), prompted us to utilise MCV-miR-M1 mimics in our comparative expression studies. RNA-Seq analysis of MCV-miR-M1 mimic-transfected 293 cells identified 70 and 111 cellular transcripts that showed significant differential expression in the presence of MCV-miR-M1-5p and MCV-miR-M1-3p, respectively (Table S1). Strikingly, gene annotation analysis using DAVID (Dennis et al., 2003) revealed an overrepresentation of gene ontologies (GO) relating to regulation of cell communication and immune system pathways (Figure 2a). Analysis of dysregulated immune-system transcripts produced a list of putative MCV-miR-M1 targets with proposed functional roles in the evasion of host-cell immune response (Figure 2b).

MCV-miR-M1 downregulates cellular immune transcripts during MCPyV replication

MCV-miR-M1-5p and MCV-miR-M1-3p targets identified via RNA-seq were initially validated by transient transfection of 293 cells with the respective MCV-miR-M1 mimic,

followed by analysis of target transcript levels by qRT-PCR. In each instance, qRT-PCR based analysis corroborated our RNA-seq data, demonstrating MCV-miR-M1-5p and MCV-miR-M1-3p specificity for several immune system-related transcripts (Figure 3a). To confirm if cellular targets identified via RNA-seq are also targeted by MCV-miR-M1 in the context of an MCPyV infection, we utilised a previously reported MCPyV replication system based on synthetic MCPyV genomes (MCVSyn) that are identical to prototypical field strain sequences (Neumann et al., 2011). Moreover, a modified MCVSyn (MCVSyn-hpko) mutated to disrupt the MCV-miR-M1 pre-miRNA hairpin structure (Theiss et al., 2015) served as a negative control. Importantly, both synthetic MCPyV genomes have been shown to undergo virus replication in 293 cells to produce increases in genome copy number that are detectable via qRT-PCR (Theiss et al., 2015). To confirm that 293 cells transfected with MCVSyn express functional MCV-miR-M1-5p and MCV-miR-M1-3p, stem-loop qRT-PCR (Figure 3b) and analysis of LT expression (Figure 3c) was carried out over a 72h period. As can be seen in Figure 3b, MCV-miR-M1-5p and MCV-miR-M1-3p expression was readily detected in cells transfected with MCVSyn, however, 293 cells transfected with MCVSyn-hpko displayed no detectable expression of MCV-miR-M1. Moreover, data in Figure 3c demonstrate that LT expression inversely correlates with expression of MCV-miR-M1, we also detected an increase in MCPyV genome copy number 72h post-transfection (Figure S2a), in agreement with previously published data characterising the MCVSyn system. We next sought to validate MCV-miR-M1-5p and MCV-miR-M1-3p mimic targets identified via RNA-seq in the context of MCPyV replication. As shown in Figure 3d, cellular transcripts identified as MCV-miR-M1 mimic targets in our RNA-seq data set and associated with immune system GO were significantly down-regulated in 293 cells harbouring replicative wild type MCVSyn but not in 293 cells transfected with MCVSyn-hpko mutant. Together these data confirm that MCV-miR-

M1 expression during MCPyV replication results in the significant decrease of several cellular transcripts associated with host-cell immune response.

The antiviral innate immunity regulator, SP100, is a direct target of MCV-miR-M1-5p

Having established that MCV-miR-M1 expression during MCPyV replication decreased the level of several host cell immune transcripts, we were keen to determine if any of these dysregulated mRNAs were direct targets of MCV-miR-M1-5p or MCV-miR-M1-3p. To address this, we initially carried out an *in silico* analysis on the 3'UTR region of each MCV-miR-M1 target listed in Figure 2b, using RNA-hybrid (Rehmsmeier et al., 2004). Interrogation of RNA-hybrid data revealed putative MCV-miR-M1 seed sequence recognition sites in the 3'UTR regions of CXCL8, RAET1G, SELPLG and SP100 (Figure 4a). To determine if transcripts containing putative MCV-miR-M1 seed sequence-binding sites were direct targets of the MCV-miR-M1, DLA assays were performed using MCV-miR-M1 mimics. As can be seen in Figure 4b, only the SP100 3'-UTR DLA construct exhibited a nominal but consistent and significant decrease in normalised luciferase signal, which was more pronounced in SP100 3'-UTR deletions (Figure S1). This decrease was observed when the SP100 3'-UTR DLA construct was co-transfected with MCV-miR-M1-5p mimic or the MCVSyn genome, however, no decrease was observed in 293 cells co-transfected with scramble mimic, MCV-miR-M1-3p mimic or the MCVSyn-hpko mutant (Figure 4c), suggesting that the observed MCV-miR-M1-dependent decrease in SP100 mRNA is due to direct targeting and translational repression of the SP100 transcript by MCV-miR-M1-5p. To confirm this, the putative MCV-miR-M1-5p recognition sequence in the SP100 3'-UTR was mutated and DLA assays repeated. Mutation of the putative MCV-miR-M1-5p seed sequence recognition site completely abolished MCV-miR-M1-5p- and MCVSyn-mediated decrease of luciferase signal, confirming that the direct targeting of the SP100 transcript by MCV-miR-M1-5p is dependent on this region of the SP100 3'-UTR (Figure 4d). Finally, we sought to establish if direct targeting of SP100 mRNA

transcript by MCV-miR-M1-5p impacted on SP100 protein levels. Figure 4e clearly shows that SP100 protein levels are significantly reduced in cells transfected with MCV-miR-M1-5p or MCVSyn, compared to negative controls. Together, these data demonstrate that SP100 is a direct target of MCV-miR-M1-5p and this targeting results in diminished SP100 protein levels during MCPyV replication.

MCV-miR-M1-dependent decrease in SP100 modulates secretion of CXCL8

CXCL8 plays an integral role in inducing neutrophil activation and migration in the skin in response to viral infection (Colditz and Watson, 1992) and is downregulated in our RNA-seq data set and during MCPyV replication (Figure 2a; Figure 3d). However, despite the presence of a putative MCV-miR-M1-3p seed sequence match in the 3'-UTR of CXCL8, DLA data shown in Figure 4b suggest that the effect of MCV-miR-M1 on the CXCL8 transcript level is indirect. Despite the indirect nature of MCV-miR-M1-induced CXCL8 transcript changes, given the importance of CXCL8 in the antiviral response in skin we were eager to determine if this resulted in diminished secretion of CXCL8. To address this question, conditioned media was collected from TNF-α stimulated 293 cells 24h post-transfection with mimics and unstimulated 293 cells transfected with either MCVSyn or MCVSyn-hpko genomes and CXCL8 secretion analysed via ELISA. We observed significantly reduced levels of CXCL8 in media collected from TNF-α-stimulated 293 cells transfected with both MCV-miR-M1-5p and MCV-miR-M1-3p mimics, compared with scramble mimic control (Figure 5a). CXCL8 levels were also significantly lower in 293 cells harbouring the wild type MCVSyn, compared with cells transfected with the MCVSyn-hpko miRNA mutant (Figure 5b).

The observation that MCV-miR-M1-5p mimic attenuated CXCL8 secretion is intriguing, as we did not observe any decrease in CXCL8 transcript levels in 293 cells transfected with this arm of the virus miRNA, either via RNA-seq (Table S1) or qRT-PCR (Figure 6a). A possible

explanation for this is that MCV-miR-M1-5p mediated reduction in SP100 expression impacts on CXCL8 secretion. While there is evidence to support a role for PML-bodies in the regulation of cytokine gene expression (Ohgiya et al., 2012), there is no evidence to support a direct role for SP100 in regulating CXCL8 expression. To test if observed decreases in secreted CXCL8 in 293 cells transfected with MCV-miR-M1-5p were related to the 5p-mediated decrease in SP100 protein expression the open reading frame of SP100 lacking the endogenous 3'-UTR was PCR cloned to generate pCDNA-SP100. Co-transfection of 293 cells with pCDNA-SP100 and either MCV-miR-M1-5p or MCVSyn prevented MCV-miR-M1-mediated downregulation of SP100 protein (Figure 5c) and led to the partial rescue of CXCL8 secretion, compared with MCV-miR-M1-3p or negative controls (Figures 5d and 5e). Together these data demonstrate that there is an MCV-miR-M1-dependent decrease in CXCL8 secretion during MCPyV replication and that this reduction is mediated in part via direct targeting of SP100 by MCV-miR-M1-5p.

Diminished migration of neutrophils towards cells harbouring MCPyV.

CXCL8 is a neutrophil chemoattractant and activator (Baggiolini et al., 1989, Harada et al., 1994) that modulates neutrophil migration (Huber et al., 1991) and is an important component of the innate immune response to virus infection in skin (de Oliveira et al., 2016). To establish if MCV-miR-M1-dependent downregulation of CXCL8 during MCPyV replication is significant in terms of attenuating neutrophil chemotaxis, trans-well migration assays were performed. Neutrophil migration was significantly impaired towards conditioned media from both MCV-miR-M1-5p and -3p mimic transfected and MCVSyn-transfected cells compared with controls (Figure 5f), suggesting that MCV-miR-M1 negatively regulates neutrophil migration, *in vitro*, towards cells harbouring replicative MCPyV. To investigate if migration was due to attenuation of CXCL8 levels, neutrophil migration assays were repeated in the presence or absence of the CXCR2 antagonist, SB265610, which has been reported to block

CXCL8-induced neutrophil migration *in vitro* and *in vivo* (Bradley et al., 2009). Treatment with SB265610 resulted in significant downregulation of neutrophil migration for both MCVSyn- and MCVSyn-hpko-transfected cells (Figure 5g). This reduction was more pronounced in MCVSyn-transfected cells, where MCV-miR-M1 induces CXCL8 downregulation. These data suggest that MCV-miR-M1 mediates downregulation of neutrophil migration during virus replication at least in part via modulation of CXCL8 secretion.

MCV-miR-M1 targets SP100 and CXCL8 secretion in MCC cell lines resulting in attenuated neutrophil migration.

Thus far data was obtained using the 293 cell line, which has been previously shown to facilitate the study of MCPyV virus-host cell interactions (Griffiths et al., 2013b, Kwun et al., 2015, Wang et al., 2012) and act as a semi-permissive cell line for the MCVSyn system (Neumann et al., 2011). However, given the proposed function of MCV-miR-M1 in the establishment of persistent infection and our data suggesting that this might be achieved in part via the dysregulation of SP100 and CXCL8 secretion we were keen to demonstrate that MCV-miR-M1 functions in the same manner in Merkel cells, where quasi-latency is hypothesised to be a functional prerequisite for transformation. Merkel cells have been cultured *in vitro* alongside keratinocytes (Fradette et al., 2003), however, no Merkel cell-specific tissue culture system is currently available. Therefore, to study MCV-miR-M1 in a Merkel cell background we utilised the MCPyV-negative MCC cell line, MCC13 and the MCPyV-positive cell line, MKL-1, which has been reported to be a representative model for MCC (Daily et al., 2015). MCC13 cells were transfected with MCV-miR-M1-5p, MCV-miR-M1-3p and scramble mimic control and an analysis of SP100 and CXCL8 transcript and protein expression carried out, as described above. We detected significant changes in both SP100 and CXCL8 mRNA (Figure 6a) and protein levels (Figures 6b and 6c) that were consistent with data gathered using 293 cells. Moreover, neutrophil migration was also significantly reduced in both MCC cell lines

expressing MCV-miR-M1 mimics (Figure 6d). These data confirm that MCV-miR-M1 modulates the expression of SP100 and CXCL8 in two MCC cell lines, resulting in reduced migration of neutrophils towards Merkel cells harbouring synthetic MCPyV genomes.

Discussion

Despite mounting evidence describing virus-encoded miRNAs as key regulators of host cell transcripts (Kincaid and Sullivan, 2012) for many virus miRNAs there is a lack of global target identification. Currently, there are no published data describing validated cellular targets of MCV-miR-M1. Using an unbiased RNA-seq-based approach we have identified numerous MCV-miR-M1-modulated cellular transcripts, including SP100, which is a constituent of Promyelocytic leukaemia protein-nuclear bodies (PML-NB), a nuclear protein complex involved in the regulation of transcription, apoptosis, cell cycle, response to stress and hormone signalling and development. While our RNA-seq data identified various cellular targets, many of which are involved in immune related processes, it is important to acknowledge that given the HEK293-cell background it is possible that some relevant targets were missed.

SP100 is an intriguing MCV-miR-M1 target, which has been implicated in the innate immune response against dsDNA viruses, including MCPyV (Gunther et al., 2014, Jiang et al., 2011, Neumann et al., 2016, Tavalai and Stamminger, 2009, Wagenknecht et al., 2015) and has been shown to be downregulated during MCPyV replication, although no mechanism for this was reported (Neumann et al., 2016). Our data suggest that MCV-miR-M1 mediates (at least in part) the observed downregulation of SP100 in cells harbouring synthetic MCPyV. The argument for MCV-miR-M1 mediated downregulation, rather than modulation by one of the MCPyV proteins is strengthened by the fact that SP100 levels are not decreased in cells transfected with MCVSyn-hpko, which lacks MCV-miR-M1 expression but exhibits

significantly increased expression of LT, sT and VP1 (Figures 3c and 4e and (Theiss et al., 2015).

A caveat of the MCV-hpko mutant in terms of its use as a negative control is that loss of MCV-miR-M1 expression results in increased levels of LT that may contribute to some of the observed effects. The use of MCV-miR-M1 mimics alongside MCVSyn has enabled us to corroborate putative MCV-miR-M1-specific effects observed using the synthetic virus in the absence of other MCPyV transcripts and proteins. While we consistently observed similar results between MCV-miR-M1 mimics and MCVSyn, we cannot rule out that elevated LT expression in MCVSyn-hpko cells may be contributing to some of our observations, in particular changes in CXCL8 secretion, where LT has been shown to increase CXCL8 expression (Richards et al., 2015). However, mimic data suggest that MCV-miR-M1 can significantly impair CXCL8 expression in the absence of LT (Figure 5a).

Our understanding of how PML-NBs and SP100 contribute to the antiviral response has improved in recent years (Nisole et al., 2013), however, mechanistically there is much still to unpick. Here we have shown that direct targeting of SP100 by MCV-miR-M1 modulates CXCL8 secretion. It has been reported that PML functions to regulate the expression of proinflammatory cytokines, including IL-6 and CXCL8 (Lunardi et al., 2011), however, it is not known if SP100 is also able to function in this manner. One explanation may be that SP100 is able to transcriptionally regulate CXCL8 either directly or in *trans*, a notion supported by previous work demonstrating that SP100 functions to activate ETS-family transcription factors (Wasylyk et al., 2002). However, as MCV-miR-M1-5p induces changes in secreted CXCL8, but does not alter CXCL8 transcript levels (Table S1 and Figure 6a), it seems more likely that MCV-miR-M1-5p-dysregulation of SP100 (or other unidentified cellular transcripts) impact on CXCL8 via a post-translational mechanism. Indeed, our observation that ectopic expression of pCDNA-SP100 only partially rescues the MCV-miR-M1-5p mediated reduction of CXCL8

secretion strongly suggests that other MCV-miR-M1 dysregulated factors are influencing CXCL8 secretion and presumably, in turn, neutrophil migration. Trans-well assays support this hypothesis, as both 5p and 3p MCV-miR-M1 mimics diminished neutrophil chemotaxis to a similar level, despite MCV-miR-M1-3p having no effect on SP100 mRNA or protein levels. We are currently investigating if other indirect MCV-miR-M1 targets impair neutrophil migration during MCPyV replication.

The functional significance of MCV-miR-M1-mediated attenuation of neutrophil chemotaxis for MCPyV remains to be established. One possibility that has been reported in γ-herpesviruses (Zhu et al., 2013) and previously discussed for MCPyV (Theiss et al., 2015) is that by reducing the immunological footprint of MCPyV during replication the virus is more likely to establish a quasi-latent infection. Such a mechanism might explain how MCPyV is able to infect Merkel cells for a duration of time that permit rare MCPyV-genome integration events and subsequent transformation and development of MCC, although the absence of a non-transformed Merkel cell culture system makes this theory difficult to test. Recently, dermal fibroblasts were identified as the likely primary host cell type naturally and productively infected by MCPyV (Liu et al., 2016b). We are currently investigating MCV-miR-M1-mediated attenuation of neutrophil chemotaxis in these cells and it will be of interest to determine how this impacts on commensal MCPyV infection in skin. In summary, we have employed an unbiased, RNA-seq based approach to identify cellular MCV-miR-M1 targets and shown that the innate immune response protein, SP100 is a direct target of MCV-miR-M1-5p. Furthermore, we have demonstrated that there is a MCV-miR-M1-dependent decrease in neutrophil migration that may be contributing to the host cell immune evasion strategy of MCPyV following infection of Merkel cells.

Materials & Methods

Tissue culture

Cells were purchased from ECACC and certified mycoplasma-free. 293 and Flp-In™ T-REx™ 293-MCVmiR were maintained in DMEM media supplemented with 10% FBS, 100 U/ml of penicillin and 100 µg/ml of streptomycin. MCC13 and MKL1 cells were maintained in RPMI 1640 media supplemented with 10% FBS, 100 U/ml of penicillin and 100 µg/ml of streptomycin. All cells were cultured at 37˚C and 5% CO2 and transfected using Lipofectamine 3000 as described (Boyne et al., 2010).

RNA sequencing (RNA-Seq)

293 cells were transfected with either MCV-miR-M1-5p, MCV-miR-M1-3p or control mimic (Thermo Fisher Scientific) prior to RNA extraction and confirmation of MCV miRNA 5p and 3p expression via stem loop qRT-PCR. Total RNA libraries were prepared using TruSeq Stranded Total RNA Sample Prep Kit (Illumina, USA) and the TruSeq cDNA libraries were analysed via Illumina HiSeq2500 paired end 100bp. Differential gene expression and gene annotation analysis were carried out using edgeR software and DAVID annotation tool, respectively. RNA-seq data have been deposited in the ArrayExpress database at EMBL-EBI (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-6526.

qRT-PCR

Total RNA was extracted from cells and 500ng used to generate cDNA prior to qRT-PCR analysis using SsoAdvanced SYBR master mix (Bio-Rad, UK) on the CFX96 system (Bio-Rad, UK).

Stem loop qRT-PCR was carried out to detect MCV miRNA (Czimmerer et al., 2013). Briefly, stem loop cDNA was generated using a stem loop primer containing a MCV miRNA specific hexamer sequence. A forward primer complementary to MCV miRNA and a universal reverse

primer were used to detect miRNA expression via qRT-PCR. qRT-PCR analysis of MCPyV genome copy number was performed as previously described (Theiss et al., 2015). A list of oligonucleotides used during this study can be found in Table S2.

Protein analysis

Human CXCL8 ELISA (eBioscience) was carried out as previously described (Bridgewood et al., 2017). SDS-PAGE and western blot analysis were performed as described (Schumann et al., 2016). Anti-SP100 and anti-LT (CM2B4) (Santa Cruz) were used at a dilution of 1:1000, GAPDH (Abcam) was used at a dilution of 1:10000.

Dual luciferase assay

psiCHECK-2 expression construct was generated by inserting the respective 3'UTR response element downstream of Renilla luciferase (hRluc) gene. 293 cells were co-transfected with psiCHECK-2/3'UTR of interest and MCV miRNA mimics, control mimic or MCVSyn genomes, prior to Dual luciferase assay (DLA).

Neutrophil isolation and migration assay

Neutrophils were isolated from peripheral blood as previously described (Lau and Hunstad, 2013). *In vitro* neutrophil migration was measured using transwell chambers. Briefly, neutrophils were seeded into the top chamber and cell supernatants were placed in the lower chamber. Plates were incubated at 37ºC and 5% CO2 for two hours prior to mixing the lower chamber contents with equal volume of CellTiter-Glo® 2.0 (Promega) and recording luminescence using a TECAN infinite M200 plate reader.

The authors state no conflict of interest.

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Figure 1: Comparative analysis of MCV-miR-M1 expression systems.

Total RNA was extracted from doxycycline-induced MCV-miR-M1-293 cells and 293 cells transiently transfected with either pCDNA3.1-MCV-miR-M1 or MCV-miR-M1 mimics and cDNA generated prior to analysis of MCV-miR-M1-5p and MCV-miR-M1-3p expression via stem-loop qPCR, n=3 (a). 293 cells were co-transfected with either psiCHECK2 harbouring the cognate MCV-miR-M1 recognition sequence or a mutated control sequence alongside pCDNA3.1-MCV-miR-M1 or MCV-miR-M1 mimics and DLA performed to determine relative miRNA activity, n=3 (b).

Figure 2: MCV-miR-M1 preferentially dysregulates cellular immune-response transcripts.

RNA-seq was performed on total RNA extracted from 293 cells that had either been transfected in biological triplicate with scramble miRNA mimic, MCV-miR-M1-5p mimic or MCV-miR-M1-3p mimic. Analysis of differential expression of cellular transcripts by edgeR and subsequent gene ontology analysis using DAVID (a) identified a set of cellular transcripts that were significantly dysregulated by MCV-miR-M1 and involved in immune response (b).

Figure 3: MCV-miR-M1 targets immune-response targets during MCPyV replication.

293 cells were transiently transfected with MCV-miR-M1-5p, MCV-miR-M1-3p or scramble mimic control and expression of target transcript determined via qRT-PCR, n=3 (a). 293 cells were transfected with MCVSyn or MCVSyn-hpko genomes and total RNA and protein extracted 24h, 48h and 72h post-transfection for analysis of MCV-miR-M1-5p and MCV-miR-M1-3p expression via stem-loop qRT-PCR (b) and LT expression by immunoblot (c), n=3. 293 cells were transfected with either MCVSyn of MCVSyn-hpko genomes and cultured for 72h prior to total RNA extraction and analysis of MCV-miR-M1 target expression by qRT-PCR, n=3 (d).

Figure 4: MCV-miR-M1 directly targets the viral innate immune response protein, SP100.

Full-length 3'UTRs of each validated target were analysed using RNA-hybrid to identify putative seed-sequence binding sites for MCV-miR-M1 (a). The full length 3'UTR regions of CXCL8, RAET1G, SELPLG and SP100 were PCR-amplified and cloned downstream of *Renilla luciferase* DLA assays performed via co-transfection of 293 cells with each recombinant 3'-UTR construct and either scramble control, MCV-miR-M1-5p or MCV-miR-M1-3p mimic, n=3 (b). DLA assays carried out via co-transfection of 293 cells with scramble control mimic, MCV-miR-M1-5p mimic, MCV-miR-M1-3p mimic, MCVSyn or MCVSyn-hpko and either wild-type SP100 3'-UTR (c) or the mutant SP100 3'-UTR (d), n=3. 293 cells were transfected with either scramble mimic control, MCV-miR-M1-5p mimic, MCV-miR-M1-3p mimic, MCVSyn or MCVSyn-hpko and cultured for 48h prior to isolation of total protein and analysis by immunoblot, n=3 (e).

Figure 5: MCV-miR-M1 mediates a reduction in the secretion of CXCL8 that impairs neutrophil chemotaxis.

Conditioned media was collected 24h post-transfection from TNF-α stimulated 293 cells transfected with either scramble mimic, MCV-miR-M1-5p mimic or MCV-miR-M1-3p mimic and relative CXCL8 levels determined via ELISA, n=3 (a). 293 cells were transfected with either MCVSyn or MCVSyn-hpko genomes (in the absence of TNF-α) and conditioned media collected 72h post-transfection for analysis of CXCL8 secretion via ELISA, n=3 (b). 293 cells were treated as described in (a) and (b) above with the addition of either pCDNA3.1 (-) or pcDNA-SP100 (SP100) to the transfection mix. Total protein was isolated and SP100 expression assessed via immunoblot (c), alongside this, conditioned media was collected for analysis of CXCL8 secretion via ELISA (d and e), n=3. 293 cells were stimulated with TNF-α

prior to transfection with either scramble mimic, MCV-miR-M1-5p mimic or MCV-miR-M1-3p and cultured for 24h before isolating conditioned growth media, alongside these 293 cells were also transfected with MCVSyn or MCVSyn-hpko and cultured for 72h prior to collection of conditioned growth media. Both sets of conditioned media obtained above were then used in neutrophil chemotaxis trans-well migration assay, n=3 (f). 293 cells were transfected with MCVSyn or MCVSyn-hpko in the presence or absence of SB265610 and cultured for 72h prior to collection of conditioned growth media and neutrophil chemotaxis trans-well migration assay, n=3 (g).

Figure 6: MCV-miR-M1 mediated attenuation of CXCL8 secretion and neutrophil chemotaxis also occurs in Merkel cells.

MCC13 or MKL-1 cells were transfected with either scramble mimic, MCV-miR-M1-5p mimic or MCV-miR-M1-3p mimic and cultured for 24h before isolating total RNA for analysis of CXCL8 and SP100 transcripts levels via qRT-PCR, n=3 (a), total protein isolation for the analysis of SP100 expression by immunoblot, n=3 (b). MCC13 or MKL-1 cells were stimulated with TNF-α prior to transfection with either scramble mimic, MCV-miR-M1-5p mimic or MCV-miR-M1-3p and cultured for 24h before the collection of conditioned growth media and analysis of secreted CXCL8 levels via ELISA, n=3 (c) and neutrophil chemotaxis by trans-well migration assay, n=3 (d).

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