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Regulation of Cellular MicroRNA Expression in Oligodendrocytes during Acute and Persistent Coronavirus Infection

Xuming Zhang*, **Yin Liu, and Jianzhong Cao**

Department of Microbiology and Immunology, University of Arkansas for Medical Sciences, Little Rock, Arkansas 72205-7199, USA

***Corresponding author:** Xuming Zhang, Department of Microbiology and Immunology University of Arkansas for Medical Sciences 4301 W. Markham Street, Slot 511 Little Rock, AR72205, USA, Tel: 501-686-7415; Fax: 501-686-5359; E-mail: zhangxuming@uams.edu

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Abstract

Genome-wide microRNA expression was profiled in oligodendrocytes during acute and persistent infection with murine coronavirus. A total of 113 microRNAs were specifically expressed in oligodendrocytes, among which 10 microRNAs [miR-23a, miR-26a, miR-30b/c/d,miR-192, miR-665, miR-709, and Let-7b/d] were significantly up-regulated while 16 microRNAs [miR-129-5p, miR-210, miR-214, miR-297, miR-297b, miR-300, miR-370, miR-466, miR-467b, miR-468, miR-669a/b, miR-672, miR-706, miR-760, and miR-801] were significantly down-regulated during infection. The biological significance of microRNA expression was further assessed for their effects on target gene expression and viral replication. Results showed that transfection of synthetic miR-214, miR-129-5p, and Let-7b specifically reduced target myelin-associated gene expression and inhibited viral replication. Importantly, there was an inverse correlation between expression of microRNAs and the level of their target genes in virus-infected cells. These results thus suggest a potential role for microRNAs in regulation of cellular gene expression in coronavirus-infected oligodendrocytes.

Keywords: Coronavirus; Mouse Hepatitis Virus; Acute infection; Persistent infection; Oligodendrocyte; MicroRNA; Expression Profiling

Introduction

MicroRNAs [miRNAs] are small RNA molecules of ≈22 nucleotides [nt] in length. All cellular miRNAs are transcribed by RNA polymerase II [pol II] as a long RNA precursor termed primary miRNA [pri-miRNA]. A pri-miRNA ranges from ≈200 nt to several thousand nt in length and may contain a single or multiple distinct miRNAs. A pri-miRNA usually undergoes step-wise processing in the nucleus, which is initiated through the recognition and cleavage of a stem-loop structure by a heterodimer consisting of a RNase III enzyme called Drosha and a cofactor called DGCR8 [1-3]. This cleavage results in three segments: a core ≈60-nt RNA hairpin intermediate with the characteristic 2-nt 3'-overhang, also called a pre-miRNA, and flanking 5' and 3' sequences [3,4]. The pre-miRNA is then exported to the cytoplasm by another heterodimer consisting of Exportin 5 and the GTP-bound form of its cofactor Ran [5,6]. Once in the cytoplasm, the pre-miRNA is released from the exporting complex by GTP hydrolysis and is then bound by a second RNase III enzyme termed Dicer and its cofactor TRBP [7-10]. Dicer binds the 2-nt 3' overhang of the pre-miRNA hairpin and removes the terminal loop, leaving a second 2-nt 3' overhang and generating the miRNA duplex intermediate [10]. Dicer then facilitates the assembly of the miRNA strand of the duplex into the RNA-induced silencing complex [RISC] containing Argonaute while the "passenger" strand is released and degraded [8,11,12]. The mature miRNA of ≈22-nt is then ready to exert its functions in the RISC assembly. miRNA plays an important role in the post transcriptional regulation of gene expression in eukaryotic cells at two levels. It acts as a guide RNA to direct RISC to complementary mRNA [13]. If the RISC contains Argonaute-2 and the mRNA has a highly complementary target sequence, then the target mRNA is cleaved and degraded by the miRNA [14-16]. If the RISC binds to an mRNA with a partially complementary sequence, it will only inhibit mRNA translation [17-19].

Virus is an obligate intracellular parasite. It requires cellular functions for its replication and survival. As miRNA generally down-regulates the expression of target genes, if the cellular target genes are critical for replication of a virus, expression of cellular miRNAs will have negative effect on that virus's replication. Cellular miRNAs may also inhibit virus replication by directly targeting viral genes. For example, human miRNA-32 can inhibit the replication of the retrovirus primate foamy virus in the human embryonic kidney cell line 293T [20]. On the other hand, cellular miRNAs may also have positive effect on viral replication and thus contribute to viral pathogenesis. An example for this type of effect is miRNA-122. It has been reported that the liver-specific miRNA-122 can directly bind to a sequence in the 5'-untranslated region of hepatitis C virus [HCV], thereby enhancing viral RNA replication [21]. In addition, viral infection often alters cellular gene expression through diverse mechanisms, one of which is through alteration of miRNA expression. Indeed, it has been shown recently that viruses can directly modulate the expression of cellular miRNAs or subvert cellular miRNAs for persistent replication and oncogenic purposes [22]. The human T cell leukemia virus [HTLV]-1 Tax protein upregulates at least two miRNAs by transactivating the miR-130b and miR-146a promoters [22-24]. Coronavirus is an important pathogen of humans and diverse species of animals. It causes respiratory, digestive, neurological and immune-mediated diseases including the severe acute respiratory syndrome [SARS]. Murine coronavirus mouse hepatitis virus [MHV] can cause acute and persistent infection and demyelination in the central nervous system [CNS] of rodents and thus has been used as a small animal model for studying multiple sclerosis [MS] and other degenerative diseases of the CNS. However, the pathogenic mechanism of MHV-caused CNS diseases and especially the

mechanism of viral persistence remain elusive. Recently, we have successfully established a persistent infection model in an oligodendroglial cell line, in which viral genome persists without the production of infectious virus [25]. This type of nonproductive persistence resembles the phenomenon observed in the mouse CNS. To facilitate our understanding of the mechanisms of viral persistence in oligodendrocytes in the absence of an adaptive immune system, in this study we investigated the effect of viral acute and persistent infection on the expression of cellular miRNAs in oligodendrocytes by DNA microarray analysis using genome-wide miRNA probes. The effect of altered miRNA expression on the expression of myelin-associated genes and its potential consequence to the biology of oligodendrocytes during coronavirus infection were also assessed.

Materials and Methods

Cell culture and virus

The N20.1 cell is a cell line clone derived from mouse primary cultures of oligodendrocyte conditionally immortalized by transformation with a temperature sensitive mutant of the simian virus-40 large T-antigen [26]. It was kindly provided by Dr. Anthony Compadre [University of California Los Angeles School of Medicine]. The N20.1 cell represents a mature oligodendrocyte because it expresses the myelin basic protein (MBP] and myelin proteolipid protein (PLP), but one that is at a very early stage of maturity based on the expression of the MBP and PLP alternatively spliced mRNA population [26]. In addition, the N20.1 cell can be recognized by the A2B5 antibody, which recognizes a surface galactolipid expressed by oligodendrocyte progenitor cells; the A007 antibody, which is specific for sulfatide and found on cells committed to the oligodendrocyte lineage as well as mature oligodendrocytes, and the anti-GC antibody, which recognizes the protein expressed specifically by mature oligodendrocytes [26]. The N20.1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 with 10% fetal bovine serum (FBS) and G418 (100 μg/ml) at the permissive temperature of 34 °C. Cells were grown either in T25 flasks or on glass coverslips coated with poly-D-lysine. Mouse fibroblast 17Cl-1 cells were cultured in DMEM. Mouse astrocytoma DBT cells were cultured in Eagle minimal essential medium (EMEM) and were used for virus propagation and plaque assay as described previously [27]. The wild-type mouse hepatitis virus strain A59 (MHV-A59), a recombinant MHV-A59 expressing GFP (MHV-A59/GFP) or luciferase (MHV-2aFLS) was used for infection. MHV-A59/GFP expresses the enhanced green fluorescence protein (GFP) that replaced the viral nonstructural protein 4 (NS4) (kindly provided by Dr. Susan Weiss, University of Pennsylvania School of Medicine) [28] and MHV-2aFLS expresses the firefly luciferase that replaced the 2b gene between 2a and spike gene in the viral genome (kindly provided by Dr. Peter Rottier, Utrecht University, The Netherlands) [29].

miRNA isolation

N20.1 cells were grown to monolayer in 100-mm Petri dishes, which were then divided into 3 groups with 3 dishes each. Cells in Group A were mock-infected with PBS as normal cell control, while cells in Groups B and C were infected with MHV-A59/GFP for 24 and 48 h, respectively. N20.1 cells that were persistently infected with MHV-A59/GFP at passage 6 (25) were cultured in parallel as Group D. miRNAs were then isolated from the 4 groups of cells using miRNeasy Mini kits (Qiagen) followed by quality control and quantification. The 12 miRNA samples were sent to Ocean Ridge Biosciences (www.oceanridgebio.com) for miRNA analysis.

Microarray-based miRNA detection and data analysis

Microarray-based miRNA expression profiling was carried out by Ocean Ridge Biosciences as a service. Briefly, the miRNA samples were hybridized with oligonucleotide probes that can detect 374 mouse miRNAs using the NCode version 2.0 miRNA microarray. Duplicate hybridization was carried out for each RNA sample. Thus, the final data represent the means of 6 hybridization results for each group. As a quality control check, NCode synthetic miRNA was spiked at 1:100,000 mass ratio into labeling reactions, and the signal intensity was detected on the arrays at well above (5x) the threshold for hybridization detection. The log-2 transformed, normalized, and averaged probe hybridization intensities were further subjected to statistical analysis (ANOVA), principal component analysis, hierachical clustering, and reproducibility assessment.

Synthesis and transfection of miRNAs

The following synthetic miRNAs were purchased from Dharmacon: mmu-miR-129-5p (Cat.#C310432-07), mmu-miR-214 (Cat.#C310573-07), mmu-miR-23a (Cat.#C310517-05), mmu-let7b (Cat.#C310504-05) and mimic negative control miRNA (Cat.#CN0010000-01-05). Synthetic miRNAs were transfected into cells using lipofectamine 2000 transfection reagent (Qiagen) according to the manufacturer's instruction.

Reverse transcription-polymerase chain reaction (RT-PCR)

For detection of mRNAs by RT-PCR, intracellular total RNAs were isolated from N20.1 cells using the Qiagen RNeasy plus Mini kit (Cat.#74134) according to the manufacturer's protocol and treated with RNase-free DNase I (Qiagen). The concentration of the RNA samples was determined with a spectrophotometer (Model U2001, Hitachi). RT-PCR was then carried out to detect cellular mRNAs as described previously (47). The following primer pairs were used:IGF1R-F (5'-AGT GTG TGC GGG AAG CGA GC-3') and IGF1R-R (5'-ATG GCG ATC CACACA GCG CC-3') for insulin growth factor-1 receptor (IGF1R) gene; Qk-F (5'-CCT GAC CATGCC TGC CTG CTG-3') and Qk-R (5'- GTT CGC TTG GGG TGG GGC ATT-3') for Quaking (Qk) gene; POU3F1-F (5'-GAG GAC GGC CAC GAG GCA CA-3') and POU3F1-R (5'-GCTGGG AGC ATC CTC GTC CG-5') for transcription factor SCIP (or POU3F1); POU3F2-F (5'-CGA GAG TCA TGG CGA CCG CA-3') and POU3F2-R (5'-TCC ACT GGT GAG CGT GGCTG-3') for transcription factor Brn-2 (or POU3F2). The PCR products were analyzed by agarose gel electrophoresis and quantified using the gel imaging system (Alpha Innotech).

Luciferase assay

The steady-Glo luciferase assay system (TM051) was carried to determine the firefly luciferase activity in MHV-2aFLS-infected cells according to the manufacturer's protocol (Promega). Briefly, N20.1 cells were reverse transfected with miRNA and cultured in 96-well white plate at approximately 5,000 cells/well/100 μl for 48h. Culture medium was then removed and cells were infected with 50 μl of MHV-2aFLS at a multiplicity of infection (MOI) of 10 at 37°C. At 16 h post infection (p.i.), the plate was transferred to 4 °C for 5 min and then kept at room temperature.

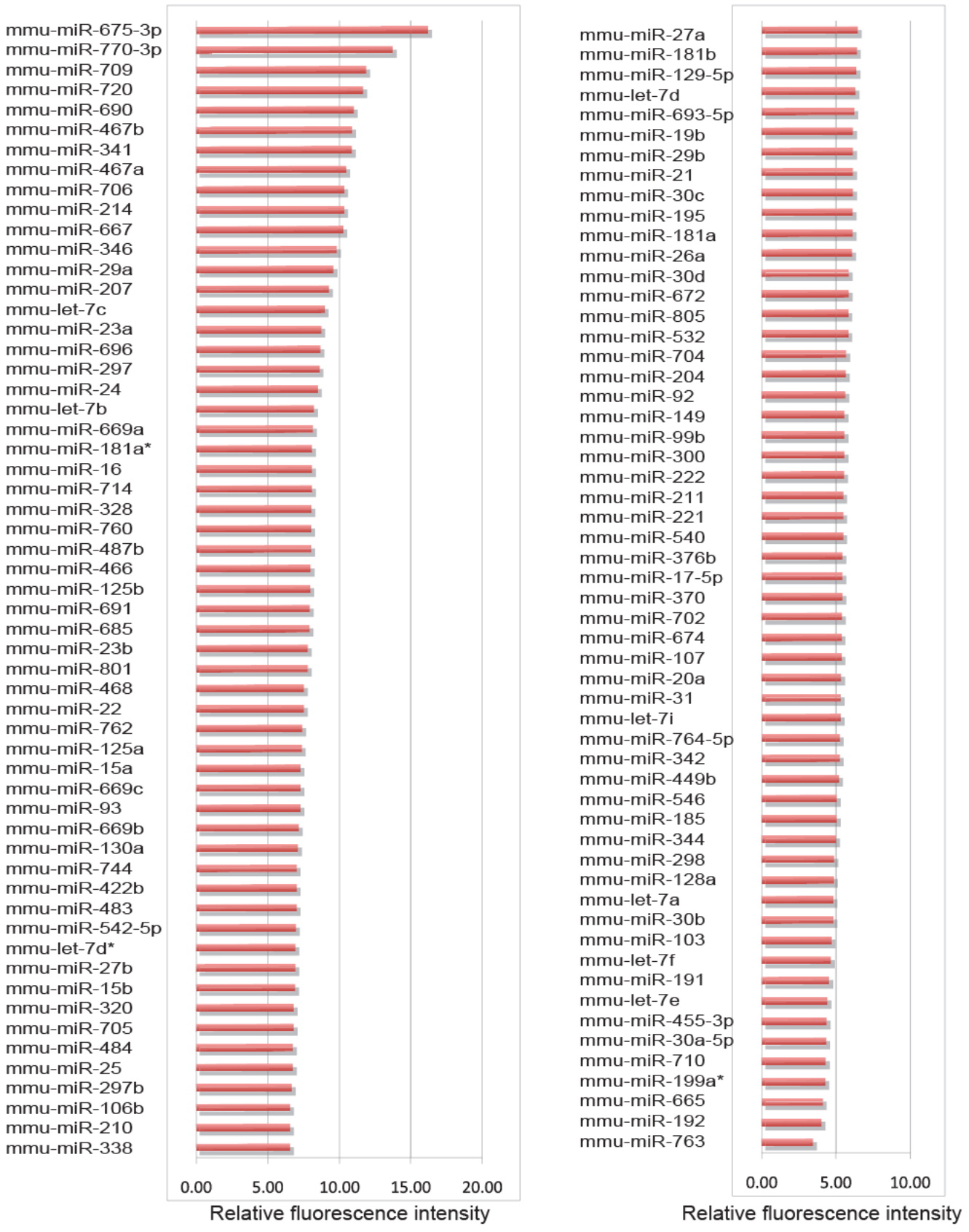

Figure 1: miRNA expression profile in normal mouse oligodendrocytic N20.1 cells. A total of 113 miRNAs were specifically expressed in N20.1 cells. The name of each miRNA detected is listed on the left while its expression level as measured in relative fluorescence intensity is shown on the right to each miRNA.

One hundred microliter Steady-Glo luciferase reagent was then added to each well of the 96-well white plate followed by shaking gently for 5 min at room temperature. Luciferase activity was read with an automatic plate reader Synergy 2 using Gen5 software (BioTek).

Results

miRNA expression profile in oligodendrocytes is altered by coronavirus infection

To determine the overall miRNA expression profile in mouse oligodendrocyte, miRNAs were isolated from mouse oligodendrocyte N20.1 cell line and were hybridized with the oligonucleotide probes using NCode version 2.0 miRNA microarray. Following statistical analysis, principal component analysis, hierarchical clustering, and reproducibility assessment, it was found that 113 probes had signal intensity above threshold. Thus, 113 miRNAs were detected in normal (uninfected) N20.1 cells with a wide-range of expression levels [Figure 1].

Name	Fold change from mock infection		
	24 hpi	48 hpi	Persistence
miR-665	2.07	2.64	3.34
miR-26a	2.36	2.68	2.97
miR-30b	2.07	2.68	2.17
Let-7d	2.14	2.14	2.23
miR-709	1.99	2.08	2.46
Let-7b	2.30	2.50	4.03
miR-30d	1.03	2.04	1.47
miR-23a	2.48	1.44	1.07
miR-30c	2.25	1.93	1.66
miR-192	1.46	2.20	2.27

Table 1: microRNAs that are increased in oligodendrocytes during acute and persistent coronavirus infection

Name	Fold change from mock infection		
	48 hpi	24 hpi	Persistence
miR-669b	-8.94*	-8.46	-4.92
miR-669a	-7.67	-8.11	-5.62
miR-300	-2.87	-3.63	-2.77
miR-297b	-6.73	-6.81	-5.13
miR-801	-2.60	-2.43	-2.01
miR-297	-8.75	-9.99	-6.54
miR-468	-4.08	-4.79	-3.29
miR-129-5p	-2.23	-2.89	-1.89
miR-760	-2.89	-2.91	-1.80
miR-466	-5.90	-8.46	-4.23
miR-706	-4.66	-3.41	-3.10
miR-210	-3.46	-3.43	-1.99
miR-370	-2.48	-2.73	-1.88
miR-214	-4.41	-3.76	-2.25
miR-672	-2.10	-2.17	-1.10
miR-467b	-5.43	-9.51	-5.39

Table 2: microRNAs that are decreased in oligodendrocytes during acute and persistent coronavirus infection. *The negative number indicates fold decreased

To further determine whether acute and persistent coronavirus infections could alter miRNA expression, we compared the miRNA expression profiles at 24 and 48 h postinfection (acute) or during persistent infection (at passage 6) with those of mock-infection in N20.1 cells. Results showed that the expression of a total of 26 miRNAs was statistically significantly altered by MHV infection [Tables 1, 2]. Specifically, 10 miRNAs (i.e. miR-23a, miR-26a, miR-30b, miR-30c, miR-30d, miR-192, miR-665, miR-709, let-7b, and let-7d) were significantly upregulated [Table 1] while 16 miRNAs (i.e. miR-129-5p, miR-210, miR-214, miR-297, miR- 297b, miR-300, miR-370, miR-466, miR-467b, miR-468, miR-669a, miR-669b, miR-672, miR-706, miR-760, and miR-801) were significantly down-regulated [Table 2] . These results demonstrate that coronavirus infection can alter cellular miRNA expression.

Dynamic changes of miRNA expressions during acute and persistent coronavirus infection

We next compared the expression of individual miRNAs at different times of acute infection and during persistence. We found that in general if a miRNA was upregulated or downregulated by virus infection, it remained so throughout the period of infection, although the expression level varied at different times. For example, all miRNAs shown in Table 1 were upregulated at 24 and 48 h after infection or during persistence. However, there were three expression patterns emerged. The first pattern is those miRNAs whose expression continuously increased from 24 h p.i. to persistent infection, such as miR-665, miR-26a, Let-7d, miR-709, Let-7d, and miR-192 (Figure 2A). The second pattern is that miRNA expression rapidly increased during acute infection (24 and 48 h p.i.) but the upregulation started to decline during persistence, such as miR-30b and miR-30d (Figure 2B). The third pattern is that the expression level rapidly increased by 24 h p.i., and then continuously declined at 48 h p.i. and during persistence (e.g. miR-30c and miR-23a) (Figure 2C).

Similarly, two expression patterns were found for those miRNAs that were down-regulated by virus infection (Figure 3). One pattern including miR-669b, miR-801, miR-706, miR-210, and miR-214 is that the expression reached to the lowest level within 24 h p.i., and began to recover at 48 h p.i. and during persistence (Figure 3A). The other pattern which includes 11 miRNAs (miR-669a, miR-300, miR-297b, miR-297, miR-468, miR-129-5p, miR-760, miR-466, miR-370, miR-672 and miR-467b) exhibited an initial decrease in expression at 24 h p.i., and further decrease at 48 h p.i.; however, during persistence

the expression level for most miRNAs recovered to a level higher than that during acute infection, but were still significantly lower as compared to that in mock-infected cells (Figure 3B).

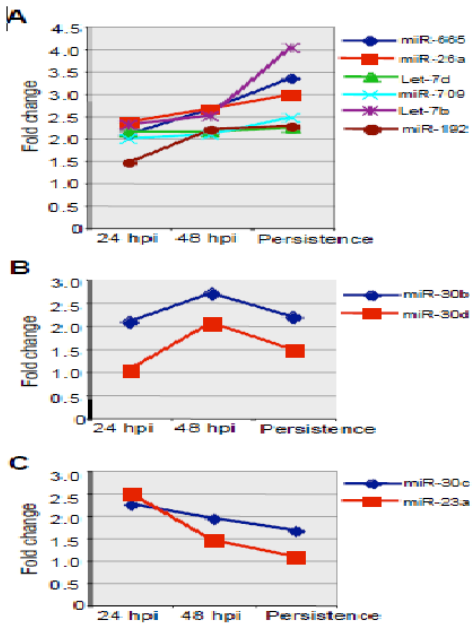

Figure 2: Graphic presentation of the patterns of miRNA expression that is up-regulated by MHV infection. (A) miRNAs whose expression increased continuously from acute (24 to 48 h) to persistent infection. (B) miRNAs whose expression increased at 24 h p.i. and peaked at 48 hp.i., but declined during persistent infection. (C) miRNAs whose expression rapidly increased and peaked at 24 h p.i., and then continuously declined at 48 h p.i. and during persistence.

Effects of altered miRNA expression on cellular gene expression

To better understand the biological relevance of the miRNA alteration in oligodendrocytes by coronavirus infection, we performed sequence analysis using the RNA22microRNA Target Detection Program (http://cbcsrv.watson.ibm.com/rna22.html) to search for potential cellular gene targets by the 26 miRNAs listed in Tables 1 and 2. We then narrowed down the target genes by selecting only those that are highly conserved among all vertebrates. Finally, we did literature search to find out whether the biological functions of these selected target genes are related to cell proliferation, differentiation and maturation, paying special attention to reports that have demonstrated a role for the genes in oligodendrocytes. The combination of these approaches allowed us to focus on 3 miRNAs, i.e. let-7b, miR-214, and miR-129-5p. Let-7b has 3 targets predicted at the 3'-UTR of insulin growth factor-1 receptor (IGF1R) gene; miR-214 has a target at the 3'-UTR of Quaking (Qk) mRNA; miR-129-5p has a target at the 3'-UTR of two related transcription factors SCIP (or POU3F1) and Brn-2 (or POU3F2) that suppress myelin-specific gene expression. To demonstrate whether these miRNAs are able to regulate the expression of these putative or established target genes, synthetic miRNAs were transfected into oligodendrocytic N20.1 cells. Cells transfected with a nonspecific miRNA were used as a negative control. At 48 h post transfection, RNAs were isolated and RT-PCR was performed to detect the target gene mRNAs of IGF1R, Qk, SCIP and Brn-2. Results showed that the expression of all these 4 genes was noticeably decreased by their respective miRNAs as compared to the controls that were transfected with the nonspecific miRNA (Figure 4 A-C). These data confirmed the specific target genes for the respective miRNAs.

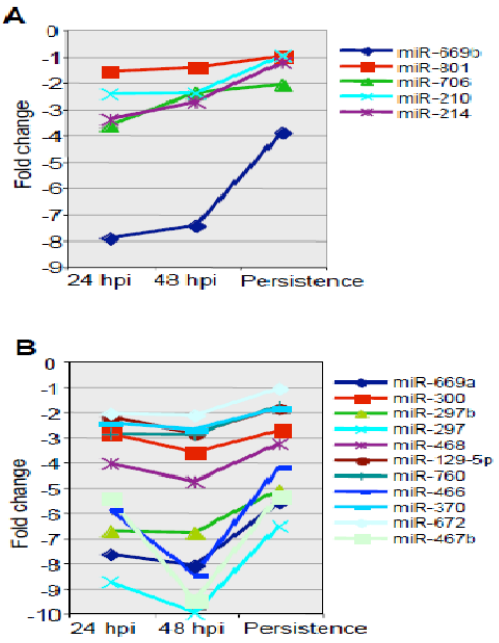

Figure 3: Graphic presentation of the patterns of miRNA expression that is down-regulated by MHV infection. (A) miRNAs whose expression decreased to the lowest level at 24 h p.i., and then began to recover at 48 h p.i. and during persistence. (B) miRNAs whose expression decreased continuously from 24 to 48 h p.i., and but began to recover during persistence.

The above results further suggest that as miRNA expression is altered by MHV infection, the expression of miRNA-target genes likely is also affected by viral infection. To test this possibility and to establish its biological significance, cellular mRNAs were isolated from virus infected N20.1 cells at 48 h p.i., or during persistent infection, and were subjected to RT-PCR amplification for IGF1R, Qk, SCIP and Brn-2. As expected, Qk, SCIP and Brn-2 mRNAs were strongly up-regulated during acute (48 h) and persistent infection as compared to those in mock infected cells (Figure 4D). In contrast, the expression of IGF1R was decreased during acute and persistent infections (Figure 4D). These results thus established the biological relevance of the altered cellular miRNAs to the target gene expression during coronavirus infection.

Role of selected cellular miRNAs on coronavirus replication

We next performed similar sequence analysis to identify whether any of the miRNAs listed in Tables 1 and 2 have a target on the 5'- and 3'-UTR of MHV genomic RNA. Our results showed that none of these miRNAs had a specific target at the 5'- and 3'-UTR of the viral

mRNAs (data not shown). We then searched for any sequence within the MHV genomic RNA and found that 8 of the upregulated miRNAs had a putative target sequence within the coding regions of the viral genome. These are Let-7b, Let-7d, miR-26a, miR-30c, miR-192, miR-665, miR-709, and miR-805.

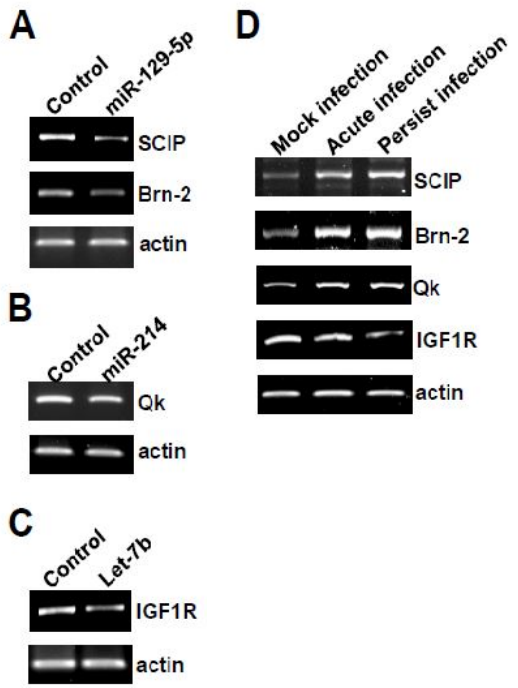

Figure 4: RT-PCR detection of selected miRNA target genes in miRNA-transfected or virusinfected cells.(A-C) Oligodendrocytic N20.1 cells were transfected with miRNAs miR214,miR-129-5p and Let-7b, respectively. Cells transfected with a negative control miRNA wereused as a control. At 24 h post transfection, intracellular mRNAs were isolated and were subject to RT-PCR amplification using gene-specific primers. (A) miR-129-5p target genes SCIP and Brn-2; (B) miR-214 target gene quaking (Qk); (C) Let-7b target gene IGF1R. (D) N20.1 cellswere acutely or persistently infected with MHV-GFP or mock-infected as a control. At 48 h p.i. (for acute infection) or at passage 6 (for persistent infection), intracellular RNAs were isolated for detecting mRNAs of SCIP, Brn-2, Qk andIGF1R as indicated. Actin was used for internal control and for normalization.

As an example to further test the biological relevance of this sequence prediction, we transfected into N20.1 cells a synthetic miRNA, Let-7b, and 3 other synthetic miRNAs (miR-23a, miR-214, and miR-129-5p) that do not have predicted targets in the viral genome. At 48 h post-transfection, cells were infected with a luciferase-expressing recombinant MHV-A59-2aFLS at an moi of 10. Cells transfected with a nonspecific miRNA or mock-transfected were used as negative controls. At 16 h p.i., luciferase activity expressed in virus-infected cells was assayed. Results showed that luciferase activities were reduced to 50.5, 76.3, and 86.8 % of the negative control for miR-129, Let-7b, and miR-214, respectively, while miR-23a had no significant effect on luciferase activity (Figure 5). These results suggest that the observed effect of the selected miRNAs on viral replication unlikely resulted from direct targeting on viral RNA genome as there was no correlation between the predicted target sequence on viral genome for a particular miRNA and virus replication.

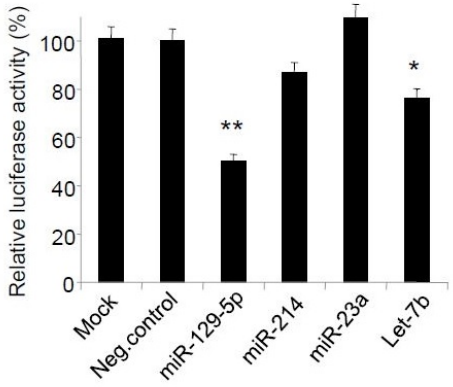

Figure 5: Effect of selected miRNAs on MHV replication. N20.1 cells were transfected with miRNAs miR-129-5p, miR-214, miR-23a, and Let-7b or the negative control miRNA. Mocktransfected cells were also used as a negative control. At 48 h post transfection, cells were infected with recombinant MHV-2aFLS at moi of 10. At 16 h p.i., luciferase activity was determined and expressed as percent relative to the negative controls. The data represent one of the two triplicate experiments. The asterisk indicates statistical significance as compared to the controls: $p<0.05$ (*); $p<0.01$ (**).

Discussion

In this study, the overall cellular miRNA expression profile and its alteration during acute and persistent MHV infection were assessed with DNA microarray analysis using genome-wide miRNA probes. To our knowledge, this is the first miRNA profiling in a mouse oligodendrocyte. Thus, the microarray data are informative for understanding the basal level expression of miRNAs in oligodendrocytes in general and mouse oligodendrocyte N20.1 cells in particular. The information is also useful for understanding the mechanisms of pathogenesis and persistence of viruses that use oligodendrocytes as host cells. It is noted, however, that the number of miRNAs that exhibited significant change in expression by virus infection is likely grossly underestimated in this study. This is because only about 25% of the N20.1 cells were infected with MHV-A59/GFP as judged by the GFP-positivity [25]. This low susceptibility of N20.1 cells to MHV infection likely results from heterogeneity of the cell subpopulations, possibly due to varying degrees of cell differentiation [30]. As the fold-change in miRNA expression in virus-infected cells was averaged by the entire cell population (including the remaining ≈75% uninfected cells), the actual fold-change in miRNA expression in virus-infected cells is likely much higher. As the cut-off threshold for statistically significant change was set to be >2 fold, the low level of cell susceptibility to virus infection has conceivably excluded a significant number of miRNAs that would be otherwise above the cut-off threshold. For this reason, the miRNAs listed in Tables 1 and 2 represent only those whose expression was highly significantly altered

by virus infection. Thus, the fold-changes in miRNA expression by virus infection as presented in these tables are most likely also under-valued.

How is expression of the miRNAs altered by coronavirus infection? While there is no single answer to this question, several possible mechanisms might offer some explanations. As described in the Introduction, the biogenesis of miRNA involves transcription by Pol II, cleavage by Drasha, transport to the cytoplasm by Exportin 5, and maturation by Dicer. Logically, alteration of any factor along this biogenesis pathway could potentially regulate miRNA expression. For example, coronavirus infection may alter miRNA expression at the transcriptional level. Indeed, the expression of a great number of cellular genes can be altered by coronavirus infection. We have shown previously that 125 genes are up-regulated while 124 genes are down-regulated in a mouse astrocytoma cell line infected by MHV in a genome-wide DNA microarray analysis [31]. Interestingly, many transcription factors such as extracellular regulatory kinase (Erk)-1/2, c-Jun, early growth regulator (Egr)-1, interferon regulatory factor (IRF)-3, nuclear factor (NF)-kB are activated in various types of cells by MHV infection [32-35]. As expression of many cellular genes is regulated by transcription factors, it is possible that expression of cellular miRNAs can be regulated by coronavirus infection through activation or suppression of the transcription factors.

The biological relevance of the observed change in miRNA expression was further assessed using synthetic miRNAs. While it is impossible to demonstrate the biological functions for each of the miRNAs identified in this study, we selectively tested a few miRNAs whose functions are known to be associated with oligodendrocyte biology as examples to establish the biological relevance. For example, Let-7b is predicted to target IGF1R. The role of IGF1 through its receptor IGF1R signaling in the CNS and in vitro-cultured CNS cells has been well established [36-39]. In particular, IGF1R signaling is required for normal in vivo oligodendrocyte development and myelination and inhibits mature oligodendrocyte apoptosis during primary demyelination [36-39]. Thus, if IGF1R is the target of miRNA Let-7b as predicted, then the up-regulation of Let-7b in MHV-infected oligodendrocytes will down regulate the expression of IGF1R, thereby promoting apoptosis of mature oligodendrocyte and preventing myelination/remyelination and differentiation. Our results show that IGF1R expression was indeed reduced in oligodendrocytic N20.1 cells when transfected with synthetic miRNA Let-7b or infected with MHV (Figure 4). On the other hand, miR-214 and miR-129-5p were down-regulated by coronavirus infection (Table 2 and Figure 3). miR-214 has predicted targets at the 3'-UTR of Quaking (Gk) gene [40]. There are 3 isoforms of Qk gene, whose expression is related to the CNS demyelination disease of mice (quaking mice, as also seen in shivering mice) [41-46].miR-129-5p targets two transcriptional repressors of myelin-specific genes, SCIP (POU3F1) and Brn-2 (POU3F2), which repress myelin-specific gene expression. Both transcription factors were also significantly up-regulated during coronavirus persistence in a rat oligodendrocyte CG-4 cell line [47] and during acute and persistent infection in mouse oligodendrocytic N20.1 cells (Figure 4D). Thus, down-regulation of miR-214 and miR-129-5p by virus infection increased the expression of Qk, and SCIP/Brn-2, respectively (Figure 4D), the latter of which results in down-regulation of myelin-specific genes expression. These data support the notion that coronavirus infection may result in inhibition of myelin-associated gene expression in part through regulation of cellular miRNAs.

The role of miRNAs in coronavirus replication was also assessed in this study using a few selected miRNAs. As miRNA usually targets the 3'-UTR of mRNA, we used a computer program for target prediction. Our results showed that none of the miRNAs listed in Tables 1-2 has a predicted target at the 3'- and 5'-UTR of the viral genomic and subgenomic mRNAs (data not shown). However, it has been shown recently that miRNA targets in the coding sequence can also regulate mRNA translation and stability [48]. Thus, we analyzed the entire coding sequence of the viral genome and found that 8 miRNAs have predicted targets in the viral genome. Among them is Let-7b, which has 5 target sites in polymerase coding sequence, 1 in the nonstructural gene 2 and 2 in the spike gene (data not shown). Our data showed that transfection of synthetic Let-7b reduced viral gene expression as determined by the luciferase reporter assay while transfection of synthetic miR-23a that does not have a predicted target on coronaviral mRNAs did not have any effect (Figure 5). Interestingly, transfection of miR-214 and miR-129-5p,which do not have a predicted target in the viral genome, decreased viral gene expression by ≈13-50% (Figure 5). The inhibitory effect appears to be specific since viral gene expression was significantly higher in mock-transfected cells or cells transfected with a non-specific control miRNA. However, the mechanism by which miR-129-5p and miR-214 inhibit coronaviral gene expression remains unknown. We speculate that the inhibitory effect on virus replication unlikely results from direct action of the miRNAs on viral genomic or subgenomic mRNAs but rather through indirect cellular targets that may be involved in coronavirus replication. While the number of miRNAs tested in the current experiments is rather limited, the results appear to suggest that miRNAs play a role in coronavirus replication. However, it is not known whether the net outcome of the altered miRNA expression contributes to restricting MHV replication as does the antiviral type I interferon response to MHV infection in N20.1 cells [32-35]. Nevertheless, our studies show that cellular miRNAs are potential regulators of viral replication and host gene expression during acute and persistent coronaviral infection that might contribute to the pathogenesis of coronavirus infection in oligodendrocytes in vitro and in vivo.

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