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Targeting macrophage- and intestinal epithelial cell-specific microRNAs against norovirus restricts replication *in vivo*

Lucy Thorne,[1,2,*] Jia Lu,[1] Yasmin Chaudhry,[1] Dalan Bailey[3] and Ian Goodfellow[1]

Abstract

Until recently, our understanding of the cellular tropism of human norovirus (HuNoV), a major cause of viral gastroenteritis, has been limited. Immune cells and intestinal epithelial cells (IECs) have been proposed as targets of HuNoV replication *in vivo*, although the contribution of each to pathogenesis and transmission is unknown. Murine norovirus (MNV) is widely used as a surrogate model for HuNoV, as it replicates in cultured immune cells. The importance of the complete MNV immune cell tropism *in vivo* has not been determined. Recent work has linked replication in IECs to viral persistence *in vivo*. MNV provides a model to assess the relative contribution of each cell tropism to viral replication in immunocompetent native hosts. Here we exploited cell-specific microRNAs to control MNV replication, through insertion of microRNA target sequences into the MNV genome. We demonstrated the utility of this approach for MNV *in vitro* by selectively reducing replication in microglial cells, using microglial-specific miR-467c. We then showed that inserting a target sequence for the haematopoietic-specific miR-142-3p abrogated replication in a macrophage cell line. The presence of a target sequence for either miR-142-3p or IEC miR-215 significantly reduced viral secretion during the early stages of a persistent infection in immunocompetent mice, confirming that both cell types support viral replication *in vivo*. This study provides additional evidence that MNV shares the IEC tropism of HuNoVs *in vivo*, and now provides a model to dissect the contribution of replication in each cell type to viral pathogenesis and transmission in a native host.

INTRODUCTION

Human noroviruses (HuNoVs) are a major cause of viral gastroenteritis worldwide, across all age groups [1], and cause between 2–6 million infections each year in the UK alone [2]. HuNoVs represent a particular problem in healthcare settings, with 4000 hospital outbreaks reported in a 2-year period in the UK, resulting in over 9000 ward closures and a significant economic cost to the National Health Service [3]. Whilst most infections are acute, chronic infections in immunocompromised individuals are a significant cause of morbidity and mortality [4]. In low- and middle-income countries (LMICs), HuNoVs account for over 200 000 deaths in children under 5 each year [5], although a full picture of the impact and prevalence in LMICs has only recently begun to emerge [6, 7].

Despite the global burden, there are still no licensed antivirals or a vaccine. Although HuNoV was identified in 1972 [8], robust *in vitro* propagation systems have only been developed in the past 3 years [9, 10]. A major hurdle in the development of a permissive cell culture system was that, until recently, the cellular tropism of HuNoVs *in vivo* remained unclear. Both immune cells and epithelial cells have been implicated as the primary cellular targets for HuNoVs [9–11]. Evidence of HuNoV antigens in immune cells has been provided by numerous animal infection models, including chimpanzees, piglets and immunocompromised mice [12–14]. These studies led to the first demonstration of HuNoV replication *in vitro*, in a cultured B cell line (BJAB) [10]. Replication in BJAB cells is modest and requires the presence of enteric bacteria expressing histo-blood group antigens (HBGAs) or soluble HBGAs, which serve as attachment factors for HuNoVs to facilitate entry. Intestinal epithelial cells (IECs) are targeted by a variety of enteric pathogens that cause gastroenteritis, and as such have also been considered to be candidate target cells for HuNoVs. However, many studies have tried and failed to find epithelial cell lines that are permissive for infection [15–17]. Evidence of HuNoV replication in IECs *in vivo* came from studies of symptomatic HuNoV infection in

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Author affiliations: [1]Division of Virology, Department of Pathology, University of Cambridge, Cambridge, UK; [2]Division of Infection and Immunity, University College London, Medical Research Council, London, UK; [3]The Pirbright Institute, Pirbright, Woking, UK.
***Correspondence:** Lucy Thorne, l.thorne@ucl.ac.uk
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Abbreviations: BMDM, bone marrow-derived macrophage; CPE, cytopathic effect; DC, dendritic cell; HBGA, histo-blood group antigens; HuNoV, human norovirus; IEC, intestinal epithelial cells; miR/miRNA, microRNA; MNV, murine norovirus; m.o.i., multiplicity of infection; MRE, microRNA response element; NS, non-structural; ORF, open reading frame; p.f.u., plaque-forming units; p.i., post-infection; $TCID_{50}$, tissue culture infectious dose 50.

gnotobiotic pigs, where viral non-structural proteins, markers of active replication, were observed in enterocytes, the most abundant type of IEC [18]. A recent seminal study using intestinal biopsies from chronically infected patients revealed the presence of non-structural proteins and genomic replication intermediates in IECs, suggesting that they are a target cell type for HuNoV *in vivo* [11], at least in immunocompromised individuals. This study paved the way for the development of a robust *in vitro* replication system using stem cell-derived enteroids, multicellular monolayers that recapitulate the natural intestinal epithelium [9]. This system supports the replication of diverse HuNoV strains in enterocytes, present in the enteroids, and allows the dissection of virus–host interactions [9]. The relative contribution of HuNoV replication in enterocytes versus B cells *in vivo* and how this links to pathogenesis and transmission in immunocompetent hosts has not yet been established. Likewise, the possibility that specialised tissue-resident immune cell subsets may also serve as target cells *in vivo* cannot be fully excluded [11].

Prior to the development and use of the B cell and enteroid propagation systems for HuNoV, understanding of norovirus replication has mainly come from studies using murine norovirus (MNV) as a surrogate model, due to the availability of efficient cell culture and reverse-genetics systems [19–22]. Like HuNoV, MNV is enteric, spread predominantly via the faecal–oral route and can cause both acute and persistent infections in the natural host, depending on the strain [23]. MNV replicates in macrophage, dendritic, B and T cells *in vitro* [10, 19, 22]. Genomic replication intermediates were recently identified predominantly in these cell types in the gut-associated lymphoid tissue of immunocompetent mice infected with an acute strain of MNV [22]. Replication in dendritic and B cells is important for acute and persistent MNV infections *in vivo*, respectively [22, 24], although the importance of the immune cell tropism as a whole has not been determined.

Specialized microfold (M-cell) IECs have also been shown to be important for MNV replication *in vivo* through the transcytosis of viral particles across the intestinal epithelium without active replication [25, 26]. Likewise, cultured IEC cell lines do not support MNV replication *in vitro* [10]. However recent work, published during this study, has established a correlation between the ability to replicate in IEC, sensitivity to interferon lambda and viral persistence *in vivo* [27]. Given its use a surrogate model, particularly for dissecting host–pathogen interactions, it is important to determine whether MNV shares the cellular tropism of HuNoV. MNV also provides the opportunity to evaluate the contribution of different cell types to norovirus replication *in vivo* in a native immunocompetent host.

To further probe the role of both immune cells and IECs in viral persistence we sought to explore use of the cellular microRNA (miRNA) machinery to control MNV tropism. This approach exploits the cell-specific nature and regulatory power of host miRNAs and has recently been used to investigate the tropism of a number of RNA viruses. miRNAs are short non-coding RNAs (18–22nt) that post-transcriptionally regulate gene expression by binding to complementary sequences known as miRNA response elements (MREs) present in the open reading frame (ORF) or 3′ UTR of messenger RNAs (mRNAs). The binding of a miRNA targets an RNA-induced silencing complex (RISC) onto the mRNA, which results in either translational silencing or mRNA cleavage, depending on whether the miRNA has partial or perfect homology, respectively. Engineering cell-specific MREs into a viral genome has been demonstrated to regulate replication in a cell-specific manner, as viral replication is targeted by the RISC complex in cells expressing the cognate miRNA [28–33]. This approach has been successfully used for poliovirus, Japanese encephalitis virus, measles virus, influenza virus and dengue virus to rationally generate live attenuated vaccine candidates, enhance existing vaccine safety and improve the safety of oncolytic viruses by preventing replication in non-tumour cell lineages and tissues where it would lead to pathogenesis [28–37]. Here, we first explored whether this approach could be applied to MNV *in vitro*. We then exploited haematopoetic- and IEC-specific miRNAs to probe the importance of each cell tropism to MNV replication during persistent infection of an immunocompetent host. The presence of a MRE for either haematopoetic miR-142–3p or IEC miR-215, but not scrambled control sequences, significantly reduced replication *in vivo*, suggesting that both cell types represent cellular targets for MNV during the early stages of persistent infection *in vivo*.

RESULTS

Insertion of miR-467c target sequences into the MNV genome restricts replication in microglial cells and BMDM cells *in vitro*

To determine the potential of targeting the miRNA machinery against MNV *in vitro,* as a proof of concept that this approach can be applied to norovirus, we set out to specifically restrict replication in one of the two permissive cell lines. We began by comparing miRNA expression in MNV-1-infected murine macrophage RAW264.7 cells and murine microglial BV-2s cells, with the aim of identifying a miRNA that was expressed in only one of these cell lines. As many microglial and macrophage miRNAs play a role in regulating the innate immune response [38–41], we analysed miRNA expression at 20 h following MNV-1 infection at a low multiplicity of infection (m.o.i.), to coincide with the peak of interferon-stimulated gene induction in RAW264.7 cells, which we had identified previously [42]. Using *Taq*-Man-based miRNA arrays, we compared the expression of 136 detectable miRNAs and identified 15 miRNAs that were only expressed in BV-2 cells and 6 that were detected solely in RAW264.7 cells (Fig. 1). We then validated three of these from each cell line – miR-10a, miR-124 and miR-125a-5p for RAW264.7 cells and miR-126–5p, miR-467a and miR-467c for BV-2 cells – using RT-qPCR. Cell-specific expression was validated for all of these miRNAs except

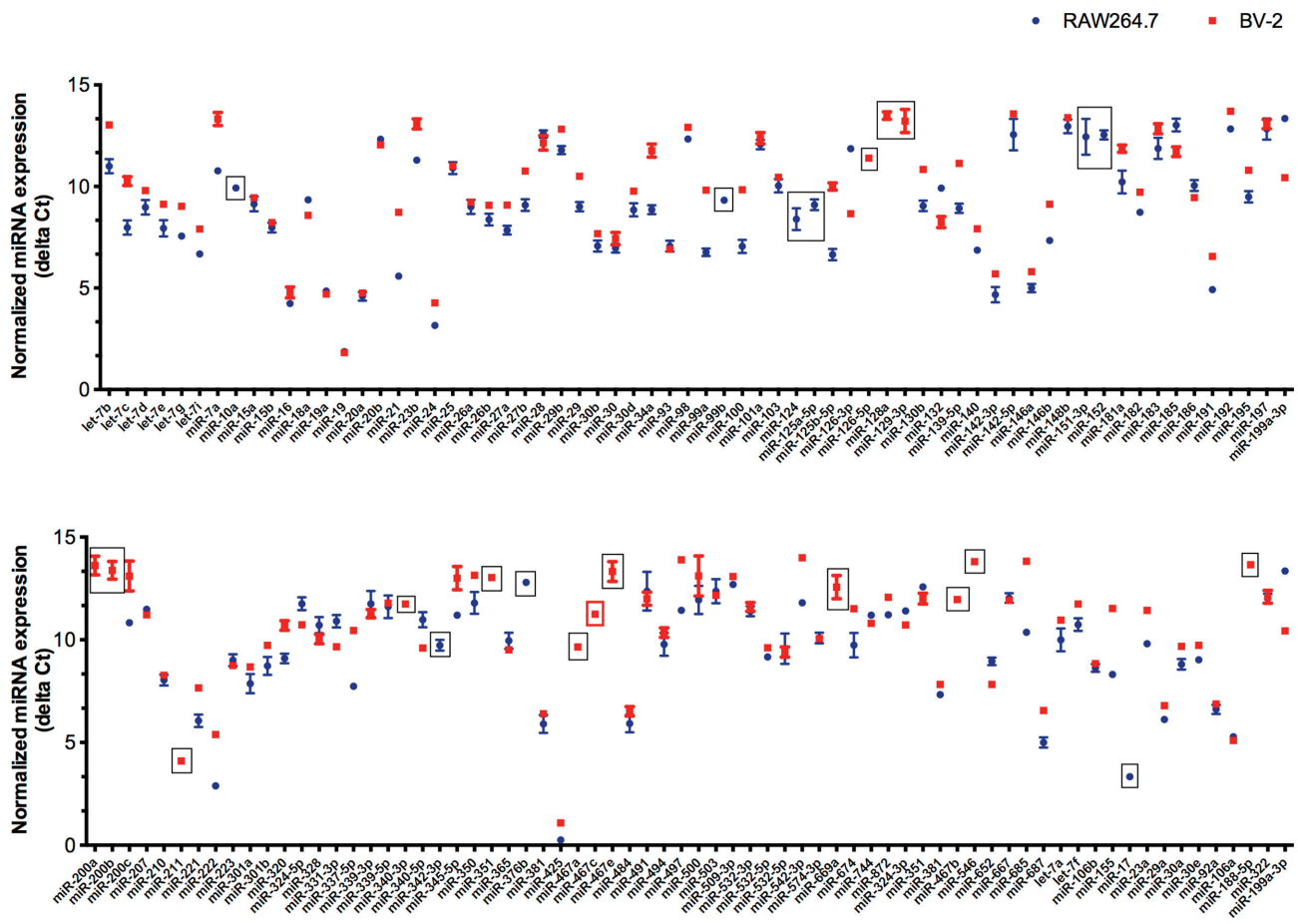

Fig. 1. Comparison of miRNA expression profiles in MNV1-infected murine macrophage RAW264.7 cells and microglial BV-2 cells. RAW264.7 and BV-2 cells were infected with MNV-1 at an m.o.i. of 0.1 $TCID_{50}$/cell. The small RNA fraction was harvested at 20 h p.i. Reverse transcription was performed using a set of primers for 380 miRNAs and control small RNAs, followed by qPCR analysis using TLDA miRNA cards. The results for the 136 detectable miRNAs are shown. The Ct for each specific miRNA was normalized against the Ct of the U6 control RNA to give the Delta Ct value shown on the *y*-axis. The higher the Delta Ct, the lower the expression. The boxed points indicate miRNAs that are only expressed in one of the cell lines during MNV-1 infection, and the red boxes indicate miR-467c.

miR-124, which was detected in both cell types (Table 1), suggesting that amplification of this miRNA failed on the arrays.

Of the validated miRNAs, we selected miR-467c to target MNV replication, on the basis that it was one of the most highly expressed BV-2-specific miRNAs. As the cellular expression profile of miR-467c is not known, we also confirmed that it is expressed in bone marrow-derived macrophages (BMDMs), but importantly not in BSR-T7 cells, which are used for viral recovery using our DNA-based reverse-genetics system, Fig. 2(b).

To target miR-467c towards the MNV genomic RNA, it was necessary to insert a cognate target sequence into the genome without compromising replication. We have previously used transposon-mediated insertional mutagenesis to identify positions across the norovirus genome that tolerate the insertion of short nucleotide sequences without compromising virus replication in cell culture [43]. Based on this study, we inserted MREs for miR-467c into the MNV-1 genome after nt 383 in the NS1-2 coding region of ORF1 (Fig. 2a, c), which we have previously shown tolerates

Table 1. Validation of the miRNA array data for a panel of differentially expressed miRNAs

Array cell line	miRNA	RT-qPCR validation	
		RAW264.7	BV-2
RAW264.7	miR-10a	+	–
	miR-124	+	+
	miR-125–5p	+	–
	miR-126–5p	–	+
BV-2	miR-467a	–	+
	miR-467c	–	+

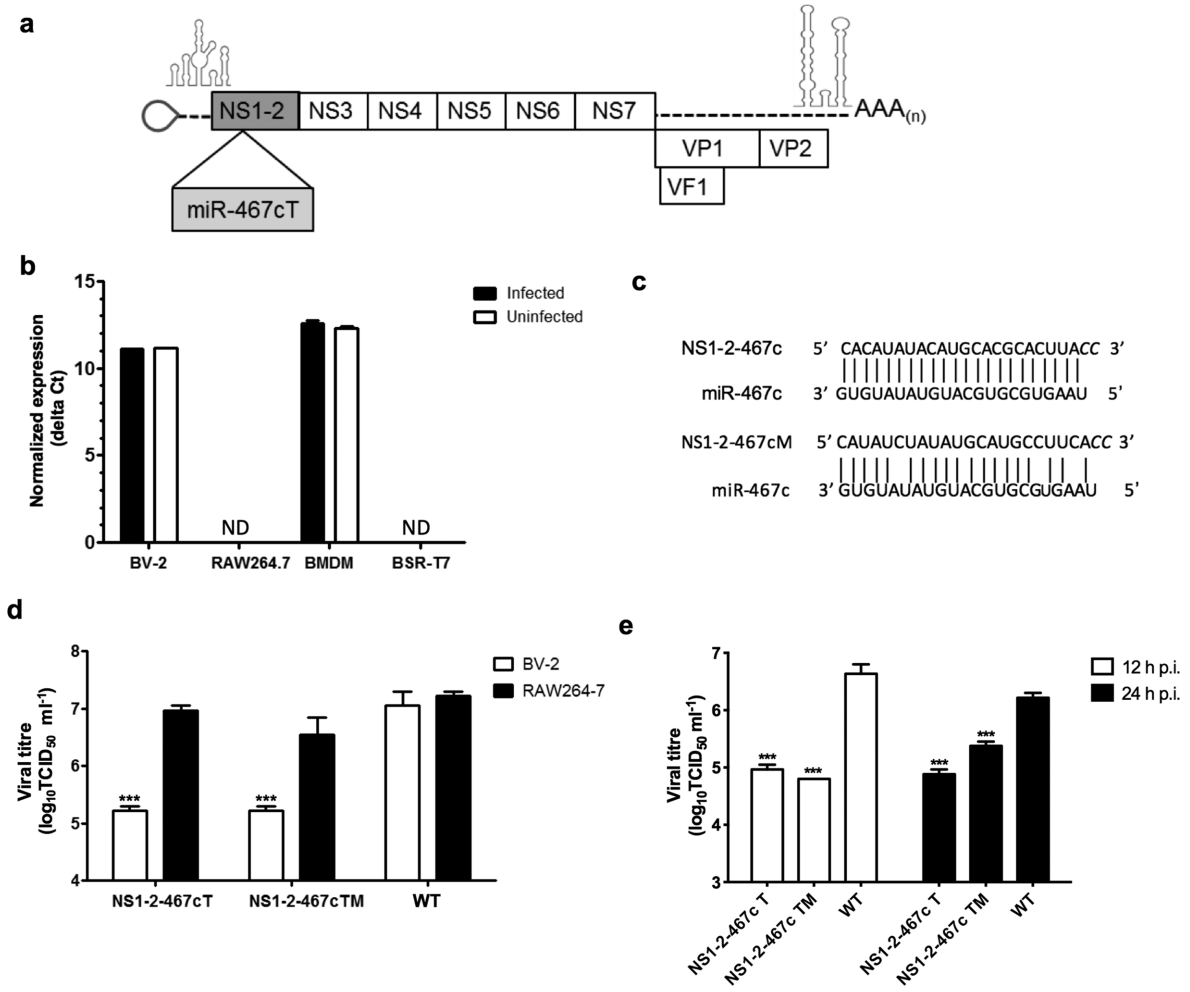

Fig. 2. Insertion of miR-467c target sequences into the MNV genome restricts replication in microglial cells and BMDMs. (a) miR-467c target sequences were inserted into the coding region for NS1-2. (b) miR_467c is expressed in BV-2 and BMDM cells, but not RAW264.7 and BSTR-T7 cells. ND, not detected, i.e. samples that fell below the limit of detection. (c) The miR-467c target sequence was inserted to be complementary to miR-467c in the positive-sense gRNA (NS1-2-467cT) and with modified (M) sequence complementarity (NS1-2-467cM), but with the same coding sequence. (d) miR-467c T or M sequences specifically reduce replication in BV-2 cells expressing miR-467c. RAW267.4 and BV-2 cells were infected with each virus at a low m.o.i. (0.01 $TCID_{50}$/cell) and titrated on RAW264.7 cells, and virus was harvested at 24 h p.i. Significance was tested using two-way analysis of variance (ANOVA) with a Dunnett post-test to compare the replication of each virus between the two cell lines. ***, $P<0.001$, $n=3$. (e) miR-467c target and modified target sequences reduce replication in BMDM cells. BMDMs were infected with each virus at an m.o.i. of 10 $TCID_{50}$/cell and titrated on RAW264.7 cells, and virus was harvested at 12 and 24 h p.i. Significance was tested using two-way ANOVA, with Sidak's multiple comparison test to compare the replication of each virus between the two cell lines. ***, $P<0.001$, $n=3$.

the insertion of a FLAG epitope tag coding sequence of the same size as the MRE insertion (24 nt) [43, 44]. We introduced a single MRE with either perfect or partial homology, to direct different effector functions of RISC against the genome. The partial homology was limited to three mismatches in order to to maintain the same amino acid sequence as the perfect MRE sequence. The viruses were designated as NS1-2-467cT or NS1-2-467cTM, where T indicates target and M indicates a modified partial complementary target sequence (Fig. 2).

As expected, replication of NS1-2-467cT carrying a perfect MRE was reduced by nearly 100-fold in BV-2 cells compared to RAW264.7 cells, whereas wild-type (WT) MNV replicated to the same level in both cell lines (Fig. 2c). Replication of the virus carrying the partially complementary target sequence (NS1-2-467cM) was also reduced in BV-2 cells compared to RAW264.7 cells, indicating that the restriction applies for both perfect and imperfect MREs. Replication of NS1-2-467cT and NS1-2-467cTM was also significantly reduced, by up to 100-fold, in BMDM cells (Fig. 2e), with

the NS1-2-467cT virus displaying the largest defect at the later time point.

Insertion of a MRE for haematopoetic-specific miR-142–3p into the MNV genome inhibits replication in macrophage cells *in vitro*

To use this approach to probe the importance of the immune cell tropism *in vivo* we selected a miRNA with a well-defined haematopoietic-specific expression profile. As targeting the genome with miR-467c did not achieve complete suppression *in vitro* (Fig. 2d, e) and its cellular expression is not well characterized, we employed one of the most highly abundant haematopoetic-specific miRNAs, miR-142–3p [45]. This miRNA has previously been used as a tool to study dengue virus replication in haematopoietic cells in a similar approach [33].

Examination of the array data (Fig. 1) and subsequent RT-qPCR (Fig. 3c) confirmed that miR-142–3p is expressed in both RAW264.7 and BV-2 cells, and at higher levels than miR-467c in BV-2 cells. To assess the importance of tropism during persistent infections in immunocompetent mice, we inserted the MREs into the genome of the persistent MNV-3 strain [21]. To avoid any impact of having the MRE in a coding sequence, this time we inserted miR-142–3p MREs into a site in the 3′UTR, which we have previously identified as being able to tolerate nucleotide insertions [43]. We inserted one (3′142T) or two (3′142DT) perfectly complementary MREs into this site, alongside scrambled control sequences of the same length and base composition (3′S and 3′DS), Fig. 3(b).

The presence of a single miR-142–3p MRE (3′142T) reduced replication to the limit of detection and a double MRE (3′142DT) completely prevented detectable viral replication in murine macrophage RAW264.7 cells (Fig. 3d). In comparison, viruses carrying the single and double scrambled control sequences (3′S and 3′DS) replicated to the level of WT MNV-3 (Fig. 3d). To determine that the loss of observed replication was due to restriction in the RAW264.7 target cells and was not a result of lower production or stability of the RNA during virus recovery, we measured the level of the viral polymerase protein (NS7) and intracellular genomes produced during virus rescue in BSR-T7 cells. The NS7 and intracellular genomes levels were not limiting for 3′142T or 3′142DT (Fig. 3e, f), which was indicative of comparable transfection efficiency and no major impact on RNA stability. To confirm that infectious virions were produced for 3′142T and 3′142DT, we determined the amount of nuclease-resistant encapsidated viral genomes produced during virus recovery. The encapsidated genome copies detected for the 3′142T virus were comparable to those for WT MNV-3 and 3′S, although the double MRE insertion resulted in a significant reduction for 3′142DT, and to a lesser extent for the double scrambled control (Fig. 3g). As we could therefore not be sure that the loss of 3′142DT replication in RAW264.7 cells was solely due to miR-142–3p targeting and not an unknown effect on genome encapsidation and viral assembly due to the size of the insertion, we did not take the 3′142DT virus forward for further characterization. We made multiple attempts to deplete miR-142–3p, using miRNA antagonists and CRIPSR-Cas9 for miR-142–3p and Dicer, a key component of the miRNA processing machinery, in order to further confirm that miR-142–3p was responsible for the restriction and to generate a cell line to be able to titre the targeted viruses in the absence of miR-142–3p. However, due to the macrophage cell lines being refractory to transfection and the necessity of the miRNA machinery for regular cellular function, it was not possible to obtain a reduction in miR-142–3p levels using these approaches (data not shown).

We also compared the effect of inserting a single perfect MRE for miR-467c into the same site in the 3′UTR, alongside a single scrambled control sequence of the same length and base composition. The presence of the miR-467c MRE reduced replication in BV-2 cells by nearly 10-fold in comparison to the scrambled control virus and WT-MNV-3, but did not affect replication in RAW264.7 cells (Fig. 3h). The differences in restriction resulting from each MRE at the same site correlate with the expression level of the cognate miRNA, where miR-142–3p is most highly expressed and results in the greatest restriction.

MNV carrying a MRE for IEC-specific miR-215 replicates in macrophage cells

As IECs have recently been shown to be a target cell for HuNoVs *in vivo* [11], to be able to confirm whether they support MNV-3 replication *in vivo,* we next inserted a perfect MRE for the most abundant miRNA in murine IECs, miR-215 [46] (Fig. 4a). miR-215 is not expressed in either RAW264.7 or BV-2 cells (Fig. 2c) and there are no reports of its expression in immune cells. Accordingly, the 3′215T virus recovered to levels that were comparable to those of the WT and 3′S control during reverse-genetics recovery from BSR-T7 cells (Fig. 4b), and replication was not restricted in RAW 264.7 cells (Fig. 4c). As MNV does not replicate in cultured IEC lines [10], and as we could not achieve sufficient transfection of either the macrophage or microglial cell lines to exogenously express miR-215 (data not shown), we were not able to further characterize the restriction by miR-215 *in vitro*.

Carrying MREs for haematopoietic miR-142–3p and IEC miR-215 reduces MNV-3 replication in the initial phase of a persistent infection *in vivo*

To determine the effect of carrying a MRE for either miR-142–3p or miR-215 on replication *in vivo*, we infected immunocompetent mice with equal doses of MNV-3 WT, 3′142T, 3′215T and 3′S, and monitored viral secretion. Equivalent levels of viral genomes were secreted 1 day post-infection (p.i.), which reflects equal inoculation doses and shedding of the excess viral inoculum prior to the peak of replication at 2 to 3 days p.i., as we have previously confirmed in this infection model [21]. At 2 days p.i. both the 3′142T and 3′215T viruses were secreted in the faeces at significantly lower levels than the WT and 3′S viruses, with a

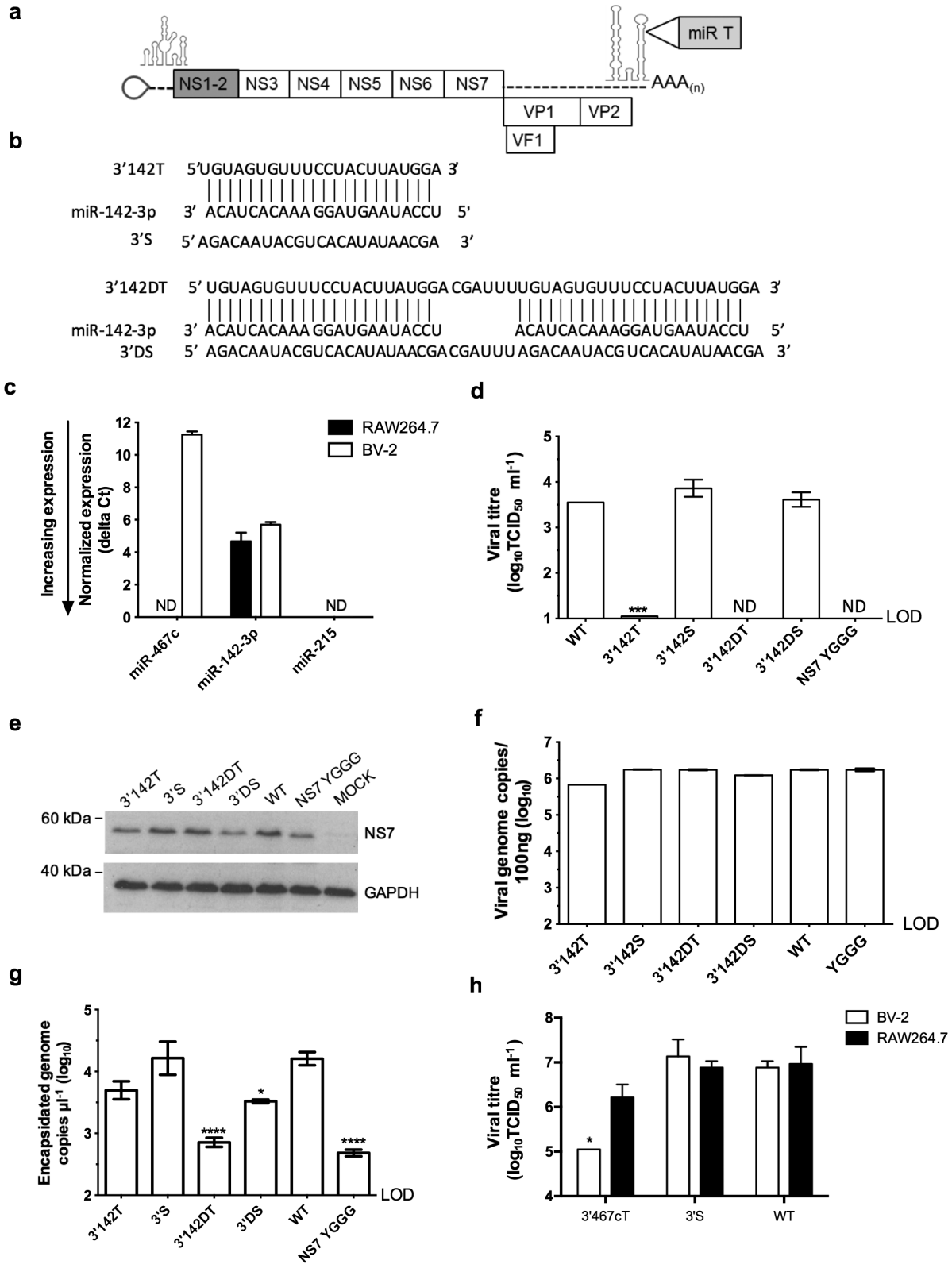

Fig. 3. Insertion of haematopoetic-specific miR-142–3p target sequences into the MNV genome inhibits replication in macrophage cells. (a) miR-142–3p target sequences were inserted into a site in the MNV-3 3′UTR that was previously found to tolerate nt insertions. (b) Single and double perfectly complementary target sequences were inserted into the MNV-3 genomes, alongside single or double scrambled control sequences. (c) miR-142–3p is expressed at higher levels than miR-467c in RAW264.7 and BV-2 cells. ND, not

detected, i.e. samples that fell below the limit of detection. (d) The insertion of single or double miR-142–3p target sequences reduces replication in RAW264.7 cells. The recovery supernatants for each virus were titred on RAW264.7 cells to determine the $TCID_{50}$ ml^{-1}. The polymerase active site mutant (NS7 YGGG) was included as a replication-defective control. ND, not detected, i.e. samples that fell below the limit of detection. (e) Production of the MNV-3 polymerase (NS7) or intracellular genomes (f) was not limiting during virus recovery of 3′142T or 3′142DT, compared to the WT and scrambled controls. (g) Encapsidated genome copies, indicating infectious virion production, were detected at WT-like levels for 3′142T and 3′S, but were significantly reduced for 3′142DT and 3′D. (h) Insertion of a single microglial miR-467c target sequence into the 3′UTR site selectively reduces replication in BV-2 cells. RAW264.7 nd BV-2 cells were infected with each virus at 0.01 $TCID_{50}$ ml^{-1} (titred on RAW264.7 cells) and virus was harvested at 24 h p.i. (d and g) Significance was tested using one-way ANOVA with Dunnett's post-test to compare replication of each virus to the WT. ****, $P<0.0001$; *, $P<0.05$, $n=3$. LOD, limit of detection. (h) Significance was tested using two-way ANOVA, with Sidak's multiple comparison test being used to compare the replication of each virus between the two cell lines. *, $P<0.05$, $n=3$.

more than 10-fold reduction in the average genome copies mg^{-1} stool being observed (Fig. 5). There was no significant difference in the level of the genomes secreted between the 3′142T and 3′215T viruses, suggesting that the replication of both was equally restricted (Fig. 5). A significant defect in the replication of these of these viruses was also observed at 3 days p.i., albeit to a lesser extent. The defect was fully recovered by 5 days p.i.; both viruses established persistent infections and were then secreted at comparable levels to the WT and 3′S for the duration of the experiment (Fig. 5). To determine whether there were any mutations in the two MREs, we sequenced the 3′UTR of the secreted virus population from each group of infected mice at 5 days p.i. We observed no mutations in the WT or 3′S 3′UTR and could not detect any changes in the 3′142 or 3′215 MREs that would compromise binding of the cognate miRNAs (data not shown).

DISCUSSION

Here we have exploited the cell-specific regulatory nature of host miRNA expression to further define the cellular tropism of MNV during persistent infection of immunocompetent hosts. Having demonstrated that this approach could be applied to MNV *in vitro*, selectively targeting

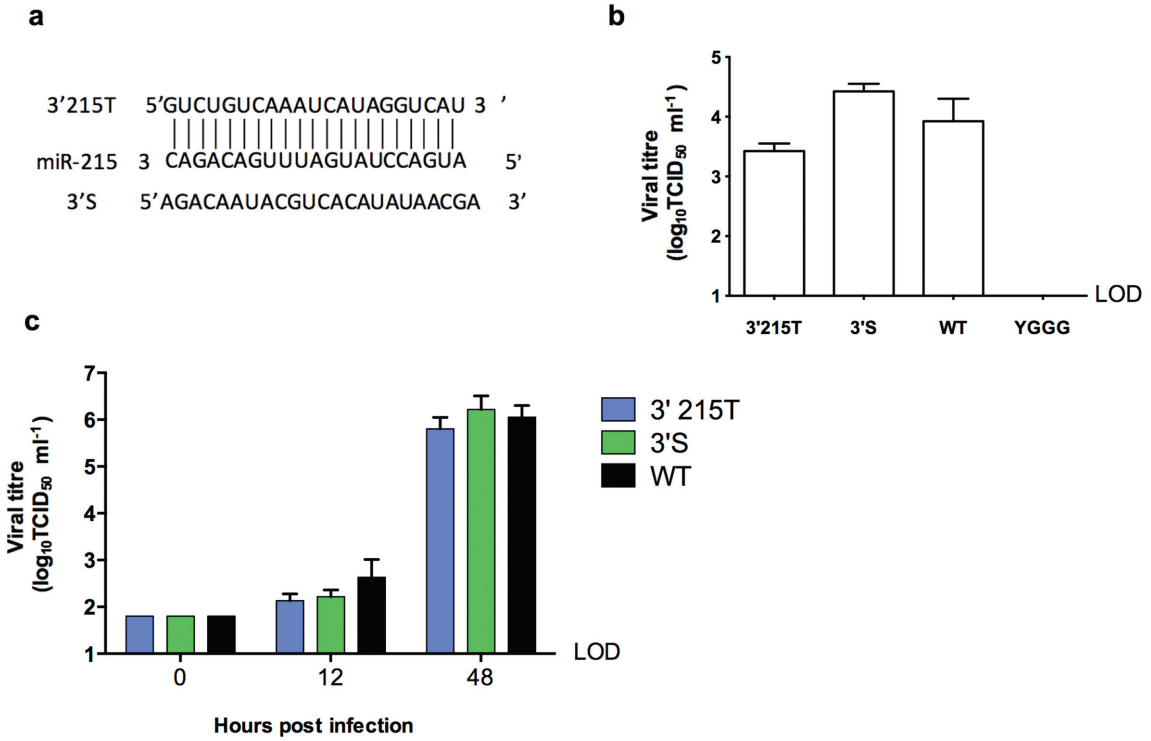

Fig. 4. MNV carrying a target sequences for intestinal epithelial cell-specific miR-215 replicates in macrophage cells. (a) A perfect target sequence for highly abundant IEC miR-215 was inserted into the 3′UTR site in the MNV-3 genome alongside the scrambled control sequence. (b) A virus carrying the miR-215 MRE (3′215T) recovered to levels that were similar to those of the WT and scrambled control virus. (c) 3′215T replicates with WT-like kinetics in RAW264.7 cells, which do not express miR-214. RAW264.7 cells were infected with each virus at a low m.o.i. (0.01 $TCID_{50}$/cell) and virus was harvested at 12 and 48 h p.i. LOD, limit of detection.

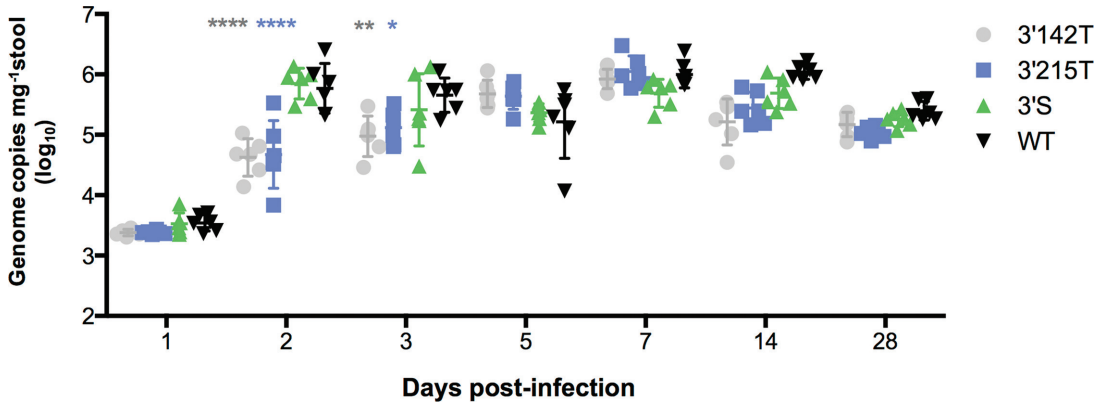

Fig. 5. Carrying a target sequence for haematopoietic miR-142–3p and intestinal epithelial miR-215 reduces MNV replication in the initial phase of a persistent infection *in vivo*. Mice were infected with 100 ul of each virus recovery supernatant containing equivalent genome copies and secreted viral genome copies were measured on the indicated days by RT-qPCR. Significance was tested using two-way Anova with Dunnett's multiple comparison test to compare each group to the WT control group. ****, $P<0.001$. $n=6$.

replication in microglial cell lines, we then employed haematopoietic-specific miR-142–3p and IEC miR-215 to probe the importance of replication in these cell types *in vivo*. The presence of a single target sequence for either miRNA reduced viral secretion during the early phase of infection in immunocompetent mice, which was indicative of reduced replication. This provides additional evidence that MNV actively replicates in IECs *in vivo*, and that in addition to having an immune cell tropism, it also shares the HuNoV tropism for IECs [11], at least during chronic infections. MNV therefore now provides a surrogate model to dissect and understand the contribution of each cell tropism to pathogenesis and transmission, which remains unknown for HuNoV infections.

During the preparation of this paper, evidence emerged that supports our finding of MNV replication in IECs *in vivo*. Lee *et al.* recently demonstrated that IECs serve as a reservoir for MNV during persistent infections *in vivo* [27]. Using a highly sensitive flow cytometry assay to detect two non-structural proteins that are indicative of active viral replication, they found that MNV maintains low-level infection of IECs during persistent infection, at a rate of approximately 20 infected cells per million IECs. These low infected cell numbers, combined with the high background signals they observed using single staining for viral proteins, suggest why the infection of IECs has not been detected before in similar studies [27]. They also show the utility of exploiting the cellular miRNA machinery and the virus itself as a tool to probe tropism as a complementary approach that does not reply on staining protocols for viral detection.

Lee *et al.* demonstrated that the ability of MNV to infect IECs correlated with an ability to persist *in vivo,* and was determined by a combination of the action of MNV non-structural (NS) protein NS1-2 and host cytokine interferon lambda (IFNλ) [27]. Studies from the same group previously demonstrated that both of these factors can determine the persistence of MNV *in vivo* [47–49], which now neatly pinpoints IECs as the cell type in which interplay between MNV and components of host innate immune response influences the outcome of persistent infections. Clearance of MNV replication in IECs by IFNλ treatment correlated with a loss of viral secretion [27], suggesting that replication in IECs results in the release of virus into the intestinal lumen, and may therefore contribute to transmission, although this has yet to be demonstrated. It is striking that the very low number of infected IECs may be solely responsible for production of the high numbers of viruses secreted during persistent MNV infection. This study did not quantify the number of infected macrophage, dendritic and B cells in the intestinal lamina propria, which would provide an important comparison. This is especially relevant as another recently published study identified immune cells of the gut-associated lymphoid tissue as the predominant cell target during acute MNV infection in immunocompetent hosts [22]. This study used RNAScope *in situ* hybridization to detect both positive- and negative-sense MNV genomic RNA, which were also observed at very low frequency in the follicule-associated epithelium, and the contribution of these cell types in acute infection is therefore not known [22]. These apparently contradictory reports may be explained by the use of different strains, suggesting that acute and persistent strains of MNV may have predominantly different tropisms [27]. It will be interesting to determine whether this is also the case for HuNoV. Our findings suggest that restricting replication in both cells types results in lower viral secretion of a persistent MNV strain. Therefore, further studies are required to determine whether newly synthesized virus in cells of the lamina propria could be secreted directly back into the intestinal lumen through the epithelium, or whether replication in immune cells indirectly affects secretion by providing more virus to infect IECs.

Our approach could be used to further investigate whether norovirus tropism is the same during acute infections and address the reported discrepancies. Using this approach in a model where the host develops a symptomatic infection would also allow assessment of the contribution of replication in each cell type to pathogenesis and disease, although symptomatic infection models involve the use of innate or adaptive immunocompromised mice, which may remove natural barriers to replication [50, 51]. It would be interesting to determine how replication in each cell type affects viral dissemination through the intestines and into extra-intestinal tissues in these models, such as the spleen and MLN. In our MNV-3 model of persistent infection, replication in these tissues peaked between days 3 and 5 p.i. [21], by which time viral replication had overcome restriction by each miRNA.

Although this approach of exploiting miRNAs has been successfully applied to selectively control viral tropism and attenuate a number of other RNA viruses [28, 33, 35], we encountered several limitations in using it as a tool to to study MNV tropism. The first was that, given that MNV only replicates in immune cells *in vitro*, which all express miR-142–3p and are resistant to transfection and genetic manipulation, there was no alternative permissive cell type to titre the miR-142–3p-restricted viruses and demonstrate replication in the absence of miR-142–3p. However, we confirmed that equal levels of encapsidated viral genomes, and therefore infectious virions, were produced for the 3′142T virus, and equivalent genome copy numbers were shed compared to the inoculation dose on day 1 post-infection *in vivo*. Conversely, as cultured IEC lines do not support MNV replication *in vitro* [10, 27], we did not have a permissive cell line with endogenous miR-215 expression to enable *in vitro* characterization of the miR-215 restriction.

A second major challenge was the difficulty in making large insertions in the compact MNV genome, which has short UTRs, without disrupting essential genomic elements or protein domains that would compromise viral replication. We were guided here by our previous studies identifying positions across the genome that tolerated 15 nt insertions [43, 44]. However, whether the same site tolerates larger insertions is size- and sequence-specific, and we have found that many sites that tolerate small epitope tags or random 15 nt insertions do not tolerate insertions of larger sizes, such as genes for fluorescent proteins of various sizes (unpublished data). We encountered the same challenge here with the insertion of MREs into a previously identified site in the 3′UTR [43]. Insertions of various single MREs (23 nt) were tolerated at this position, without replication being compromised by the insertion itself, as evidenced by the WT-like replication of the scrambled control viruses, and differential replication of the miRNA-targeted viruses correlating with differences in expression of the cognate miRNA. However, inserting a double miR-142–3p MRE with a spacer region (51 nt) into the same site reduced the production of infectious virions, measured as encapsidated viral genomes. The norovirus 3′UTR is known to contain structures that are important for viral replication [52]. This finding suggests that the 3′UTR may also impact on genome encapsidation and viral production, although further studies are required to confirm this. In the majority of other studies using this approach, restriction has been achieved *in vivo* by inserting at least a double MRE, and in some cases by the insertion of a cassette containing up to six MREs [28, 31–33, 53]. A larger number of MREs likely facilitates complete restriction early in infection and in the face of high levels of RNA virus genome replication during infection. It has been suggested that a minimum miRNA copy number threshold must be exceeded for gene silencing to occur [54]. Therefore, if the restriction is not complete then it is likely that the number of newly synthesized genomes could soon outnumber the copy number of the miRNA in each cell and thereby overcome restriction. As such, replication of MNV-3 carrying single MREs overcame each miRNA restriction in the early stages of infection *in vivo*. The lack of resistance mutations in the MRE sequences at the point at which restriction was overcome (5 days p.i.) suggests that a single MRE did not exert sufficient selection pressure on each virus, which is also in line with high levels of secreted virus production from relatively few infected cells *in vivo*, at least for IECs [27]. To exert more selective pressure, some studies have inserted MREs at multiple sites in the genome [32], which could be a possibility for taking this approach further for MNV, however, given its short UTRs, the additional insertions would need to be in coding regions, which can be complicated by effects at the protein level.

As both the miR-142–3p- and miR-215-targeted viruses overcame restriction during the acute phase of infection [21], we could not examine the importance of each cellular tropism for maintaining persistent infection and shedding. This may also reflect the fact that several cell types are permissive *in vivo*, so restriction in one cell type may only have a minor impact. This approach has so far only been applied to the study of tropism in acute viral infections, and it also suggests that maintaining restriction in the face of high rates of mutagenic RNA virus replication may not applicable to persistent viruses.

Finally, the finding that MNV replicates in IECs *in vivo* raises a number of interesting questions and possibilities. Firstly, a proteinaceous receptor for MNV has recently been identified, namely CD300lf [55], an Ig domain-containing molecule expressed on myeloid cells with low expression on epithelial cells [22]. This suggests that MNV may use a different proteinaceous receptor to enter IECs. As *in vitro* replication in cell culture of MNV and HuNoVs is predominantly at the level of entry [55, 56], identification of this receptor could by comparison shed light on the long-sought-after receptor for HuNoV and facilitate the development of further *in vitro* propagation systems for both viruses.

METHODS

Cells

The murine macrophage cell line RAW264.7 was maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 10 % foetal calf serum (FCS), penicillin (P) (100 SI units ml^{-1}) and streptomycin (S) (100 µg ml^{-1}) and 10 mM HEPES (pH 7.6) at 37 °C with 10 % CO_2. The murine microglial BV-2 cell line [45] was provided by Jennifer Pocock (University College London). BV-2 cells were maintained in DMEM supplemented with 10 % FCS (Biosera), 2 mM L-glutamine, 0.075 % sodium bicarbonate (Gibco) and the antibiotics penicillin and streptomycin as above. BHK cells engineered to express T7 RNA polymerase (BSR-T7 cells, obtained from Karl-Klaus Conzelmann, Ludwid Maximillians University, Munich, Germany) were maintained in DMEM containing 10 % FCS, penicillin (100 SI units ml^{-1}) and streptomycin (100 µg ml^{-1}), and 0.5 mg ml^{-1} G418. For the preparation of bone marrow-derived macrophage (BMDM), bone marrow cells were harvested from female C57BL/6 mice and cultured in DMEM containing 10 % FCS, penicillin (100 SI units ml^{-1}) and streptomycin (100 µg ml^{-1}). For BMDM differentiation, supernatant collected from CMG14 cells, which contains macrophage colony-stimulating factor (M-CSF), was added to the media for 5–7 days.

Viral reverse genetics

The cDNA clone pT7:MNV 3′ RZ, containing the WT MNV-1 genome under the control of a truncated T7 polymerase promoter was constructed previously [20]. The cDNA clone pT7:MNV3 3′RZ (MNV3-WT) contains the MNV-3 sequence under the control of the T7 promoter, as described in [21]. The insertion of MREs into each genome was achieved by overlap mutagenic PCR (primer details available upon request). Virus was rescued from cDNA clones using the reverse-genetics system based on recombinant Fowlpox expressing T7 RNA polymerase, as previously described [20]. Briefly, 1 µg of each cDNA clone was transfected, using Lipofectamine 2000 transfection reagent (Invitrogen), into BSR-T7 cells previously infected with recombinant Fowlpox virus expressing T7 RNA polymerase at an m.o.i. of approximately 0.5 p.f.u. per cell. After 48 h post-transfection, the cells were freeze-thawed at −80 °C to release virus particles.

Viral $TCID_{50}$ and growth analysis

Fifty per cent tissue culture infectious dose ($TCID_{50}$) titrations were performed on BV-2 or RAW264.7 cells using 10-fold serial dilutions, typically over a range of neat to 10^{-7}. The viral $TCID_{50}$ ml^{-1} was determined by scoring for signs of cytopathic effect (CPE) at 5 days post-infection by visual inspection. Multistep growth curve analysis of miRNA-targeted viruses was performed by infecting RAW264.7 cells with sequence-verified virus at an m.o.i. of 0.01 $TCID_{50}$/cell. BMDM cells were infected at an m.o.i. of 10 $TCID_{50}$/cell, as titred on RAW 264.7 cells. Virus was harvested at various time points post-infection through a single freeze-thaw cycle and virus titre was determined by $TCID_{50}$ on RAW264.7 cells.

miRNA extraction and expression analysis

To analyse miRNA expression, RAW264.7 and BV-2 cells were infected with MNV-1 WT at an m.o.i. of 0.1 $TCID_{50}$/cell or were mock-infected, while BMDM cells were infected at an m.o.i. of 10 $TCID_{50}$/cell, and the small cellular RNA fraction was harvested at 20 h p.i. A DNA-based recovery of MNV-1 was performed in BSR-T7 cells and small RNA species were extracted at 24 h post-transfection. In each case the small cellular RNA fraction (<200 bp) was harvested using the miRVana RNA isolation kit, as per the manufacturer's instructions. This retains RNA molecules as small as 10 nt. For cDNA synthesis, the *Taq*Man miRNA reverse-transcription kit was used (Life Technologies), according to the manufacturer's instructions. A thousand nanograms of RNA extracted from infected and uninfected RAW264.7 cells and BV-2 cells were used with Megaplex RT primers, Rodent Pool A (Life Technologies). The pool contains primers specific to 335 and 238 mature unique mouse and rat miRNAs, respectively, alongside primers for four specific endogenous controls. The cDNA was then used for qPCR using *Taq*Man Rodent miRNA Array A cards (TDLA, Life Technologies). The *Taq*Man Rodent miRNA Array A is a preconfigured 384 well card that can amplify and detect 381 mature rodent miRNAs and 3 control small RNAs (that are common to those in the Megaplex primer pools). As recommended in the manufacturer's instructions, 6 µl of cDNA was mixed with *Taq*Man Universal PCR 2× Master Mix (no UNG) (Life Technologies) into a final volume of 900 µl. Then 100 µl of each mix was loaded into a port on the TLDA card, which covered two rows of the 384-well card. The TLDA cards were run on the 7900HT T Fast Real-Time PCR System and the data were visualized and analysed for the Ct value of each miRNA using RQ manager software (Life Technologies). Further analysis was performed using Microsoft Excel. The Ct value for each miRNA was normalized against the Ct value for the endogenous control small non-coding nuclear RNA U6, which did not appear to be altered with infection. RT-qPCR validation was performed using RT and PCR primer probes specific to a selection of miRNAs. The RT-qPCR was performed using the same enzymes and master mixes, but with individual primers for miR-467a, miR-467c, miR-126, miR-125–5p, miR-124, miR-10a and the control RNA U6.

Quantification of viral RNA by RT-qPCR

Quantification of viral genome copies from infected cells or faecal samples was performed by two-step RT-qPCR. Nuclease-resistant encapsidated genomes were produced as previously described [20], prior to RNA extraction using 100 µl of treated recovery supernatants. Twenty nanograms of of RNA from infected cell samples was used for reverse transcription. The amount of RNA used from faecal samples corresponded to that extracted from 0.5 mg of stool. Viral RNA of known copy number was generated by *in vitro* transcription of the MNV-1 or MNV-3 cDNA clones and was

serially diluted to generate a standard curve. Reverse transcription was performed with M-MLV using random hexamers, as per the manufacturer's instructions. qPCR was performed on the cDNA using a *Taq*Man Low Rox qPCR Master Mix (Primer design), with an initial denaturation step of 8 min at 95 °C, followed by 50 cycles of 95 °C for 10 s and 60 °C for 1 min. The genome copy number was interpolated from the standard curve and was calculated per ng of RNA or per mg of stool, depending on the sample, using Microsoft Excel. All graphs were produced using GraphPad Prism version 5 software.

In vivo infections

Three-to-four week old female C57BL/6 mice were inoculated by oral gavage with 100 µl of recovery supernatant of MNV-3 3′142T, 3′215T, 3′S and WT viruses, which contained equivalent encapsidated viral genome copy numbers. Each group contained six mice and a control group was mock-infected. Mice were weighed and faecal samples were collected on days 0–7, 14, 21 and 28 post-infection, while mice were euthanized on day 28. Faecal pellets were homogenized in PBS (100 mg ml^{-1}), followed by centrifugation at 4000 r.p.m. for 5 min, 4 °C. RNA was extracted from 100 µl of supernatant using the GenElute mammalian total RNA kit and viral genome copy numbers were determined as described.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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