Biochemical and Biophysical Research Communications 395 (2010) 11–16

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Analysis of microRNAs induced by Venezuelan equine encephalitis virus infection in mouse brain

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ARTICLE INFO

Article history:
Received 2 March 2010
Available online 19 March 2010

Keywords:
MicroRNA profiling
Venezuelan equine encephalitis virus
Alphavirus infection
Real-time PCR
mRNA expression

ABSTRACT

MicroRNAs (miRNA) are small RNA (~22nts) molecules that are expressed endogenously in cells and play an important role in regulating gene expression. Recent studies have shown that cellular miRNA plays a very important role in the pathogenesis of viral infection. Venezuelan equine encephalitis virus (VEEV) is an RNA virus and is a member of the genus *Alphavirus* in the family *Togaviridae*. VEEV is infectious in aerosol form and is a potential biothreat agent. In this study, we report for the first time that VEEV infection in mice brain causes modulation of miRNA expression. Pathway analyses showed that majority of these miRNAs are involved in the neuronal development and function. Target gene prediction of the modulated miRNAs correlates with our recently reported mRNA expression in VEEV infected mice brain.

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1. Introduction

Venezuelan equine encephalitis virus (VEEV) is an arthropod borne virus of the genus *Alphavirus* in the family *Togaviridae*. The VEEV genome consists of a single 11.4 kb, positive sense, single-stranded RNA [1]. VEEV has caused periodic outbreaks of febrile and neurological disease primarily in Latin America during the past century [2]. There are no effective antiviral drugs to treat or safe vaccine for prophylaxis against VEEV infection [3]. VEEV can be easily propagated and aerosolized which also makes it a potential biothreat agent [2]. VEEV infection is characterized by high viremia, rash, fever and encephalitis that can lead to death in immunocompromised individuals [4]. In central nervous system (CNS), VEEV targets neurons and glial cells and induces inflammation resulting in neurodegeneration [5]. Inflammation in CNS plays an important role in the disease outcome and is often believed to be associated with the secondary neuronal damage in brain [5–7].

MicroRNAs (MiRNAs) are a class of small (~22nts) endogenous RNA molecules that negatively regulate gene expression by translational repression [8]. MiRNA binds to the complementary sequences in the mRNA and blocks the translation and/or accelerates mRNA decay [9]. MiRNAs play an important role in cellular processes such as development, differentiation, cell proliferation, tumorigenesis, neuronal development and hematopoiesis [10].

There is a growing body of evidence that demonstrates key regulatory roles for cellular miRNA during viral infection and altered cellular miRNA expression during the virus infection may be an important determinant of virulence [11–13]. Cellular miRNAs have been shown to be either necessary for progression or suppression of viral replication. Mir-122 has been shown to positively regulate hepatitis C virus (HCV) replication by enhancing colony formation efficiency of HCV [14]. Moreover, knocking down mir-122 in non-human primate model leads to long lasting suppression of HCV viremia [15]. On the other hand, mir-28, mir-125b, mir-150, mir-223, and mir-382 were reported to inhibit human immunodeficiency virus (HIV) replication in $CD4^+$ T cells. Inhibition of these miRNAs was shown to result in the active replication of HIV in $CD4^+$ T cells [16]. Other viruses including primate foamy virus (PFV) and human cytomegalovirus have been also been shown to utilize host miRNAs for their benefit [17,18]. Though studies have reported host miRNAs modulation during DNA viruses such as Epstein Barr virus [19] and retroviruses such as HIV and PFV [18,16], only a few studies have shown host miRNA modulation in RNA virus infections [20].

Earlier we have shown that VEEV causes modulation of genes that are involved in host innate immune responses such as apoptosis, inflammation and antigen presentation [21,22]. However, the regulatory pathways which control the expression of these genes

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0006-291X/$ - see front matter Published by Elsevier Inc.
doi:10.1016/j.bbrc.2010.03.091

are poorly understood. Since, miRNAs have been shown to play a critical role in neurodegenerative disorders [23–26,27], we have evaluated expression profile of miRNAs in VEEV infected mouse brain and a computational correlation of the modulated miRNAs with earlier reported genes expression studies was carried out.

Data reported in this study have demonstrated the modulation of several miRNAs in VEEV infected mice brain. Computational analysis of the predicted targets of these miRNAs revealed a possible regulation of the downstream genes by the miRNAs. To our knowledge, this is the first study to evaluate the miRNA expression in the mice brain following alphavirus infection.

2. Material and methods

2.1. Animals

Six to eight weeks old male CD-1 mice were obtained from Charles River Laboratories, Wilmington, MA. All the experiments with live virus challenge were carried out at the Uniformed Services University of the Health Sciences (USUHS) bio-safety level 3 (BSL-3) facilities in accordance with the Guide for the Care and Use of Laboratory Animals (Committee on Care And Use of Laboratory Animals of The Institute of Laboratory Animal Resources, National Research Council, NIH Publication No. 86–23, revised 1996).

Table 1
Modulation of miRNAs in mouse brain upon VEEV infection.

miRBase Accession Number	Up-regulated microRNAs	Fold increase	miRBase Accession Number	Down-regulated microRNA	Fold decrease
(a) MiRNAs modulated at 48 h post-VEEV infection					
MIMAT 0000165	mmu-miR-155	5.05	MIMAT 0003742	mmu-miR-455	13.97
MIMAT 0003171	mmu-miR-542–5p	4.83	MIMAT 0003388	mmu-miR-376b*	3.14
MIMAT 0004578	mmu-miR-300*	3.75	MIMAT 0004544	mmu-miR-193*	2.66
MIMAT 0000236	mmu-miR-203	3.33	MIMAT 0003469	mmu-miR-690	2.57
MIMAT 0004842	mmu-miR-879	3.29	MIMAT 0000209	mmu-miR-129–5p	2.35
MIMAT 0000666	mmu-miR-320	2.73	MIMAT 0004520	mmu-let-7i*	2.33
MIMAT 0004633	mmu-miR-27a*	2.69	MIMAT 0000660	mmu-miR-181a-1*	2.12
MIMAT 0000148	mmu-miR-136	2.69	MIMAT 0004537	mmu-miR-154*	2.09
MIMAT 0004531	mmu-miR-135a*	2.68	MIMAT 0003199	rno-miR-381	2.02
MIMAT 0001542	mmu-miR-449a	2.38	MIMAT 0004624	mmu-miR-15a*	1.83
MIMAT 0003735	mmu-miR-672	2.32	MIMAT 0000537	mmu-miR-27a	1.45
MIMAT 0003480	mmu-miR-297b-5p	2.23		#mmu-miR-801	3.61
MIMAT 0004746	mmu-miR-409–3p	2.19			
MIMAT 0000138	mmu-miR-126–3p	2.18			
MIMAT 0004522	mmu-miR-27b*	2.18			
MIMAT 0004893	mmu-miR-574–3p	2.12			
MIMAT 0000133	mu-miR-101a	1.96			
MIMAT 0000612	mmu-miR-135b	1.89			
MIMAT 0004643	mmu-miR-331–3p	1.87			
MIMAT 0000584	mmu-miR-339–5p	1.72			
(b) MiRNAs modulated at 72 h post-VEEV infection. Statistical analysis was done using Statminer (Integromics Inc.). MicroRNAs with $p < 0.05$ and fold change of >1.5 were considered significant					
MIMAT 0000165	mmu-miR-155	10.66	MIMAT 0004706	rno-let-7e*	8.34
MIMAT 0003494	mmu-miR-704	9.59	MIMAT 0003732	mmu-miR-668	5.99
MIMAT 0005334	rno-miR-743a	6.81	MIMAT 0004582	mmu-miR-106b*	5.27
MIMAT 0004746	mmu-miR-409–5p	2.24	MIMAT 0000239	mmu-miR-206	4.38
MIMAT 0003509	mmu-miR-501–3p	1.81	MIMAT 0000569	mmu-miR-330*	4.09
			MIMAT 0000746	mmu-miR-381	3.93
			MIMAT 0000249	mmu-miR-30e*	3.76
			MIMAT 0000129	mmu-miR-30a*	3.73
			MIMAT 0000548	mmu-miR-322	3.61
			MIMAT 0004636	mmu-miR-93*	3.15
			MIMAT 0003484	mmu-miR-720	3.13
			MIMAT 0004942	mmu-miR-598	2.93
			MIMAT 0004537	mmu-miR-154*	2.93
			MIMAT 0003202	rno-miR-382*	2.92
			MIMAT 0000648	mmu-miR-10a	2.87
			MIMAT 0004666	mmu-miR-33*	2.82
			MIMAT 0004722	rno-miR-30d*	2.81
			MIMAT 0000136	mmu-miR-125b*	2.61
			MIMAT 0004630	mmu-miR-26b*	2.36
			MIMAT 0000537	mmu-miR-27a	2.22
			MIMAT 0004583	mmu-miR-130b*	2.18
			MIMAT 0000534	mmu-miR-26b	2.10
			MIMAT 0003450	mmu-miR-488	2.04
			MIMAT 0000248	mmu-miR-30e	1.96
			MIMAT 0000137	mmu-miR-126–5p	1.90
			MIMAT 0003199	rno-miR-381	1.80
			MIMAT 0000679	mmu-miR-217	1.80
			MIMAT 0000224	mmu-miR-194	1.78
			MIMAT 0000225	mmu-miR-195	1.72
			MIMAT 0000219	mmu-miR-24	1.68
				#mmu-miR-801	4.62

* miRNA strands that are the complementary strands of the functional single-stranded miRNAs.
\# mmu-miR-801 is no longer present in miRBase database since miR-801 appears to be a fragment of U11 spliceosomal RNA and hence removed from the database.

2.2. Virus challenge

V3000, derived from an infectious cDNA clone of the wild type Trinidad donkey strain subtype IA/B was used for these experiments. Virus challenge was done as described before [21]. Six animals were used for each group. Briefly, mice were anesthetized with 5% isoflurane and 1000 plaque forming units (pfu) of V3000 were injected in the left rear footpad. Control animals were injected with saline.

2.3. Brain isolation

Following the infection with VEEV, animals were sacrificed at 48 and 72 h post infection (p.i) and brains were isolated and immediately snap-freezed over dry ice and stored at −80 °C.

2.4. RNA isolation

Frozen brain section were minced over ice and total RNA was isolated using TriZol reagent (Invitrogen Inc., Carlsbad, CA) according to the manufacturer's protocol. RNA was quantitated using Nanodrop (Thermo Scientific, Wilmington, DE) and the presence and integrity of miRNA in the total RNA samples was analyzed on 6% Tris borate EDTA urea gel (Supplementary Fig. 1).

2.5. Real-time PCR

cDNA pool was synthesized from the total RNA by reverse transcription reaction using a stem-loop multiplex miRNA primer pool as per the manufacturer's protocol (Applied Biosystems Inc., Foster city, CA). The stem-loop multiplex miRNA primer pool specifically binds only to miRNA to generate a corresponding miRNA-cDNA pool. The expression profile of miRNAs in the infected tissues and the uninfected control tissues were measured by quantitative real-time polymerase chain reactions (qPCR) using the Low Density miRNA Taqman array (Applied Biosystems Inc., Foster city, CA) as per manufacturer's protocol. From here onwards these arrays are referred as miRNA array cards. The miRNA array card contains 821 unique rodent miRNAs including six endogenous controls and one negative control. Briefly, 6 μl of total cDNA was mixed with 450 μl Taqman PCR master mix without Uracil N-glycosylase (UNG) (Applied Biosystems Inc., Foster city, CA) and 444 μl nuclease free water. 800 μl of this mix was loaded on the miRNA array cards (Applied Biosystems Inc., Foster city, CA) and the cards were sealed following centrifugation at 2000 rpm for 2 min and qPCR was carried out on Applied Biosystem 7900HT real-time PCR machine.

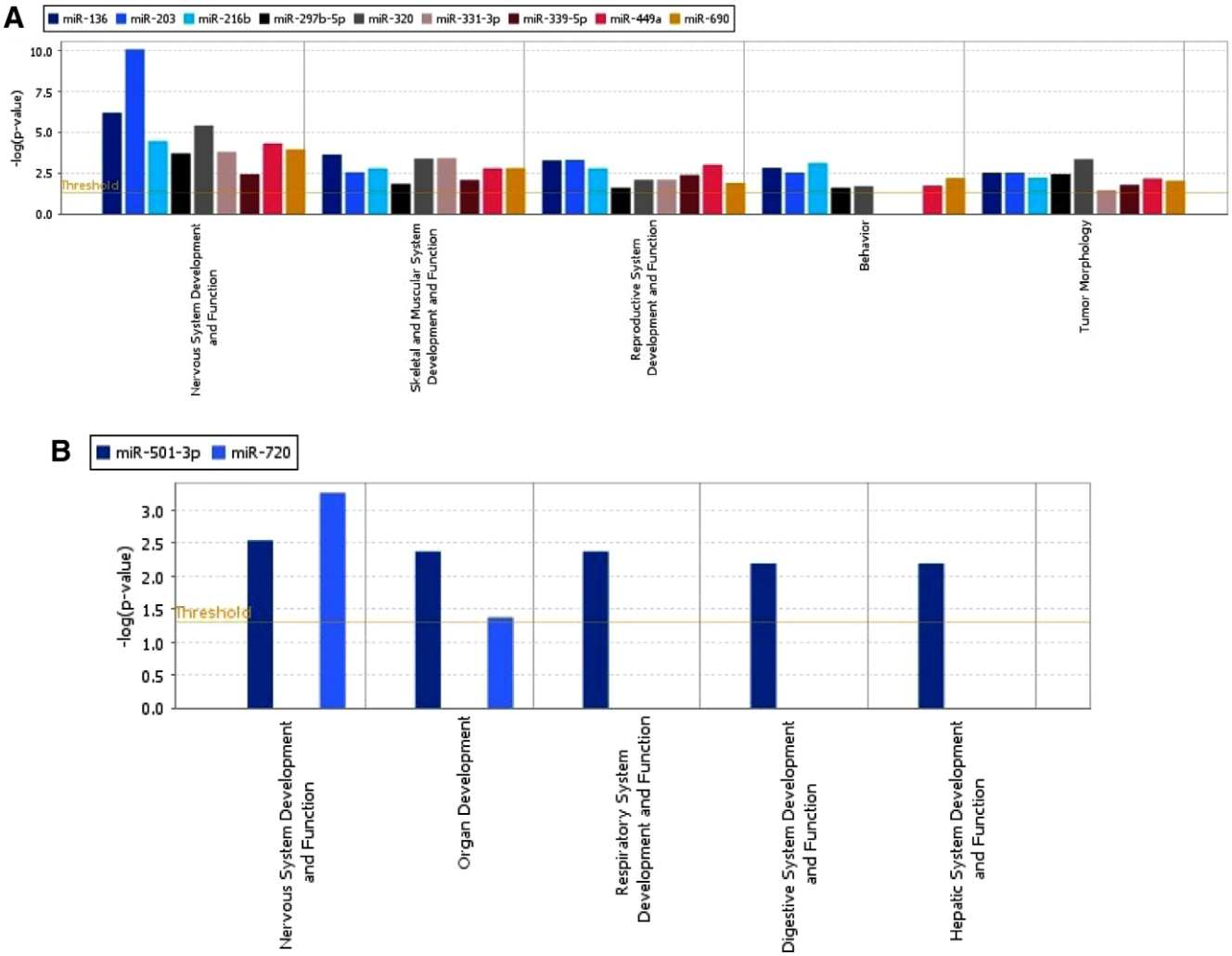

Fig. 1. Biofunctional analysis using modulated miRNAs upon VEEV infection showed nervous system development and function as topmost biofunction both at 48 h (A) and 72 h (B) p.i in physiological systems and development and functions category. The analysis was done based on target prediction by TargetScan and Ingenuity pathway analysis.

2.6. Statistical analysis

qPCR data obtained from the miRNA array cards was analyzed for the modulation of statistically significant miRNAs using Statminer software (Integromics Inc., Philadelphia, PA). Cycle threshold (Ct) values of >35 was considered to be non-specific [28] and miRNAs with raw Ct value of >35 were excluded from the analysis. Detectability threshold of miRNAs in all the samples was set as $\geqslant 4$. A computationally predicted endogenous control calculated using Genorm scoring method was used for normalization. All the six endogenous controls present on the miRNA cards were used for Genorm scoring. A parametric limma test was used to compute the statistically significant miRNAs in infected and control samples. MicroRNAs with a p value score of $\leqslant 0.05$ and fold change of $\geqslant 1.5$ were considered statistically significant.

2.7. Target prediction and pathway analysis

MiRNA target prediction was done by using TargetScan Mouse 5.1. These predicted gene target of miRNAs were then used for network and functional analysis through the use of Ingenuity Pathways Analysis (IPA) (Ingenuity Systems Inc., Redwood City, CA).

3. Results and discussion

3.1. MicroRNA modulation upon VEEV infection

VEEV have been shown to cause severe neurodegeneration in the CNS, which is also considered to be the cause of death following VEEV infection. Modulation of various genes involved in the host cellular and immune regulatory pathways such as apoptosis, antigen presentation and inflammation in the brain following VEEV infection play a major role in neurodegeneration [2,21,22]. VEEV has been shown to appear in the brain at around 36–48 h following footpad infection and rapidly spreads throughout the brain [29]. Therefore, to understand the miRNA changes occurring at or around the time of VEEV appearance and during its active replication in brain, we evaluated miRNAs expression at 48 and 72 h post infection.

Overall, 32 miRNAs were found to be significantly modulated upon VEEV infection at 48 h p.i, out of which 20 were up-regulated and 12 were down-regulated (Table 1a). At 72 h p.i, a total of 36 miRNAs were significantly modulated, however, in contrast to 48 h p.i, 31 of these miRNAs were down-regulated and only five miRNAs were up-regulated (Table 1b). MicroRNAs such as mir-155, mir-27a, rno-mir-381, mir154*, and mir 801 were found to be modulated at both the time points. Since miRNA negatively regulate gene expression, this pattern corroborate with our previously reported gene expression study [21] where, the number of genes up-regulated increased from 48 to 72 h p.i.

3.2. Functional analysis of miRNAs modulated in VEEV infected brain and their target prediction

A biological functional analysis was done for significantly modulated miRNAs at 48 and 72 h p.i. Genes regulated by the miRNAs that were found to be modulated at 48 and 72 h p.i were predicted using TargetScan Mouse 5.1 software. Ingenuity pathway analysis (IPA) was done for these miRNAs using the predicted gene targets to analyze their potential biological function. IPA analysis revealed that 9 (Mir-136, 203, 216b, 297b-5p, 320, 331-3p, 339-5p, 449a, and 690) of the 33 miRNAs that were modulated at 48 h p.i and two (Mir-501-3p and 720) of the 36 miRNAs that were modulated at 72 h p.i were found to play a significant role in nervous system

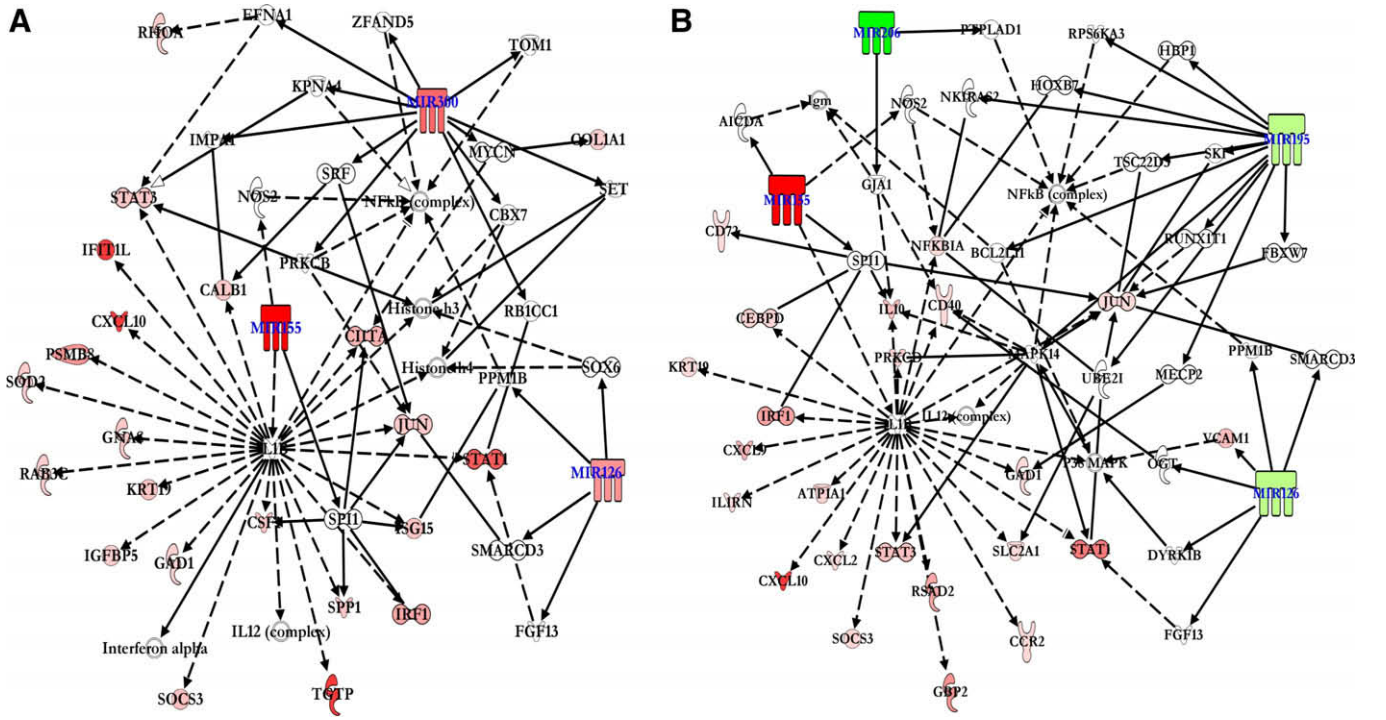

Fig. 2. Prediction of mRNA regulation by miRNAs using IPA; (A) Network analysis with mRNA expression data of topmost network molecules with modulated miRNAs at 48 h p.i with VEEV. (B) Network analysis with mRNA expression data showing topmost network molecules with modulated miRNAs at 72 h p.i with VEEV. Solid lines indicate a direct interaction whereas a broken line indicates an indirect interaction. Green color of the molecules indicates low expression or down regulation whereas red color indicates upregulation. The expression levels indicated here are based on the experimental data of miRNA and mRNA expression analyses (complete details of the genes involved in these networks are included in Supplementary Table 3). (For interpretation of color mentioned in this figure legend the reader is referred to the web version of the article.)

development and function (NSDF) category (Fig. 1). Since VEEV primarily results in the neurodegeneration, these miRNAs may play an important role in neurodegeneration and modulation of gene expression in brain following VEEV infection.

Functional analysis of predicted genes also revealed involvement in various cellular and biological processes which can be broadly classified into disease and disorders, molecular and cellular functions, and physiological systems and development and functions (Supplementary Table 1). As mentioned before, VEEV infection in brain induces apoptosis and inflammation resulting in severe encephalitis, therefore, several of these biological functions such as cell death, immune and neurological disorders and, nervous system development and function are relevant to VEEV induced disease.

We have previously reported a complete gene expression profile of mouse brain during VEEV infection [21]. In this study, we compared the predicted targets of significantly modulated miRNAs with the gene expression profile of VEEV infected mouse brain. This comparison showed that some of the predicted miRNA targets correlate with the mRNA expression profile reported earlier. Some genes that were found to be common can be classified under biological functional categories of antigen presentation, inflammation and, nervous system development and function (Supplementary Table 2). These analyses suggest that miRNAs may regulate the downstream gene expression during VEEV infection either directly or indirectly.

To analyze these direct and indirect miRNA-gene interactions, we performed IPA with mRNA expression data and significantly modulated miRNAs. The miRNAs involved in these networks were mir-155, mir-126, and mir-300 at 48 h p.i and mir-155, mir-126, mir-195, and mir-206 at 72 h p.i. The miRNA-gene networks showed that miR-155 targets IL1β indirectly through intermediate molecules. IL1β then regulates many other downstream genes which were observed to be modulated upon VEEV infection (Fig. 2). These miRNA-gene networks clearly showed that miRNA may indirectly regulate gene expression during VEEV infection through intermediate molecules. Moreover, mir-155 was up-regulated at 48 h p.i and its expression increased by 72 h p.i, whereas miR-126 was up-regulated at 48 h p.i and its expression was down-regulated at 72 h p.i. These differentially regulated miRNAs thus may play an important role in VEEV infection of brain.

To our knowledge, this is the first report on differential regulation of miRNAs in the brains of VEEV infected mice. A profile of miRNA modulation will help to understand the interaction of the host miRNAs and the viral genome. Biological functional and target gene analysis of miRNAs showed that these miRNAs may play an important role in pathways such as cell death or apoptosis, antigen immune and neurological disorders, antigen presentation and inflammation. These findings corroborated with the previous reports from our laboratory which have implicated the above mentioned pathways in VEEV infection of the brain. These results suggest that miRNAs may indeed play an important role in the VEEV infection of brain and improve our present understanding of VEEV pathogenesis.

Studies are in progress to screen these miRNAs and restoring them to the physiological levels either by using miRNA inhibitors or mimics. This will help in designing better antivirals and miRNA based vaccines.

Acknowledgments

These studies were supported by JSTO-CBD/DTRA Contract/Grant/Intergovernmental Project Order/Project # 4.10019_07_US_B, USU intramural funds and cystic fibrosis foundation. Authors would like to acknowledge Dr. Thazepadath Sreevalsan for critically reviewing the manuscript. Opinions expressed are those of the authors and should not be construed as official or reflecting the views of Uniformed Services University of Health Sciences, Bethesda, MD.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.03.091.

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