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

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13 **Abstract**

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

31 **Introduction**

32 Merkel cell polyomavirus (MCPyV) is the aetiological agent in MCPyV-positive Merkel cell
33 carcinoma (MCC), a rare but aggressive skin cancer that typically occurs in
34 immunocompromised individuals (recently reviewed by (Grundhoff and Fischer, 2015, Liu et
35 al., 2016a)). Following the discovery of MCPyV in 2008 (Feng et al., 2008), research has
36 principally focused on the role of the large and small tumour antigens (LT and sT,
37 respectively). To this end, the last decade has seen a dearth of studies demonstrating how LT
38 and sT usurp or perturb an array of host cell mechanisms to promote virus replication, create
39 a conducive environment for persistent infection and promote transformation and MCC
40 metastasis (Abdul-Sada et al., 2017, Arora et al., 2012, Cheng et al., 2013, Griffiths et al.,
41 2013a, Houben et al., 2012, Knight et al., 2015, Kwun et al., 2013, Kwun et al., 2017, Liu et
42 al., 2011, Shuda et al., 2008, Verhaegen et al., 2017). MCPyV-mediated attenuation of the
43 innate immune response following infection and during virus replication is thought to enable
44 a quasi-latent MCPyV infection in Merkel cells, an event presumed to be a pre-requisite for
45 the development of MCC. Several elegant studies have demonstrated that LT and sT function
46 to diminish the immunological footprint of MCPyV by targeting host innate immune
47 components, including TLR9 (Shahzad et al., 2013) and the NF-κB essential modulator,
48 NEMO (Abdul-Sada et al., 2017, Griffiths et al., 2013a). This approach is representative of
49 convergent and divergent mechanisms that have evolved in most, if not all, DNA viruses. For
50 example, similar immune evasion strategies have been reported in the pathogenic human
51 polyomaviruses, JC and BK (Bauman et al., 2011) and in human papillomavirus (Tummers et
52 al., 2015). The master exponents of host-immune evasion are herpesviruses, which utilise a
53 plethora of approaches to manipulate the host cell immune response, including;
54 destabilisation of host cell mRNA (Rowe et al., 2007), proteasome-mediated degradation of
55 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).

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

91 To date, the only proposed cellular targets for MCV-miR-M1 were derived via *in silico*
92 analysis of the MCV-miR-M1-5p seed sequence (Lee et al., 2011). However, a meticulous
93 miRNA-seq-based study of MCV-miR-M1 expression has since called into doubt the validity
94 of these targets, due to a discrepancy in the seed sequence used in their identification (Theiss
95 et al., 2015). Herein, we describe an unbiased RNA-seq-based analysis of MCV-miR-M1-5p
96 and MCV-miR-M1-3p cellular targets. These data demonstrate that MCV-miR-M1 alters the
97 expression of numerous cellular transcripts. Specifically, direct targeting of the intrinsic
98 antiviral protein, SP100, during MCPyV replication leads to a reduction in the secretion of
99 CXCL8 and a significant decrease in neutrophil chemotaxis towards host-cells harbouring
100 replicative MCPyV. Together, these data describe a mechanism for MCV-miR-M1-mediated
101 subversion of the host cell immune response that is likely to contribute to the reported role of
102 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

126 MCV-miR-M1-5p and MCV-miR-M1-3p targets identified via RNA-seq were initially
127 validated by transient transfection of 293 cells with the respective MCV-miR-M1 mimic,
128 followed by analysis of target transcript levels by qRT-PCR. In each instance, qRT-PCR
129 based analysis corroborated our RNA-seq data, demonstrating MCV-miR-M1-5p and MCV-
130 miR-M1-3p specificity for several immune system-related transcripts (Figure 3a). To confirm
131 if cellular targets identified via RNA-seq are also targeted by MCV-miR-M1 in the context of
132 an MCPyV infection, we utilised a previously reported MCPyV replication system based on
133 synthetic MCPyV genomes (MCVSyn) that are identical to prototypical field strain
134 sequences (Neumann et al., 2011). Moreover, a modified MCVSyn (MCVSyn-hpko) mutated
135 to disrupt the MCV-miR-M1 pre-miRNA hairpin structure (Theiss et al., 2015) served as a
136 negative control. Importantly, both synthetic MCPyV genomes have been shown to undergo
137 virus replication in 293 cells to produce increases in genome copy number that are detectable
138 via qRT-PCR (Theiss et al., 2015). To confirm that 293 cells transfected with MCVSyn
139 express functional MCV-miR-M1-5p and MCV-miR-M1-3p, stem-loop qRT-PCR (Figure
140 3b) and analysis of LT expression (Figure 3c) was carried out over a 72h period. As can be
141 seen in Figure 3b, MCV-miR-M1-5p and MCV-miR-M1-3p expression was readily detected
142 in cells transfected with MCVSyn, however, 293 cells transfected with MCVSyn-hpko
143 displayed no detectable expression of MCV-miR-M1. Moreover, data in Figure 3c
144 demonstrate that LT expression inversely correlates with expression of MCV-miR-M1, we
145 also detected an increase in MCPyV genome copy number 72h post-transfection (Figure
146 S2a), in agreement with previously published data characterising the MCVSyn system. We
147 next sought to validate MCV-miR-M1-5p and MCV-miR-M1-3p mimic targets identified via
148 RNA-seq in the context of MCPyV replication. As shown in Figure 3d, cellular transcripts
149 identified as MCV-miR-M1 mimic targets in our RNA-seq data set and associated with
150 immune system GO were significantly down-regulated in 293 cells harbouring replicative

151 wild type MCVSyn but not in 293 cells transfected with MCVSyn-hpko mutant. Together
152 these data confirm that MCV-miR-M1 expression during MCPyV replication results in the
153 significant decrease of several cellular transcripts associated with host-cell immune response.

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

155 Having established that MCV-miR-M1 expression during MCPyV replication decreased the
156 level of several host cell immune transcripts, we were keen to determine if any of these
157 dysregulated mRNAs were direct targets of MCV-miR-M1-5p or MCV-miR-M1-3p. To
158 address this, we initially carried out an *in silico* analysis on the 3'UTR region of each MCV-
159 miR-M1 target listed in Figure 2b, using RNA-hybrid (Rehmsmeier et al., 2004).
160 Interrogation of RNA-hybrid data revealed putative MCV-miR-M1 seed sequence
161 recognition sites in the 3'UTR regions of CXCL8, RAET1G, SELPLG and SP100 (Figure
162 4a). To determine if transcripts containing putative MCV-miR-M1 seed sequence-binding
163 sites were direct targets of the MCV-miR-M1, DLA assays were performed using MCV-miR-
164 M1 mimics. As can be seen in Figure 4b, only the SP100 3'-UTR DLA construct exhibited a
165 nominal but consistent and significant decrease in normalised luciferase signal, which was
166 more pronounced in SP100 3'-UTR deletions (Figure S1). This decrease was observed when
167 the SP100 3'-UTR DLA construct was co-transfected with MCV-miR-M1-5p mimic or the
168 MCVSyn genome, however, no decrease was observed in 293 cells co-transfected with
169 scramble mimic, MCV-miR-M1-3p mimic or the MCVSyn-hpko mutant (Figure 4c),
170 suggesting that the observed MCV-miR-M1-dependent decrease in SP100 mRNA is due to
171 direct targeting and translational repression of the SP100 transcript by MCV-miR-M1-5p. To
172 confirm this, the putative MCV-miR-M1-5p recognition sequence in the SP100 3'-UTR was
173 mutated and DLA assays repeated. Mutation of the putative MCV-miR-M1-5p seed sequence
174 recognition site completely abolished MCV-miR-M1-5p- and MCVSyn-mediated decrease of
175 luciferase signal, confirming that the direct targeting of the SP100 transcript by MCV-miR-

176 M1-5p is dependent on this region of the SP100 3'-UTR (Figure 4d). Finally, we sought to
177 establish if direct targeting of SP100 mRNA transcript by MCV-miR-M1-5p impacted on
178 SP100 protein levels. Figure 4e clearly shows that SP100 protein levels are significantly
179 reduced in cells transfected with MCV-miR-M1-5p or MCVSyn, compared to negative
180 controls. Together, these data demonstrate that SP100 is a direct target of MCV-miR-M1-5p
181 and this targeting results in diminished SP100 protein levels during MCPyV replication.

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

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

198 The observation that MCV-miR-M1-5p mimic attenuated CXCL8 secretion is intriguing, as
199 we did not observe any decrease in CXCL8 transcript levels in 293 cells transfected with this

200 arm of the virus miRNA, either via RNA-seq (Table S1) or qRT-PCR (Figure 6a). A possible
201 explanation for this is that MCV-miR-M1-5p mediated reduction in SP100 expression
202 impacts on CXCL8 secretion. While there is evidence to support a role for PML-bodies in the
203 regulation of cytokine gene expression (Ohgiya et al., 2012), there is no evidence to support a
204 direct role for SP100 in regulating CXCL8 expression. To test if observed decreases in
205 secreted CXCL8 in 293 cells transfected with MCV-miR-M1-5p were related to the 5p-
206 mediated decrease in SP100 protein expression the open reading frame of SP100 lacking the
207 endogenous 3'-UTR was PCR cloned to generate pCDNA-SP100. Co-transfection of 293
208 cells with pCDNA-SP100 and either MCV-miR-M1-5p or MCVSyn prevented MCV-miR-
209 M1-mediated downregulation of SP100 protein (Figure 5c) and led to the partial rescue of
210 CXCL8 secretion, compared with MCV-miR-M1-3p or negative controls (Figures 5d and
211 5e). Together these data demonstrate that there is an MCV-miR-M1-dependent decrease in
212 CXCL8 secretion during MCPyV replication and that this reduction is mediated in part via
213 direct targeting of SP100 by MCV-miR-M1-5p.

214 **Diminished migration of neutrophils towards cells harbouring MCPyV.**

215 CXCL8 is a neutrophil chemoattractant and activator (Baggiolini et al., 1989, Harada et al.,
216 1994) that modulates neutrophil migration (Huber et al., 1991) and is an important
217 component of the innate immune response to virus infection in skin (de Oliveira et al., 2016).
218 To establish if MCV-miR-M1-dependent downregulation of CXCL8 during MCPyV
219 replication is significant in terms of attenuating neutrophil chemotaxis, trans-well migration
220 assays were performed. Neutrophil migration was significantly impaired towards conditioned
221 media from both MCV-miR-M1-5p and -3p mimic transfected and MCVSyn-transfected cells
222 compared with controls (Figure 5f), suggesting that MCV-miR-M1 negatively regulates
223 neutrophil migration, *in vitro*, towards cells harbouring replicative MCPyV. To investigate if
224 migration was due to attenuation of CXCL8 levels, neutrophil migration assays were repeated

225 in the presence or absence of the CXCR2 antagonist, SB265610, which has been reported to
226 block CXCL8-induced neutrophil migration *in vitro* and *in vivo* (Bradley et al., 2009).
227 Treatment with SB265610 resulted in significant downregulation of neutrophil migration for
228 both MCVSyn- and MCVSyn-hpko-transfected cells (Figure 5g). This reduction was more
229 pronounced in MCVSyn-transfected cells, where MCV-miR-M1 induces CXCL8
230 downregulation. These data suggest that MCV-miR-M1 mediates downregulation of
231 neutrophil migration during virus replication at least in part via modulation of CXCL8
232 secretion.

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

235 Thus far data was obtained using the 293 cell line, which has been previously shown to
236 facilitate the study of MCPyV virus-host cell interactions (Griffiths et al., 2013b, Kwun et al.,
237 2015, Wang et al., 2012) and act as a semi-permissive cell line for the MCVSyn system
238 (Neumann et al., 2011). However, given the proposed function of MCV-miR-M1 in the
239 establishment of persistent infection and our data suggesting that this might be achieved in
240 part via the dysregulation of SP100 and CXCL8 secretion we were keen to demonstrate that
241 MCV-miR-M1 functions in the same manner in Merkel cells, where quasi-latency is
242 hypothesised to be a functional prerequisite for transformation. Merkel cells have been
243 cultured *in vitro* alongside keratinocytes (Fradette et al., 2003), however, no Merkel cell-
244 specific tissue culture system is currently available. Therefore, to study MCV-miR-M1 in a
245 Merkel cell background we utilised the MCPyV-negative MCC cell line, MCC13 and the
246 MCPyV-positive cell line, MKL-1, which has been reported to be a representative model for
247 MCC (Daily et al., 2015). MCC13 cells were transfected with MCV-miR-M1-5p, MCV-miR-
248 M1-3p and scramble mimic control and an analysis of SP100 and CXCL8 transcript and
249 protein expression carried out, as described above. We detected significant changes in both

250 SP100 and CXCL8 mRNA (Figure 6a) and protein levels (Figures 6b and 6c) that were
251 consistent with data gathered using 293 cells. Moreover, neutrophil migration was also
252 significantly reduced in both MCC cell lines expressing MCV-miR-M1 mimics (Figure 6d).
253 These data confirm that MCV-miR-M1 modulates the expression of SP100 and CXCL8 in
254 two MCC cell lines, resulting in reduced migration of neutrophils towards Merkel cells
255 harbouring synthetic MCPyV genomes.

256 **Discussion**

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

268 SP100 is an intriguing MCV-miR-M1 target, which has been implicated in the innate immune
269 response against dsDNA viruses, including MCPyV (Gunther et al., 2014, Jiang et al., 2011,
270 Neumann et al., 2016, Tavalai and Stamminger, 2009, Wagenknecht et al., 2015) and has
271 been shown to be downregulated during MCPyV replication, although no mechanism for this
272 was reported (Neumann et al., 2016). Our data suggest that MCV-miR-M1 mediates (at least
273 in part) the observed downregulation of SP100 in cells harbouring synthetic MCPyV. The

274 argument for MCV-miR-M1 mediated downregulation, rather than modulation by one of the
275 MCPyV proteins is strengthened by the fact that SP100 levels are not decreased in cells
276 transfected with MCVSyn-hpko, which lacks MCV-miR-M1 expression but exhibits
277 significantly increased expression of LT, sT and VP1 (Figures 3c and 4e and (Theiss et al.,
278 2015).

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

289 Our understanding of how PML-NBs and SP100 contribute to the antiviral response has
290 improved in recent years (Nisole et al., 2013), however, mechanistically there is much still to
291 unpick. Here we have shown that direct targeting of SP100 by MCV-miR-M1 modulates
292 CXCL8 secretion. It has been reported that PML functions to regulate the expression of
293 proinflammatory cytokines, including IL-6 and CXCL8 (Lunardi et al., 2011), however, it is
294 not known if SP100 is also able to function in this manner. One explanation may be that
295 SP100 is able to transcriptionally regulate CXCL8 either directly or in *trans*, a notion
296 supported by previous work demonstrating that SP100 functions to activate ETS-family
297 transcription factors (Wasylyk et al., 2002). However, as MCV-miR-M1-5p induces changes
298 in secreted CXCL8, but does not alter CXCL8 transcript levels (Table S1 and Figure 6a), it

299 seems more likely that MCV-miR-M1-5p-dysregulation of SP100 (or other unidentified
300 cellular transcripts) impact on CXCL8 via a post-translational mechanism. Indeed, our
301 observation that ectopic expression of pCDNA-SP100 only partially rescues the MCV-miR-
302 M1-5p mediated reduction of CXCL8 secretion strongly suggests that other MCV-miR-M1
303 dysregulated factors are influencing CXCL8 secretion and presumably, in turn, neutrophil
304 migration. Trans-well assays support this hypothesis, as both 5p and 3p MCV-miR-M1
305 mimics diminished neutrophil chemotaxis to a similar level, despite MCV-miR-M1-3p
306 having no effect on SP100 mRNA or protein levels. We are currently investigating if other
307 indirect MCV-miR-M1 targets impair neutrophil migration during MCPyV replication.

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

323 decrease in neutrophil migration that may be contributing to the host cell immune evasion

324 strategy of MCPyV following infection of Merkel cells.

325 **Materials & Methods**

326 *Tissue culture*

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

333 *RNA sequencing (RNA-Seq)*

334 293 cells were transfected with either MCV-miR-M1-5p, MCV-miR-M1-3p or control mimic
335 (Thermo Fisher Scientific) prior to RNA extraction and confirmation of MCV miRNA 5p and
336 3p expression via stem loop qRT-PCR. Total RNA libraries were prepared using TruSeq
337 Stranded Total RNA Sample Prep Kit (Illumina, USA) and the TruSeq cDNA libraries were
338 analysed via Illumina HiSeq2500 paired end 100bp. Differential gene expression and gene
339 annotation analysis were carried out using edgeR software and DAVID annotation tool,
340 respectively. RNA-seq data have been deposited in the ArrayExpress database at EMBL-EBI
341 (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-6526.

342 *qRT-PCR*

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

346 Stem loop qRT-PCR was carried out to detect MCV miRNA (Czimmerer et al., 2013).
347 Briefly, stem loop cDNA was generated using a stem loop primer containing a MCV miRNA
348 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% CO_2 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.

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

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

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

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

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

398 293 cells were transiently transfected with MCV-miR-M1-5p, MCV-miR-M1-3p or scramble
399 mimic control and expression of target transcript determined via qRT-PCR, n=3 (a). 293 cells
400 were transfected with MCVSyn or MCVSyn-hpko genomes and total RNA and protein
401 extracted 24h, 48h and 72h post-transfection for analysis of MCV-miR-M1-5p and MCV-
402 miR-M1-3p expression via stem-loop qRT-PCR (b) and LT expression by immunoblot (c),
403 n=3. 293 cells were transfected with either MCVSyn of MCVSyn-hpko genomes and

404 cultured for 72h prior to total RNA extraction and analysis of MCV-miR-M1 target
405 expression by qRT-PCR, n=3 (d).

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

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

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

421 Conditioned media was collected 24h post-transfection from TNF-α stimulated 293 cells
422 transfected with either scramble mimic, MCV-miR-M1-5p mimic or MCV-miR-M1-3p
423 mimic and relative CXCL8 levels determined via ELISA, n=3 (a). 293 cells were transfected
424 with either MCVSyn or MCVSyn-hpko genomes (in the absence of TNF-α) and conditioned
425 media collected 72h post-transfection for analysis of CXCL8 secretion via ELISA, n=3 (b).
426 293 cells were treated as described in (a) and (b) above with the addition of either pCDNA3.1
427 (-) or pcDNA-SP100 (SP100) to the transfection mix. Total protein was isolated and SP100

428 expression assessed via immunoblot (c), alongside this, conditioned media was collected for
429 analysis of CXCL8 secretion via ELISA (d and e), n=3. 293 cells were stimulated with TNF-
430 α prior to transfection with either scramble mimic, MCV-miR-M1-5p mimic or MCV-miR-
431 M1-3p and cultured for 24h before isolating conditioned growth media, alongside these 293
432 cells were also transfected with MCVSyn or MCVSyn-hpko and cultured for 72h prior to
433 collection of conditioned growth media. Both sets of conditioned media obtained above were
434 then used in neutrophil chemotaxis trans-well migration assay, n=3 (f). 293 cells were
435 transfected with MCVSyn or MCVSyn-hpko in the presence or absence of SB265610 and
436 cultured for 72h prior to collection of conditioned growth media and neutrophil chemotaxis
437 trans-well migration assay, n=3 (g).

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

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

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