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Research Article

Screening and validation of differentially expressed microRNAs and target genes in hypertensive mice induced by cytomegalovirus infection

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Introduction: Multiple studies have suggested an association between cytomegalovirus (CMV) infection and essential hypertension (EH). MicroRNAs (miRNAs) play a critical role in the development of EH by regulating the expression of specific target genes. However, little is known about the role of miRNAs in CMV-induced EH. In the present study, we compared the miRNA expression profiles of samples from normal and murine cytomegalovirus (MCMV)-infected C57BL/6 mice using high-throughput sequencing analysis. **Methods:** We collected the thoracic aorta, heart tissues, and peripheral blood from 20 normal mice and 20 MCMV-infected mice. We identified differentially expressed miRNAs in the peripheral blood samples and predicted their target genes using bioinformatics tools. We then experimentally validated them using quantitative reverse transcription polymerase chain reaction (qRT-PCR) and the target genes with double luciferase reporter gene assay. **Results:** We found 118 differentially expressed miRNAs, among which 9 miRNAs were identified as potential MCMV infection-induced hypertension regulators. We then validated the expression of two candidate miRNAs, mmu-miR-1929-3p and mcmv-miR-m01-4-5p, using qRT-PCR. Furthermore, the dual-luciferase reporter gene assay revealed that the 3′-untranslated region (UTR) of endothelin A receptor (*Ednra*) messenger RNA (mRNA) contained a binding site for mmu-miR-1929-3p. Collectively, our data suggest that MCMV infection can raise the blood pressure and reduce mmu-miR-1929-3p expression in C57BL/6 mice. Moreover, we found that mmu-miR-1929-3p targets the 3′-UTR of the *Ednra* mRNA. **Conclusion:** This novel regulatory axis could aid the development of new approaches for the clinical prevention and control of EH.

Introduction

Hypertension is one of the most common chronic diseases, affecting more than 1 billion people worldwide [1]. Chronically elevated blood pressure is a common cause of chronic renal failure, myocardial infarction, stroke, heart failure, and death [2]. Approximately 9095% of all the hypertension cases are classified as essential hypertension (EH) [3,4]. Although the prevention and treatment of hypertension have made great progress in the past decade, the etiology and pathogenesis of hypertension have not been fully elucidated yet. A recent study showed that hypertension is not only related to the interaction between genetic and environmental factors but also with viral infections and non-coding RNAs, such as microRNA (miRNA) [5,6]. Another population association study showed that the human cytomegalovirus (HCMV) and miRNAs encoded by HCMV

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are correlated to hypertension [7]. HCMV is a member of the cytomegalovirus (CMV) genus, and the prevalence of HCMV infection has been estimated in the 40–100% range [8]. Moreover, a European study has shown an independent correlation between HCMV infection and blood pressure [9]. However, at present, the relationship between HCMV infection and hypertension remains unclear. Some studies suggested that the mechanism of CMV infection-induced hypertension may be correlated to oxidative stress and endothelial dysfunction [10]. Alternatively, HCMV could promote hypertension through developmental and metabolic disorders to vascular smooth muscle cells [11]. Due to the high prevalence of hypertension and widespread HCMV infection, it is particularly important to clarify the role of HCMV in hypertension pathogenesis.

HCMV is a ubiquitous β herpes virus that has the largest known genome among human herpesviruses (230 kb) encoding a variety of factors that modulate the host immune response [12,13]. HCMV infection can cause latent infection and toxic lytic infection, depending on the host's response to the infection. In latent infection, the virus does not replicate, although the viral genome and proteins remain in the host cells. When the immune system is normal, HCMV infection will not cause any uncomfortable symptoms [14]. However, when the host defenses are impaired—in fetuses, infants, young children, and people with low immunity—the virus immediately gets activated, causing a toxic lytic infection which can lead to extensive damage [15,16]. A recent study has shown that HCMV infection is associated with increased systolic blood pressure (SBP) in healthy old people [17]. The murine cytomegalovirus (MCMV) has been also associated with an increase in blood pressure. Interestingly, MCMV infection increased the expression of renin in renal cells, suggesting that the renin-mediated increase in the blood volume may contribute to hypertension [18]. The molecular mechanisms underlying the correlation between HCMV infection and elevated blood pressure have not been elucidated. We hypothesized that miRNAs, including virus-encoded miRNA, could be involved in HCMV-mediated hypertension.

MiRNAs are highly conserved, single-stranded, non-coding small RNAs (1925 nucleotides in length) that negatively regulate the expression of their target genes at the post-transcriptional level, by directly binding to the 3′-untranslated region (UTR) or 5′-UTR of their target messenger RNAs (mRNAs) [19,20]. Previous studies have shown that several miRNAs are involved in the development of primary hypertension [21,22]. Inhibition of miRNA-126 leads to bleeding, suggesting that miRNA-126 is involved in the maintenance of vascular endothelium integrity [21]. Moreover, miRNA-155 is up-regulated in the plasma of hypertensive subjects, where it inhibits the angiotensin II type I receptor [22]. Notably, Cai et al. have shown that the HCMV-encoded miRNA hcmv-miR-UL112 was up-regulated in hypertensive patients, and the blood pressure of the mice significantly increased 912 days after adenoviral transfection of hcmv-miR-UL112 [7]. It has been reported that HCMV can encode at least 26 different mature miRNAs (www.mirbase.org) [23] and can change the miRNA expression profile in the host [19]. Given that hypertension is regulated by a complex miRNA-gene network, it is implausible that hcmv-miR-UL112 is the only miRNA involved in HCMV-mediated regulation of hypertension. Thus, further efforts are needed to identify novel miRNAs involved in HCMV-mediated hypertension.

Our previous studies found that HCMV infection is associated with EH in patients of Kazakh and Han ethnicity in Xinjiang, and is associated with EH progression and target organ damage in patients of Han ethnicity, further supporting the correlation between HCMV and hypertension [24,25]. We thus hypothesized that MCMV may be correlated to hypertension in mice through similar miRNA-induced mechanisms. Therefore, in the present study, we screened differentially expressed miRNAs in mice infected with MCMV, identified the miRNAs potentially involved in hypertension, and experimentally validated our findings to elucidate the role of MCMV in the pathogenesis of hypertension.

Materials and methods

Mice

All experiments in the present study were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal model was established as previously reported. A total of 40 C57BL/6 mice (4 weeks of age) were provided by the Institutional Animal Research Committee of Shihezi University School of Medicine. All mice were housed under pathogen-free conditions in individually ventilated cages, placed in a temperature-controlled environment with a 12-h day/12-h night cycle. Mice received food and water *ad libitum*, and were kept at the Shihezi University School of Medicine animal facility. After adaptive feeding for 1 week, the mice were divided into the following two groups: the MCMV infection group (1×10^5 pfu/ml, 1 ml, intraperitoneal injection) and the control group (equivalent volume of saline solution, intraperitoneal

injection). All animal experiments were conducted at the Medical College of Shihezi University and performed according to the protocols approved by the Animal Experimental Ethical Inspection of First Affiliated Hospital, Shihezi University School of Medicine (approval number: A2018-038-01).

Virus and cell culture

MCMV was maintained in NIH 3T3 cells. NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum and 1% antibiotic solution (100 U/ml of penicillin and 100 μg/ml of streptomycin). The virus was collected from the supernatant of infected NIH 3T3 (infection rate: 100%) by centrifugation (15000×***g*** for 20 min) and then further purified with ultracentrifugation (20000 rpm for 1 h) through a 15% sucrose cushion in a virus standard buffer (VSB, 50 mM Tris/HCl pH 7.8, 12 mM KCl, and 5 mM EDTA). The virus pellet was resuspended in 0.5–1.0 ml of VSB, aliquoted, and stored at −80°C. Viral titers were determined with standard plaque assay in NIH 3T3 cells. Both the MCMV Smith strain and NIH 3T3 cells were donated by the Department of Neurovirology, Wuhan Institute of Virology (Hubei, China).

Blood pressure measurement

The mice were anesthetized using sodium pentobarbital (P3761, Sigma–Aldrich, St. Louis, MO, U.S.A.) through intraperitoneal injection (30 mg/kg), after which they were fixed on the operating table. An incision was made along the midline of the neck and the neck tissues were separated to expose the common carotid artery. The vagus nerves surrounding the carotid artery were separated carefully. A silicone tube (internal diameter: 0.3 mm) was implanted near the artery (diameter: ∼0.5 mm) and a cannula was ligated using 6-0 silk threads to monitor the blood pressure. Then, the carotid artery was put back into position. Finally, the mice were killed by decapitation under pentobarbital anesthesia.

RNA isolation and sequencing

The total RNA was isolated from thoracic aorta samples using miRNeasy Mini Kit (Qiagen, Germany, 217004). The total RNA isolation from peripheral blood monocytes for sequencing and validation analysis was performed using the miRcute miRNA isolation kit with the enrichment for small RNAs, following the manufacturer's instructions (TIANGEN, DP501). RNA quality was measured using the Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, U.S.A.). Paired-end libraries were synthesized by using the QIAseq miRNA Library Kit (Qiagen, Germany) following the QIAseq miRNA Library Kit Guide. The products were then purified and enriched by PCR to create the final cDNA library. Purified libraries were quantified by Qubit® 2.0 Fluorometer (Life Technologies, U.S.A.) and validated using the Agilent 2100 bioanalyzer (Agilent Technologies, U.S.A.) to confirm the insert size and calculate the library concentration. A cluster was generated by cBot with the library diluted to 10 pM and then sequenced on the Illumina Hiseq Xten (Illumina, U.S.A.).

The library construction and sequencing were performed by Sinotech Genomics Co., Ltd (Shanghai, China).

Small RNA-seq data analysis and target prediction

After removing the adoptor sequences and the contaminated reads, the clean reads were processed for letter anlysis. Sequence files (fastq) were mapped to the reference genome (GRCm38) using Bowtie. Small RNA was classified by Unitas software. The miRNA abundance was expressed as counts of exon model per million mapped reads. Differential expression analysis for mRNA was performed using DESeq software. A minimum of two-fold difference between groups and a P-value ≤ 0.05 were used as criteria for the identification of differentially expressed miRNA genes. We then performed gene ontology (GO) enrichment analysis to annotate gene functions, according to three categories: biological process (BP), cellular component (CC), and molecular function (MF). Next, we performed an enrichment analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) to systematically analyze, annotate, and visualize gene functions. Potential targets of the differentially expressed miRNAs were predicted using miRanda (http://www.microrna.org/microrna/home.do), miRDB (http://www.mirdb.org/), and TargetScan (http://www.targetscan.org/). Only the genes identified by all three software were selected as target genes.

Quantitative reverse transcription polymerase chain reaction

The integrity and purity of total RNA samples were evaluated using PCR and agarose gel electrophoresis. Total RNA samples of suitable quality were reverse transcribed into cDNA using the miRcute Plus miRNA First-Strand cDNA kit (TIANGEN, KR211). The qPCR conditions were the following (Supplementary Table S1): 95°C for 15 min; 5 cycles at 94°C for 20 s, 65°C for 30 s, and 72°C for 34 s; 45 cycles at 94°C for 20 s and 60°C for 34 s. All primers were

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synthesized by Shanghai Living Creature (China) (Supplementary Table S2). Gene expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method [26].

Plasmid construction and dual-luciferase activity assay

The full-length 3′-UTR of the endothelin A receptor (*Ednra*) gene was amplified by PCR and cloned downstream the pmirGLO Luciferase (Shanghai GenePharma Co., Ltd, China) multiple cloning site by digestion with the SpeI and HindIII restriction enzymes. The resulting construct was called pEdnra-wild-type (Wt). The miR-1929-3p binding site on the Ednra mRNA was mutated and cloned in the pmirGLO Luciferase plasmid to create the pEdnra-Mutant (Mut) vector. The Wt and Mut vectors were each transfected with and without miR-1929-3p into the HEK293 cells. Additionally, a dual-luciferase reporter gene assay was performed using the dual-luciferase reporter assay system (Shanghai GenePharma Co., Ltd, China).

The mmu-miR-1929-3p mimics, corresponding negative control and siRNA targeting Ednra were synthesized from GenePharma (Shanghai GenePharma Co., Ltd, China). Ednra sense 5′-CCTGGCAGGAAACAATGTCAAAGT GGCCAAATGAGCTGATTGTGTTAAGTGAGATGTAGTTACACC-3′ antisense 5′-TCGAGGTGTAACTACATC TCACTTAACACAATCAGCTCATTTGGCCACTTTGACATTGTTTCCTGCCAGGAGCT-3′; si-Ednra sense 5′-CGCAGAACAUCAAAGAAGATT-3′, antisense 5′-UCUUCUUUGAUGUUCUGCGTT-3′; negative control siRNA sense 5′-UUCUCCGAACGUGUCACGUTT-3′, antisense 5′-ACGUGACACGUUCGGAGAATT-3′.

Statistical analysis

The SPSS 21.0 statistical software (IBM Corp., Armonk, NY, U.S.A.) was used for statistical analysis. Data were presented throughout the study as mean ± standard deviation. The *t* test was used for comparing the differences between the two groups. A P-value <0.05 indicated a statistically significant difference.

Results

MCMV infection raises blood pressure in C57BL/6 mice

To confirm the MCMV infection, we measured with quantitative reverse transcription polymerase chain reaction (qRT-PCR) the MCMV IE1 gene expression in the thoracic aorta of mice infected with the virus (MCMV IE Forward primer: 5′-ATC AAT CAG CCA TCA ACT CTG CTA CCA CAC-3′; MCMV IE Reverse primer: 5′-ATG GTG AAG CTA TCA AAG ATG TGC ATC TCA-3′). The results confirmed that the infection was successful (Figure 1A). Then, we measured the blood pressure and observed that it was significantly higher in mice infected with MCMV after 10 weeks ($P<0.01$; Figure 1B–D). However, the heart rate and weight were not significantly different between the two groups (Figure 1E,F).

Differential miRNA expression upon MCMV infection

We compared miRNA expression profiles in the peripheral blood of mice infected with MCMV with uninfected mice using a high-throughput sequencing approach. We found 118 significantly differentially expressed miRNAs, of which 91 were up-regulated and 27 were down-regulated (absolute value fold-change ≥ 1, $P<0.05$; Figure 2A and Supplementary Table S3). Scatter plots and volcano plots of the standardized expression data showed the miRNA expression trend between the two groups (Figure 2B,C). The GO function enrichment analysis and the KEGG enrichment analysis of the top 20 target genes regulated by differentially expressed miRNAs are shown in Figure 2D (Supplementary Table S8) and 2E (Supplementary Table S9), respectively.

Screening of hypertension-related miRNAs and target genes prediction

We further analyzed the small RNA-seq data to identify differentially expressed miRNAs involved in hypertension. After removing the unknown and weak signal samples from each group, we performed cluster analysis and found a cluster containing 36 up-regulated and 11 down-regulated miRNAs (Figure 3A and Supplementary Table S4). Then, we predicted the miRNA target genes using bioinformatics methods. We found 90 hypertension-related target genes that were potentially targeted by 32 differentially regulated miRNAs, among which 26 were up-regulated and 6 down-regulated (Figure 3B) (Supplementary Table S5). The GO function and the KEGG pathway enrichment analysis of the top 30 hypertension-related target genes are shown in Figure 3C,D and Supplementary Table S6).

To further identify miRNAs closely related to hypertension, we performed a ranking analysis between genes correlated with hypertension and high blood pressure with 29 miRNAs, 2 of them of CMV origin (Table 1). Then, we built an miRNA–mRNA regulatory interaction network (Figure 3E and Supplementary Table S7) and selected 9 candidate miRNAs for experimental validation of their role in MCMV-induced hypertension (Table 2).

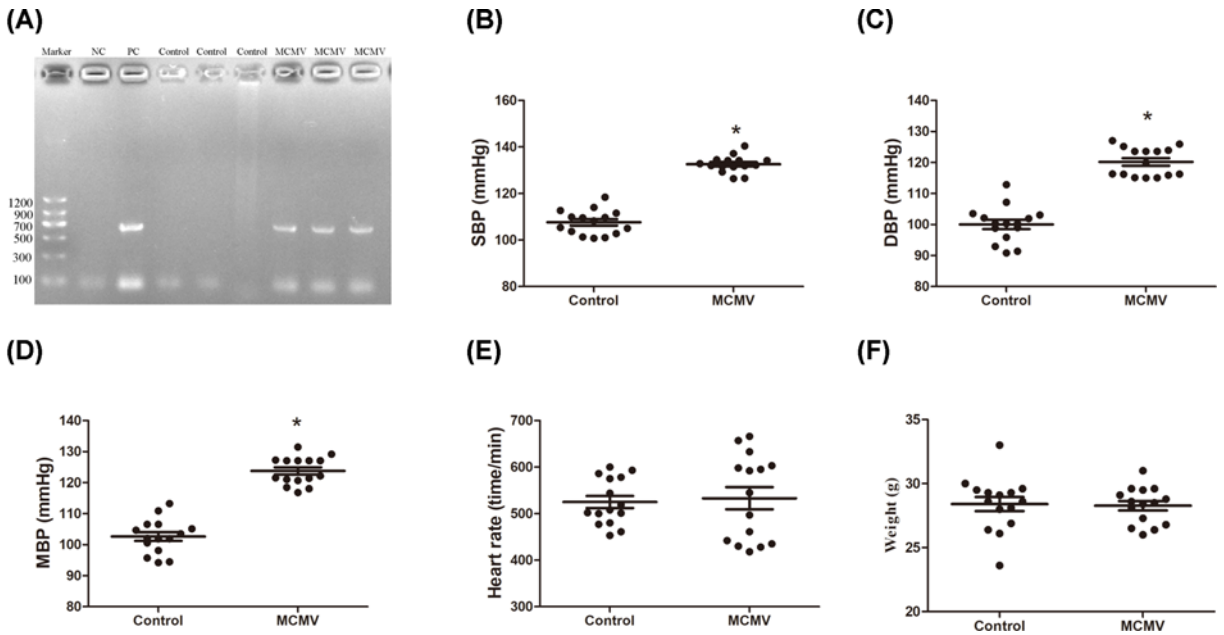

Figure 1. The MCMV infection raised blood pressure in C57BL/6 mice

(**A**) PCR analysis of MCMV IE gene in the thoracic aorta of male C57BL/6 mice. NC, negative control group. PC, positive control group. M, marker. 1∼4, thoracic aorta of different mice infected with MCMV for 21 days. (**B**) SBP. (**C**) Diastolic blood pressure. (**D**) Mean arterial pressure. (**E**) Heart rate. (**F**) Weight. The data are expressed as the means ± SEM, (n=15), *P<0.05 vs. the control group.

Table 1 Interaction analysis of MCMV-induced hypertension-related differential expression mRNA target genes and GCBI (top 20 diseases)

Up-regulated miRNAs	Down-regulated miRNAs
mmu-miR-211-5p	mmu-miR-328-3p
mmu-miR-379-5p	mmu-miR-486a-3p
mmu-miR-204-5p	mmu-miR-1929-3p
mmu-miR-183-5p	
mmu-miR-143-3p	
mmu-miR-133b-3p	
mmu-miR-182-5p	
mmu-miR-411-5p	
mmu-miR-1a-3p	
mmu-miR-203-3p	
mmu-miR-429-3p	
mmu-miR-3473c	
mmu-miR-145a-5p	
mmu-miR-145a-3p	
mmu-miR-200b-3p	
mmu-miR-709	
mmu-miR-206-3p	
mmu-miR-9-5p	
mmu-miR-148a-3p	
mmu-miR-200a-3p	
mmu-miR-133a-3p	
mmu-let-7f-5p	
mmu-let-7i-5p	
mmu-let-7a-5p	
mcmv-miR-m01-4-5p	
mcmv-miR-m88-1-3p	

Abbreviation: GCBI, Gene-cloud of Biotechnology Information.

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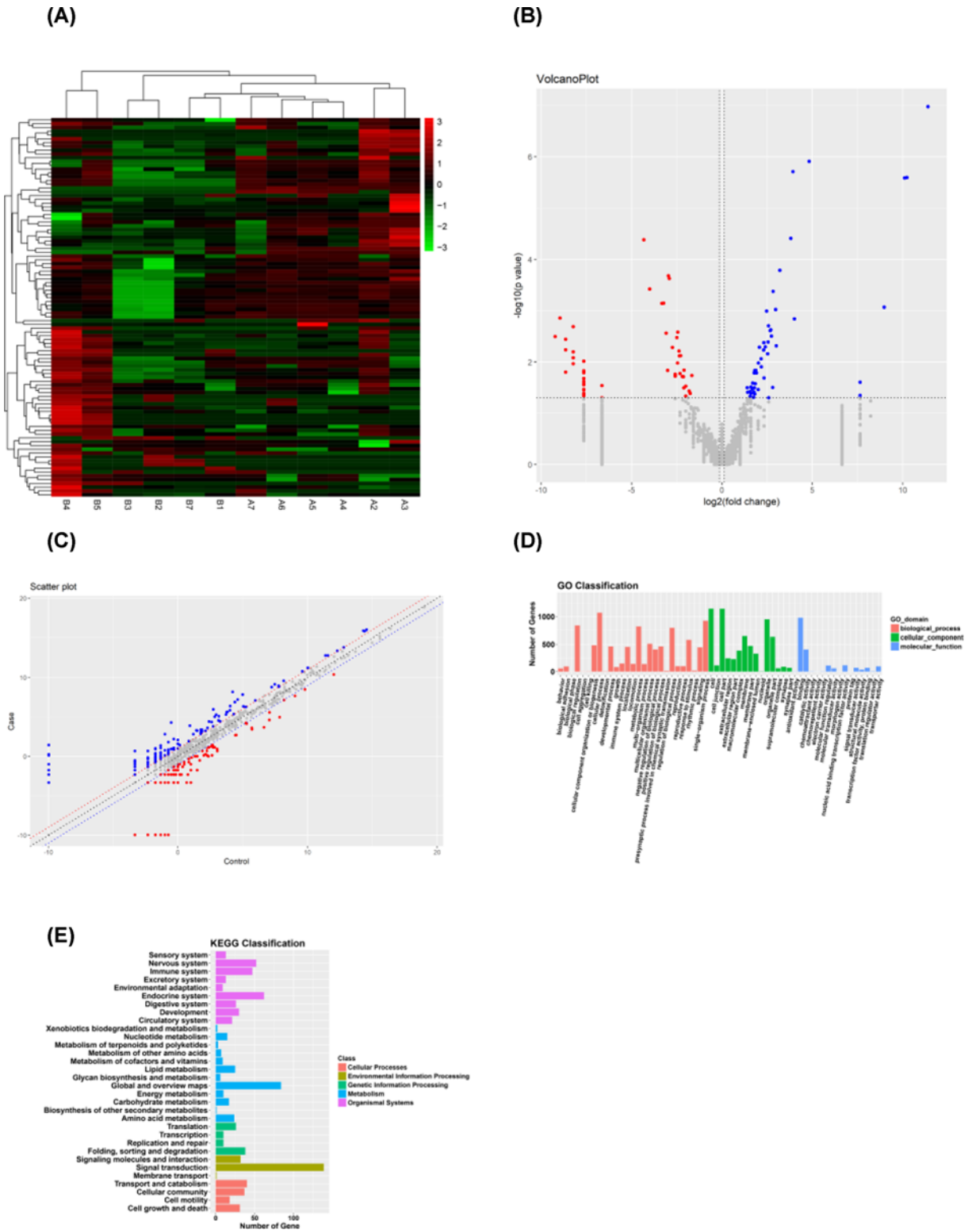

Figure 2. MiRNA expression changes by MCMV infection and bioinformatics analysis

x-axis: Sample.ID. the gray lines represent a log2 difference of 1 in the 2C. (**A**) Heat map of all 118 significantly changed miRNAs in the peripheral blood identified by sequencing (n=6). A2∼A7: MCMV group; B1∼B7: Control group. Red (up) and green (down) indicate relative fold-changes for each comparison. (**B**) Scatter plot of mean expression between MCMV group and control group. (**C**) Volcano map of differentially expressed miRNAs. (**D**) Top 20 genes for GO function enrichment analysis of target genes regulated by differential miRNAs. (**E**) KEGG Pathway enrichment analysis of hypertension-related target genes (top 20).

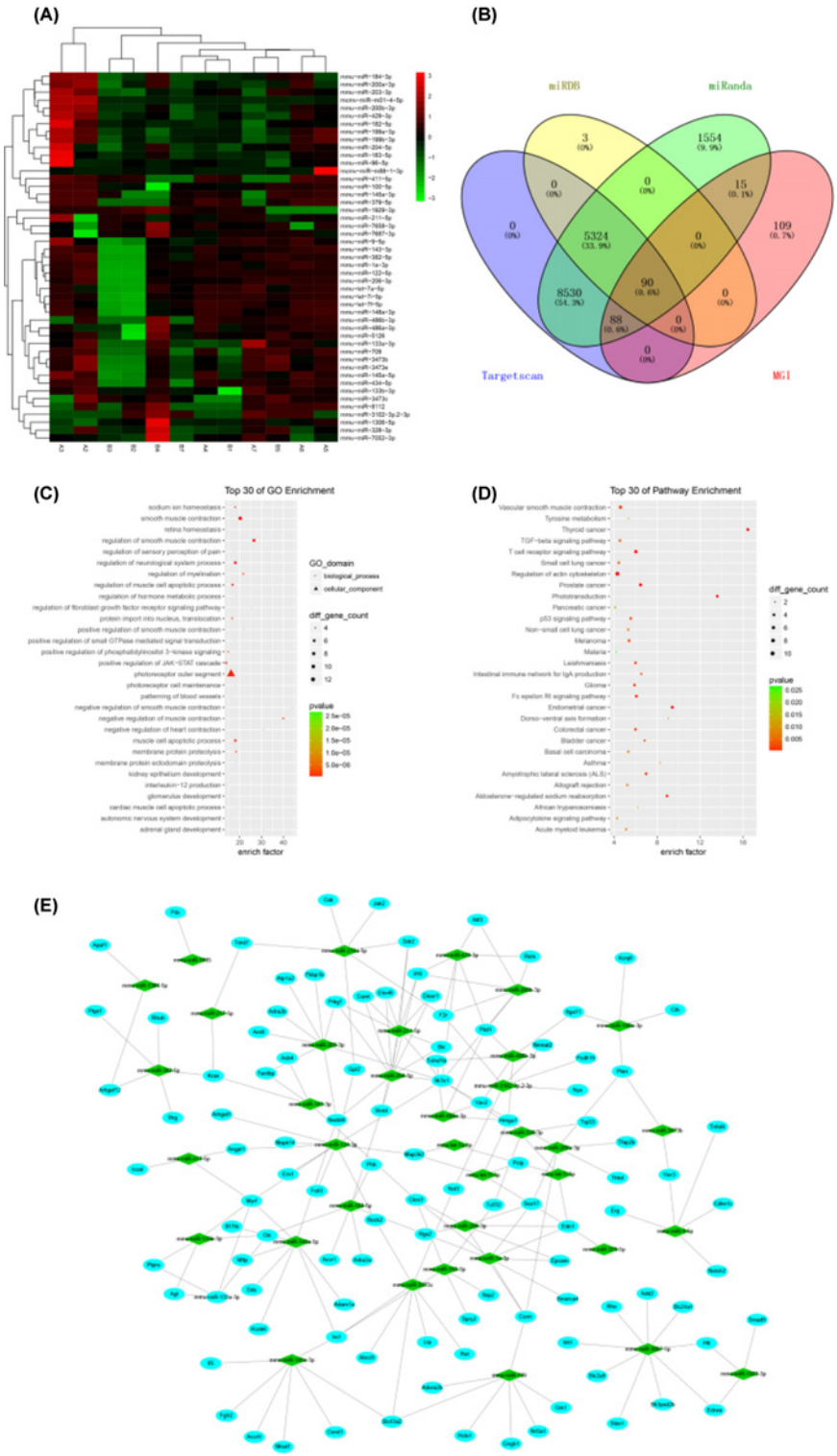

Figure 3. Screening of hypertension-related miRNAs and prediction of target genes

x-axis: Sample.ID. (**A**) Heat map of all 47 significantly changed miRNAs in the peripheral blood identified by sequencing (n=6). A2∼A7: MCMV group; B1∼B7: Control group. Red (up) and green (down) indicate relative fold-changes for each comparison. (**B**) Venn diagrams of target genes and mouse hypertension-related genes predicted by three databases of differentially expressed miRNAs. (**C**) Top 30 genes for GO function enrichment analysis of hypertension-related target genes regulated by differentially expressed miRNAs. (**D**) KEGG Pathway enrichment analysis of hypertension-related target genes (top 30). (**E**) MCMV-induced hypertension-related differential expression of miRNAs and target gene network regulation.

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Table 2 Interaction analysis of MCMV-induced hypertension-related mRNA target genes of differentially expressed miRNAs and GCBI

Up-regulated miRNAs	Target genes associated with hypertension	Down-regulated miRNAs	Target genes associated with hypertension
mmu-miR-133b-3p	*Agt*	mmu-miR-328-3p	*Nr3c1*, *Tcf7l2*
mmu-miR-143-3p	*Asb4*	mmu-miR-1929-3p	*Ednra*, *Smad9*
mmu-miR-182-5p	*Adra2a*, *Rgs2*		
mmu-miR-204-5p	*Nr3c1*, *Prkg1*, *Nedd4l*		
mmu-miR-211-5p	*Nr3c1*, *Prkg1*		
mcmv-miR-m01-4-5p	*Slc12a6*		
mcmv-miR-m88-1-3p	*KLHL3*		

Abbreviations: Adra2a, adrenergic receptor, α 2a; Agt, angiotensinogen (serpin peptidase inhibitor, clade A, member 8); Asb4, ankyrin repeat and SOCS box-containing 4; *Ednra*, endothelin receptor type A; GCBI, Gene-cloud of Biotechnology Information; KLHL3, Kelch-like 3; Nedd4l, neural precursor cell expressed, developmentally down-regulated gene 4-like; Nr3c1, nuclear receptor subfamily 3, group C, member 1; Prkg1, protein kinase, cGMP-dependent, type I; Rgs2, regulator of G-protein signaling 2; Slc12a6, solute carrier family 12 (potassium/chloride transporters), member 6; Smad9, SMAD family member 9; Tcf7l2, transcription factor 7 like 2, T-cell specific, HMG box.

Validation of miRNA differential expression with qRT-PCR

qRT-PCR was used to validate the expression levels of the nine differentially expressed miRNAs in independent samples from different tissues (n=10). We isolated monocytes by performing Ficoll density gradient centrifugation and found that in peripheral blood monocytes of mice infected with MCMV [27], mcmv-miR-m01-4-5p increased, while mmu-miR-133b-3p, mmu-miR-1929-3p, and mmu-miR-204-5p decreased (Figure 4A). In the thoracic aorta, mcmv-miR-m01-4-5p increased and mmu-miR-1929-3p decreased (Figure 4B). In the cardiac tissue mcmv-miR-m01-4-5p increased, while mcmv-miR-m88-1-3p, mmu-miR-1929-3p, mmu-miR-328-3p, and mmu-miR-182-5p decreased (Figure 4C). Thus, mmu-miR-1929-3p was the only host-derived miRNA consistently down-regulated in all three tissues. Moreover, we observed that the expression levels of mmu-miR-1929-3p in the peripheral blood monocytes were significantly higher than those in the thoracic aorta and cardiac tissue (Figure 4D). At the same time, in our experimental validation using MCMV to infect NIH3T3 cells *in vitro*, we still found that MCMV infection is able to down-regulate the expression of mmu-miR-1929-3p in infected cells (Supplementary Figure S1B).

Ednra is a target of mmu-miR-1929-3p

Using a bioinformatics approach, we found that endothelin receptor type A (*Ednra*) could be a potential target of mmu-miR-1929-3p (Figure 5A). Dual-luciferase reporter assay showed that the luciferase activity significantly dropped when we transfected the mednra-miR1929-WT vector ($P<0.05$), while no significant difference was observed upon transfecting the mednra-miR1929-MUT vector or an NC mimic ($P>0.05$) (Figure 5B). At the same time, we found that in vascular tissues, the expression of *Ednra* mRNA in the MCMV infection group was higher than that in the control group (Supplementary Figure S1A). Thus, we experimentally validated that *Ednra* is a target of mmu-miR-1929-3p.

Discussion

In the present study, we observed that MCMV infection caused the differential expression of various miRNA, originating both from the host and the virus, which could play a role in the pathogenesis of hypertension. In particular, we found that the MCMV-encoded mcmv-miR-m01-4-5p miRNA increased, while the host-encoded mmu-miR-1929-3p miRNA decreased. However, as the target genes regulated by mcmv-miR-m01-4-5p did not rank among the top ten hypertension-related genes in the bioinformatic analysis, we focused on mmu-miR-1929-3p for the subsequent analysis. CMV infection is usually latent, although it undergoes periodic reactivation events, by bypassing the host immune response [28]. Although HCMV infection is usually asymptomatic, congenital HCMV infection can cause severe birth defects, including hearing loss, cognitive impairment, and microcephaly [16,29]. As MCMV is a natural mouse pathogen, it is not necessary to manipulate the host. Thus, the study of MCMV provides a unique model for studying *in vivo* the biological mechanism of viruses of the CMV genus. MCMV has been extensively used in cardiovascular studies [30]. Much of the information derived from studies using this model has been translated into clinical practice [31]. A previous study has shown that CMV infection causes an increase in arterial blood pressure [18]. Similarly, we reported that CMV infection may be correlated to hypertension in patients belonging to two

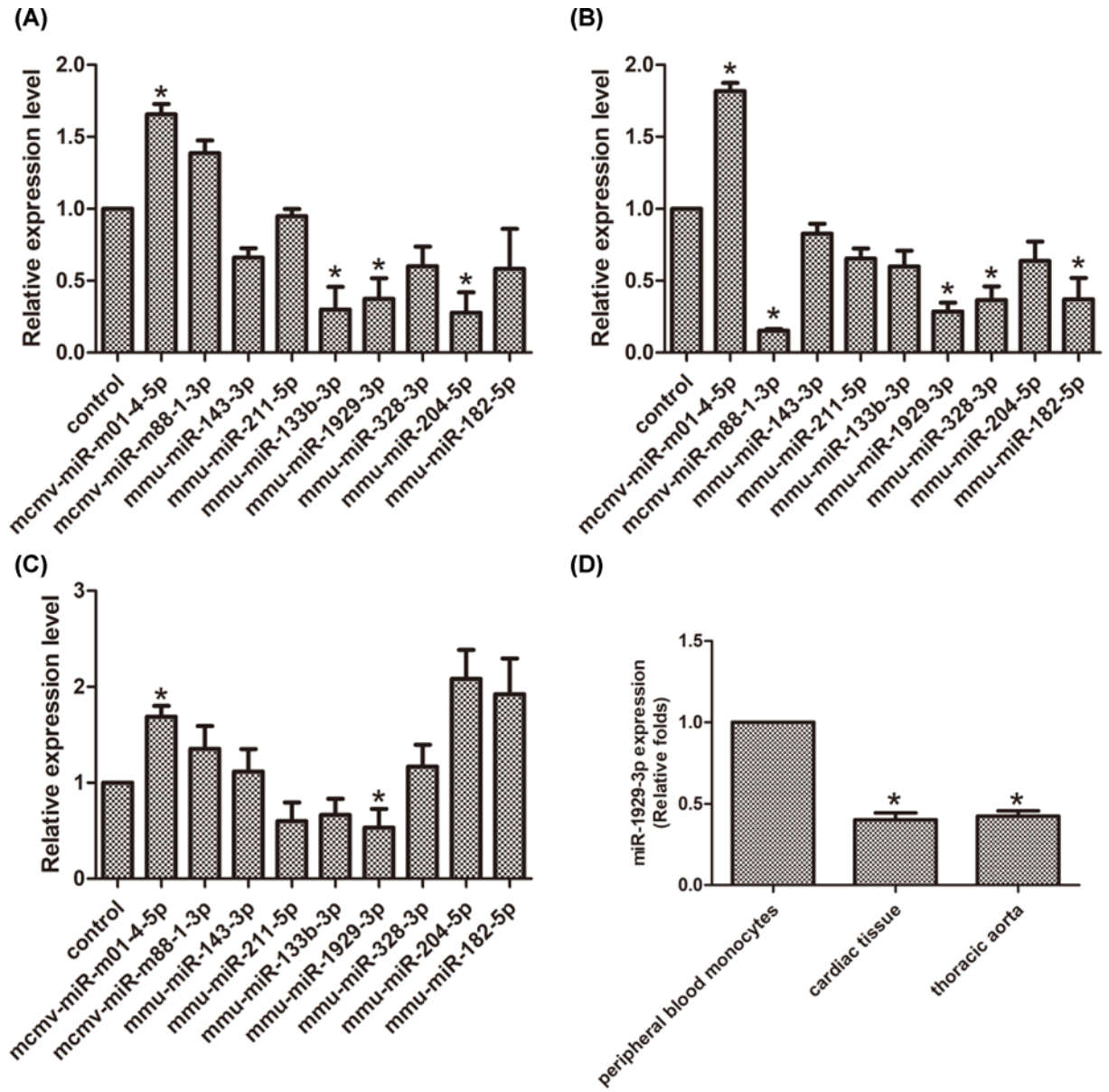

Figure 4. The value of each group of differentially expressed miRNA in MCMV-infected C57BL/6 mice

Control: value observed with uninfected control. (**A**–**C**) Differential expression of miRNA in peripheral blood monocytes, cardiac tissue and the thoracic aorta by RT-qPCR. The value at each group was normalized to the value observed with control, which was set to 1. (**D**) mmu-miR-1929-3p expression in peripheral blood monocytes, cardiac tissue and the thoracic aorta by RT-qPCR. The value at each group was normalized to the value observed with peripheral blood monocytes, which was set to 1. The data are expressed as the means ± SEM, (n=15), *P<0.05 vs. the control group or peripheral blood monocytes group.

different ethnicities living in the Xinjiang region [32]. In this study, we infected C57BL/6 mice with MCMV to establish a model presenting elevated blood pressure. Our results confirmed that, after 10 weeks of infection, the blood pressure is significantly higher in infected mice than that in the uninfected mice.

It has been widely recognized that miRNAs play an important role in regulating cardiovascular functions [33,34]. Patients with coronary heart disease are characterized by a high serum level of miR-486, miR-92a, and miR-122 [35]. Moreover, the level of circulating miR-126 is increased in patients with acute myocardial infarction and angina [36], while plasma miR-126 levels are down-regulated in patients with diabetes [37], heart failure [38], and cancer [39]. Recently, miR-126 has been identified as an effective biomarker for endothelial cell detection and purification [40]. Many studies reported a functional role for miRNAs in hypertension. According to *in vitro* studies, CMV-encoded

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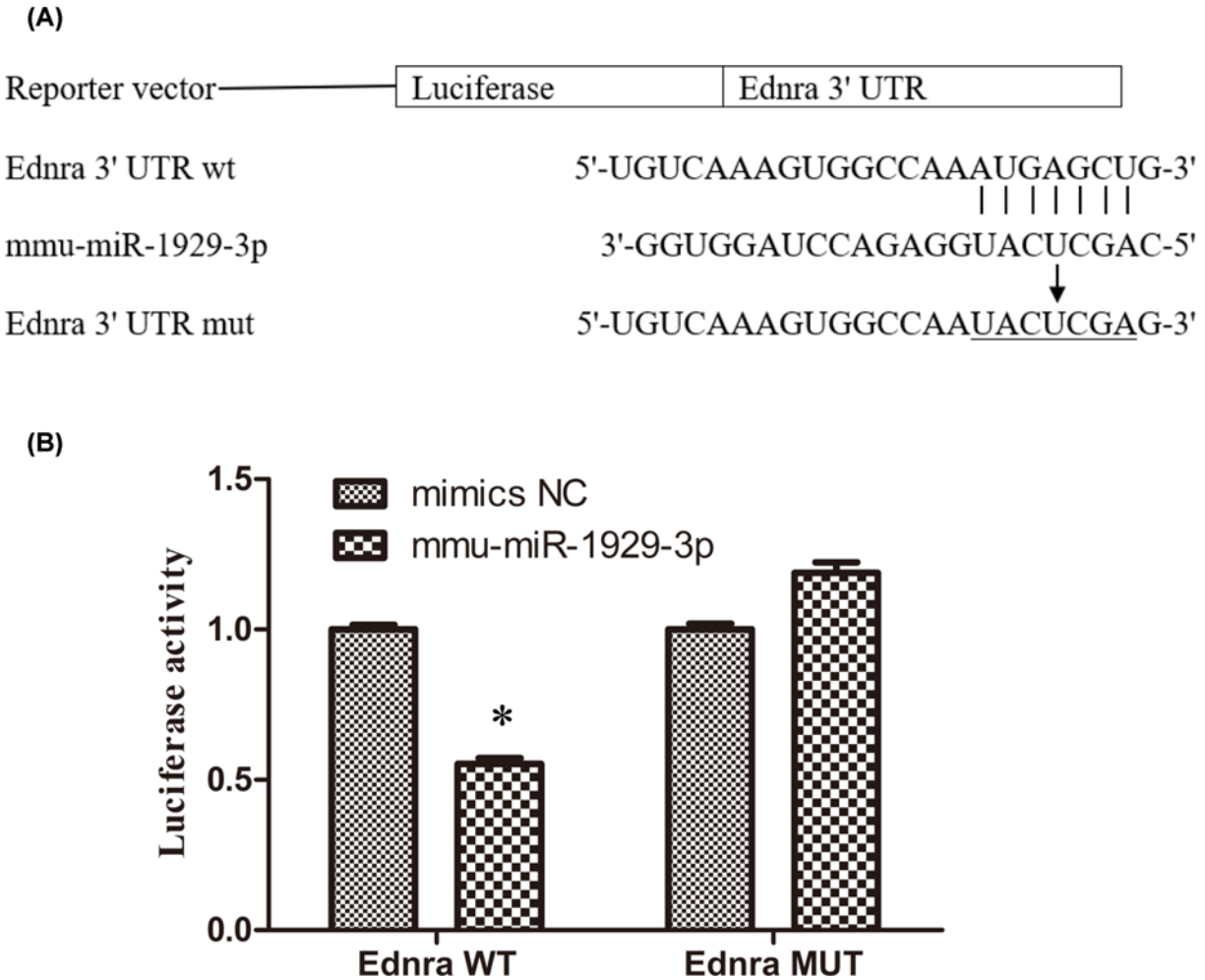

Figure 5. *Ednra* is determined to be a target of mmu-miR-1929-3p

NC, negative control. (**A**) Sequences of the 3′-UTR of the *Ednra* mRNA binding with mmu-miR-1929-3p. *Ednra*, endothelin receptor type A. (**B**) Luciferase activity in the Ednra-WT and Ednra-MUT group. *$P<0.05$ vs. the mimics NC group.

miRNAs play an important regulatory role in viral replication, but the association between host-encoded miRNAs, HCMV-encoded miRNAs, and hypertension remain unclear. Here, we identified 118 differentially expressed miRNAs. Among them, the expression of two miRNA encoded by MCMV was up-regulated. These results suggest that both MCMV- and host-encoded miRNA may be involved in the insurgence of MCMV-mediated hypertension.

MiRNAs are known to be effective regulators of gene expression, by binding to target mRNA, blocking their translation by degrading the mRNA or inhibiting the translation process [34]. ACE inhibitors and angiotensin 1 receptor blockers have been reported to partially reverse vascular dysfunction and normalize gene expression in miR-143/145-deleted mice [41]. Marques et al. demonstrated that miR-181a-5p binds to the 3′-UTR of the renin mRNA to reduce its gene expression. In hypertensive individuals, high expression of renin mRNA is accompanied by a decreased expression of miR-181a in the kidney [42]. In rats with hypertension complicated with heart failure, miR-16, miR-20b, miR-93, miR-106b, miR-223, and miR-423-5p circulating levels increases significantly [43]. By inhibiting miR-208a, the circulating levels of these miRNA decrease, suggesting that plasma miRNA changes can be used as biomarkers of disease progression and manipulated to treat hypertension in the context of heart disease [43]. In this study, we validated with qRT-PCR the expression of mmu-miR-1929-3p and mcmv-miR-m01-4-5p in peripheral blood monocytes, blood vessels, and myocardial tissues samples. Although the exact role of viral-encoded miRNAs is not known in many cases, it has been proposed that they could be a common mechanism by which viruses modulate transcriptional changes in the host and their genome during toxic infection and/or incubation. For instance, the simian virus 40-encoded miR-s1 miRNA mediates the *cis*-degradation of cytotoxic t-antigen mRNA, which encodes

for a major viral regulator, in a process thought to help to evade the host immune response [44]. The HCMV-encoded miR-ul112-3p targets the 3′-UTR of gene 72 (IE72), an early major *trans*-activator gene. The removal of miR-ul112-3p target sites in the IE72 mRNA increased IE72 expression [45]. One of the differentially expressed miRNAs detected in this study was mcmv-miR-m01-4, which is derived from a pre-miRNA located in the 3′-UTR of the m01 transcript and accumulates to detectable levels *in vivo* [46]. However, we did not find any potential hypertension-related target genes. In contrast, for mmu-miR-1929-3p, we predicted a hypertension-related target gene, *Ednra*, and experimentally validated the finding with the dual-luciferase assay.

In mammals, the endothelin signaling system (ligand plus receptor) consists of three ligands (encoded by *EDN1*, *EDN2*, and *EDN3*) and two receptors (encoded by *Ednra* and *Ednrb*), belonging to the seven-fold transmembrane domain receptor family of G protein-conjugated receptors [46]. The *Ednra* gene spans over 40 kb and contains 8 exons and 7 introns [47]. In hypertensive patients, *Ednra* increases vasoconstriction tension and may be associated with an increase in endothelin-1 (ET-1) [48]. ET-1 is the most effective vasoconstrictor in the human cardiovascular system and has very long-lasting effects. In the present study, we showed that mmu-miR-1929-3p targets *Ednra* mRNA and thus might be involved in MCMV-induced hypertension. Future studies are required to identify potential biomarkers of CMV-mediated hypertension.

A previous study identified different virus- and host-encoded miRNAs whose target genes are related to the inflammatory response, vasoconstriction, and vascular remodeling (although their identified target genes are not among the top ten genes involved in the incidence of hypertension) [34]. The discrepancy with our results can be explained by various factors, such as the use of different models, research objects, research methods, and CMV specificity in different species. However, both the present study and our support after the idea that MCMV infection promotes the development of hypertension through the miRNA-mediated regulation. Both studies also suggest that antiviral therapy can be a novel approach for the prevention and treatment of hypertension.

The present study presents some limitations. In particular, the effect of mmu-miR-1929-3p in the occurrence and development of hypertension and target organ damage still need to be further validated *in vivo* and elucidated at the cellular and molecular level.

In conclusion, we identified mmu-miR-1929-3p as a novel miRNA down-regulated in mice infected with MCMV. We also experimentally validated that *Ednra* may be the target gene of mmu-miR-1929-3p. Thus, mmu-miR-1929-3p can be promising drug targets and/or potential biomarkers for the prevention and management of hypertension.

Data Availability

The data associated with the present paper are stored in NCBI SRA (accession number: PRJNA679174). Other data that were used to support the findings of the present study are included within the supplementary information files.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Author Contribution

S.Y.S., F.H., and D.M.X. conceived and designed the experiments. S.Y.S., D.M.X., and H.Z. performed the experiments. S.Y.S., N.T., Y.M.L., L.M.W., and Y.T. analyzed the data. S.Y.S., X.N.Z., H.Z., and F.H. wrote or modified the paper. All authors contributed to and approved the final draft of the manuscript.

Ethics Approval

The study was approved by the Ethics Committee of Shihezi Medical University (Shihezi, China) (approval number: A2018-038-01).

Abbreviations

ACE , angiotensin; CMV , cytomegalovirus; *Ednra*, endothelin A receptor; EH, essential hypertension; ET-1, endothelin-1; GO, gene ontology; HCMV, human cytomegalovirus; KEGG, Kyoto Encyclopedia of Genes and Genomes; MCMV, murine cytomegalovirus; miRNA, microRNA; mRNA, messenger RNA; PFU, plaque forming unit; qRT-PCR, quantitative reverse transcription polymerase chain reaction; UTR, untranslated region; VSB, virus standard buffer.

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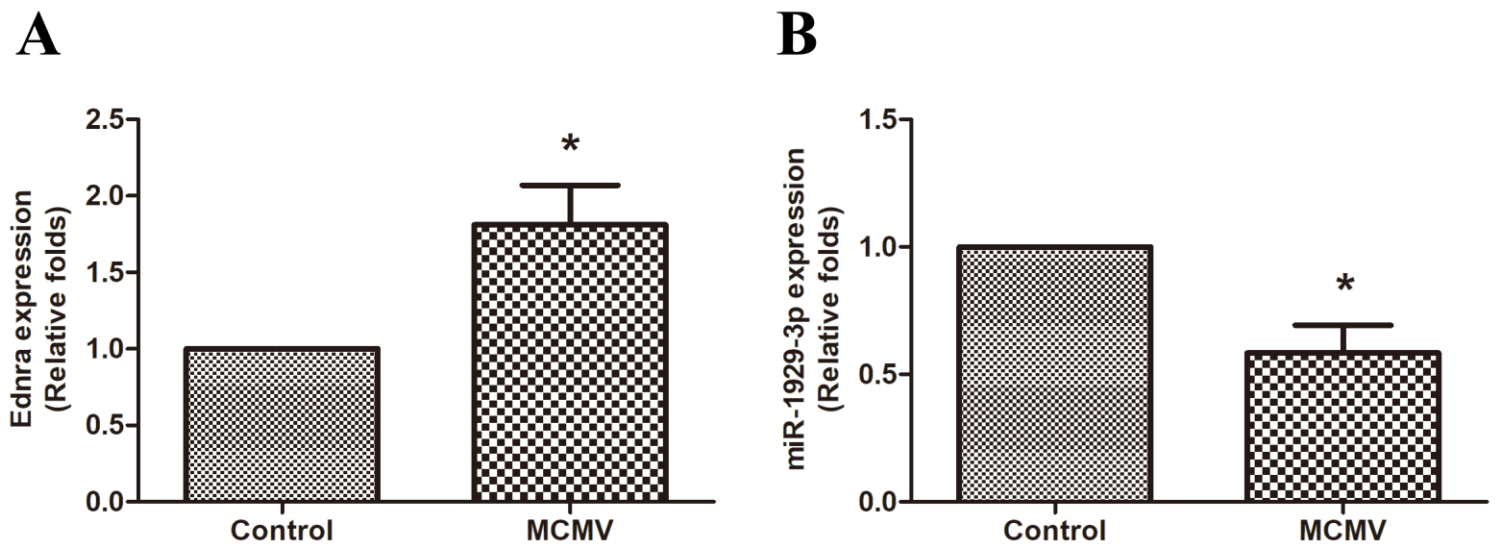
Supplementary Figure 1. The expression levels of *Ednra* in tissues and miR-1929-3p in cells



Supplementary Table 1. The qRT-PCR system

Reaction Component	Concentration	Volume(μL)
2×miRcute Plus miRNA PreMix（SYBR＆ROX）	2×	10
Forward primer（U6）	200 nM	0.4
Reverse primer	200 nM	0.4
ddH2O	-	8.2
miRNA the first chain cDNA	-	1
Total Volume	-	20

Supplementary Table 2. The primer sequences for qRT-PCR

microRNA	Sequence	Accession number
mmu-miR-204-5p	5'-UUCCCUUUGUCAUCCUAUGCCU-3'	MIMAT0000237
mmu-miR-182-5p	5'-UUUGGCAAUGGUAGAACUCACACCG-3'	MIMAT0000211
mmu-miR-143-3p	5'-UGAGAUGAAGCACUGUAGCUC-3'	MIMAT0000247
mmu-miR-211-5p	5'-UUCCCUUUGUCAUCCUUUGCCU-3'	MIMAT0000668
mmu-miR-133b-3p	5'-UUUGGUCCCCUUCAACCAGCUA-3'	MIMAT0000769
mmu-miR-328-3p	5'-CUGGCCCUCUCUGCCCUUCCGU-3'	MIMAT0000565
mmu-miR-1929-3p	5'-CAGCUCAUGGAGACCUAGGUGG-3'	MIMAT0022729
mcmv-miR-m01-4-5p	5'-UCCUAUGCUAACACGUGCGCGUG-3'	MIMAT0005538
mcmv-miR-m88-1-3p	5'-CAGAAGUCGAUGUCGGGGUCU-3'	MIMAT0005552
U6 F	5'-GCTTCGGCAGCACATATACTAAAAT-3'	-
U6 R	5'-CGCTTCACGAATTTGCGTGTCAT-3'	-

Supplementary Table 3: Characteristics of differentially expressed miRNAs in mice induced by MCMV infection

Upregulated miRNAs	log2(FC)	P	Downregulated miRNAs	log2(FC)	P
mmu-miR-211-5p	4.81	<0.001	mmu-miR-7658-3p	-4.32	<0.001
mmu-miR-379-5p	4.00	0.001	9_21079	-4.00	<0.001
mmu-miR-122-5p	3.92	<0.001	11_25868	-3.32	<0.001
mmu-miR-204-5p	3.81	<0.001	mmu-miR-7687-3p	-3.22	<0.001
mmu-miR-183-5p	3.20	<0.001	mmu-miR-486b-3p	-3.09	<0.001
2_3620	3.00	0.005	mmu-miR-8112	-3.00	0.015
mmu-miR-96-5p	2.96	<0.001	15_33426	-2.95	<0.001
mmu-miR-143-3p	2.82	<0.001	mmu-miR-1306-5p	-2.91	<0.001
mmu-miR-434-5p	2.81	0.031	1_2396	-2.74	0.001
17_35459	2.74	0.003	6_12883	-2.59	0.019
16_34132_star	2.70	0.002	8_18027	-2.58	0.017
2_3619	2.66	0.002	7_15813	-2.49	0.013
mmu-miR-133b-3p	2.58	0.050	7_14875	-2.46	0.01
mmu-miR-182-5p	2.57	0.002	2_5312	-2.45	0.003
mmu-miR-382-5p	2.54	0.004	9_20785	-2.38	0.006
mmu-miR-100-5p	2.52	0.007	7_15814	-2.35	0.008
mmu-miR-184-3p	2.47	0.001	mmu-miR-1929-3p	-2.32	0.017
17_35460	2.39	0.005	mmu-miR-5126	-2.28	0.008
mmu-miR-411-5p	2.32	0.020	10_22165_star	-2.19	0.019
mmu-miR-1a-3p	2.31	0.004	7_14962	-2.13	0.019
mmu-miR-203-3p	2.30	0.006	mmu-miR-486a-3p	-2.11	0.032
16_34879	2.17	0.012	11_26101	-2.09	0.014
mmu-miR-429-3p	2.16	0.009	mmu-miR-3102-3p	-2.00	0.030
mmu-miR-3473c	2.07	0.005	mmu-miR-7052-3p	-2.00	0.046
mmu-miR-145a-5p	2.01	0.010	1_639	-1.82	0.037
mmu-miR-145a-3p	2.00	0.035	15_33614	-1.75	0.041
mmu-miR-200b-3p	1.90	0.016	mmu-miR-328-3p	-1.66	0.018
mmu-miR-3473e	1.87	0.015			
mmu-miR-199b-3p	1.83	0.026			
mmu-miR-709	1.81	0.042			
mmu-miR-206-3p	1.80	0.015			
mmu-miR-199a-3p	1.79	0.032			
11_25514	1.78	0.016			
11_25831	1.77	0.039			
mmu-miR-9-5p	1.74	0.040			
5_10470	1.74	0.049			
X_40860	1.70	0.026			
mmu-miR-148a-3p	1.70	0.034			
mmu-miR-200a-3p	1.60	0.041			

mmu-miR-133a-3p	1.60	0.031
mmu-let-7f-5p	1.55	0.047
mmu-miR-3473b	1.54	0.038
mmu-let-7i-5p	1.42	0.040
mmu-let-7a-5p	1.39	0.032
1_335	Inf	0.016
10_21727_star	Inf	0.045
10_22793	Inf	0.021
10_23162	Inf	0.042
11_24008	Inf	0.045
11_24173	Inf	0.015
11_25908	Inf	0.045
11_26241_star	Inf	0.046
14_30406	Inf	0.006
14_30821	Inf	0.027
14_30846	Inf	0.003
14_30905	Inf	0.010
14_31532	Inf	0.045
14_31674	Inf	0.028
15_33456	Inf	0.035
16_34299	Inf	0.006
19_39006	Inf	0.021
2_3169	Inf	0.002
2_4983_star	Inf	0.042
2_5181	Inf	0.027
2_5474_star	Inf	<0.001
3_6204	Inf	0.027
3_6376	Inf	0.027
4_7885	Inf	0.029
4_8698	Inf	0.035
4_9362	Inf	0.041
5_10623	Inf	0.035
5_10674	Inf	0.011
6_13582	Inf	0.021
6_13591	Inf	0.050
6_14044	Inf	0.003
7_15083_star	Inf	0.048
7_15376	Inf	0.034
8_18816	Inf	0.045
9_20233	Inf	0.027
9_21014	Inf	0.045
mmu-miR-124-3p	Inf	<0.001
mmu-miR-1895	Inf	0.045
mmu-miR-216a-5p	Inf	<0.001

mmu-miR-217-5p	Inf	<0.001
mmu-miR-3087-5p	Inf	0.024
mmu-miR-6959-3p	Inf	0.001
mmu-miR-6968-3p	Inf	0.008
mmu-miR-7071-3p	Inf	0.016
mmu-miR-7072-3p	Inf	0.025
mcmv-miR-m01-4-5p		<0.005
mcmv-miR-m88-1-3p		<0.005

Supplementary Table 4: Characteristics of differentially expressed miRNAs in mice induced by MCMV infection

Upregulated miRNAs	log2(FC)	*P*	Downregulated miRNAs	log2(FC)	*P*
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mmu-miR-379-5p	4.00	0.001	mmu-miR-3102-3p	-2.00	0.030
mmu-miR-122-5p	3.92	<0.001	mmu-miR-7052-3p	-2.00	0.046
mmu-miR-204-5p	3.81	<0.001	mmu-miR-486a-3p	-2.11	0.032
mmu-miR-183-5p	3.20	<0.001	mmu-miR-5126	-2.28	0.008
mmu-miR-96-5p	2.96	0.001	mmu-miR-1929-3p	-2.32	0.017
mmu-miR-143-3p	2.82	<0.001	mmu-miR-1306-5p	-2.91	<0.001
mmu-miR-434-5p	2.81	0.032	mmu-miR-8112	-3.00	0.015
mmu-miR-133b-3p	2.58	0.050	mmu-miR-486b-3p	-3.09	0.003
mmu-miR-182-5p	2.57	0.002	mmu-miR-7687-3p	-3.22	0.001
mmu-miR-382-5p	2.54	0.004	mmu-miR-7658-3p	-4.32	<0.001
mmu-miR-100-5p	2.52	0.007			
mmu-miR-184-3p	2.47	0.001			
mmu-miR-411-5p	2.32	0.020			
mmu-miR-1a-3p	2.32	0.004			
mmu-miR-203-3p	2.30	0.006			
mmu-miR-429-3p	2.16	0.009			
mmu-miR-3473c	2.07	0.005			
mmu-miR-145a-5p	2.01	0.010			
mmu-miR-145a-3p	2.00	0.035			
mmu-miR-200b-3p	1.90	0.016			
mmu-miR-3473e	1.87	0.015			
mmu-miR-199b-3p	1.83	0.026			
mmu-miR-709	1.81	0.042			
mmu-miR-206-3p	1.80	0.015			
mmu-miR-199a-3p	1.79	0.032			
mmu-miR-9-5p	1.74	0.040			
mmu-miR-148a-3p	1.67	0.034			
mmu-miR-200a-3p	1.61	0.041			
mmu-miR-133a-3p	1.60	0.031			
mmu-let-7f-5p	1.55	0.047			
mmu-miR-3473b	1.54	0.038			
mmu-let-7i-5p	1.42	0.039			
mmu-let-7a-5p	1.39	0.032			
mcmv-miR-m01-4-5p		<0.005			
mcmv-miR-m88-1-3p		<0.005			

Supplementary Table 5: Characteristics of differentially expressed miRNAs related to hypertension in mice induced by MCMV infection

Upregulated miRNAs	log2(FC)	*P*	Downregulated miRNAs	log2(FC)	*P*
mmu-miR-211-5p	4.81	<0.001	mmu-miR-328-3p	-1.66	0.018
mmu-miR-379-5p	4.00	0.001	mmu-miR-3102-3p	-2.00	0.030
mmu-miR-204-5p	3.81	<0.001	mmu-miR-486a-3p	-2.11	0.032
mmu-miR-183-5p	3.20	<0.001	mmu-miR-1929-3p	-2.32	0.017
mmu-miR-143-3p	2.82	<0.001	mmu-miR-1306-5p	-2.91	<0.001
mmu-miR-133b-3p	2.58	0.050	mmu-miR-486b-3p	-3.09	0.003
mmu-miR-182-5p	2.57	0.002			
mmu-miR-382-5p	2.54	0.004			
mmu-miR-411-5p	2.32	0.020			
mmu-miR-1a-3p	2.32	0.004			
mmu-miR-203-3p	2.30	0.006			
mmu-miR-429-3p	2.16	0.009			
mmu-miR-3473c	2.07	0.005			
mmu-miR-145a-5p	2.01	0.010			
mmu-miR-145a-3p	2.00	0.035			
mmu-miR-200b-3p	1.90	0.016			
mmu-miR-709	1.81	0.042			
mmu-miR-206-3p	1.80	0.015			
mmu-miR-9-5p	1.74	0.040			
mmu-miR-148a-3p	1.67	0.034			
mmu-miR-200a-3p	1.61	0.041			
mmu-miR-133a-3p	1.60	0.031			
mmu-let-7f-5p	1.55	0.047			
mmu-miR-3473b	1.54	0.038			
mmu-let-7i-5p	1.42	0.039			
mmu-let-7a-5p	1.39	0.032			

Supplementary Table 6: Enrichment analysis of hypertension-related target genes

Description	pathwayID	p value	genes	enrich_factor
Thyroid cancer	mmu05216	2.072e-06	KRAS TCF7L2 CCND1 RET TRP53	16.66
Phototransduction	mmu04744	3.277e-05	SLC24A1 GRK1 CNGB1 RHO	13.79
Endometrial cancer	mmu05213	4.575e-05	KRAS TCF7L2 CCND1 PTEN TRP53	9.61
Dorso-ventral axis formation	mmu04320	3.261e-03	KRAS NOTCH2	9.09
Aldosterone-regulated sodium reabsorption	mmu04960	2.303e-04	KRAS IRS1 ATP1A2 NEDD4L	9.09
Asthma	mmu05310	4.168e-03	IL10 IL5	8.33
Amyotrophic lateral sclerosis (ALS)	mmu05014	6.913e-04	TNFRSF1B APAF1 MAPK14 TRP53	7.14
Bladder cancer	mmu05219	2.289e-03	KRAS CCND1 TRP53	6.97
Intestinal immune network for IgA production	mmu04672	2.700e-03	IL10 ICOSL IL5	6.66
Prostate cancer	mmu05215	1.192e-04	KRAS TCF7L2 FGFR2 CCND1 PTEN TRP53	6.66
T cell receptor signaling pathway	mmu04660	6.776e-05	IL10 KRAS VAV2 VAV3 IFNG IL5 MAPK14	6.25
Fc epsilon RI signaling pathway	mmu04664	4.681e-04	KRAS VAV2 VAV3 IL5 MAPK14	6.25
African trypanosomiasis	mmu05143	9.282e-03	IL10 IFNG	6.25
Colorectal cancer	mmu05210	1.345e-03	KRAS TCF7L2 CCND1 TRP53	6.15
Leishmaniasis	mmu05140	1.345e-03	IL10 IFNG JAK2 MAPK14	6.15
Glioma	mmu05214	1.439e-03	KRAS CCND1 PTEN TRP53	6.06
p53 signaling pathway	mmu04115	1.864e-03	APAF1 CCND1 PTEN TRP53	5.71
Melanoma	mmu05218	2.108e-03	KRAS CCND1 PTEN TRP53	5.55
Basal cell carcinoma	mmu05217	5.541e-03	TCF7L2 WNT4 TRP53	5.45
Non-small cell lung	mmu05223	5.541e-03	KRAS CCND1	5.45

cancer			TRP53	
Tyrosine metabolism	mmu00350	1.380e-02	DDC COMT	5.40
Allograft rejection	mmu05330	5.905e-03	IL10 IFNG IL5	5.36
Acute myeloid leukemia	mmu05221	6.286e-03	KRAS TCF7L2 CCND1	5.26
Vascular smooth muscle contraction	mmu04270	8.527e-04	EDNRA ADORA2B PRKG1 ARHGEF1 ARHGEF12 ROCK2	4.80
TGF-beta signaling pathway	mmu04350	4.311e-03	SMAD9 IFNG ACVR1 ROCK2	4.70
Small cell lung cancer	mmu05222	4.759e-03	APAF1 CCND1 PTEN TRP53	4.60
Regulation of actin cytoskeleton	mmu04810	6.759e-05	F2R KRAS CSK VAV2 VAV3 FGFR2 ARHGEF1 ARHGEF12 ROCK2 ITGA11	4.59
Adipocytokine signaling pathway	mmu04920	1.162e-02	TNFRSF1B IRS1 JAK2	4.41
Malaria	mmu05144	2.618e-02	IL10 IFNG	4.25
Pancreatic cancer	mmu05212	1.347e-02	KRAS CCND1 TRP53	4.22
Leukocyte transendothelial migration	mmu04670	3.697e-03	VAV2 VAV3 RHOH MAPK14 ROCK2	4.17
Chronic myeloid leukemia	mmu05220	1.550e-02	KRAS CCND1 TRP53	4.05
Melanogenesis	mmu04916	8.879e-03	EDN1 KRAS TCF7L2 WNT4	3.96
B cell receptor signaling pathway	mmu04662	1.696e-02	KRAS VAV2 VAV3	3.95
Jak-STAT signaling pathway	mmu04630	2.698e-03	IL10 SPRY2 IFNG CCND1 IL5 JAK2	3.92
Wnt signaling pathway	mmu04310	2.798e-03	TCF7L2 CCND1 SOX17 WNT4 ROCK2 TRP53	3.89
Neurotrophin signaling pathway	mmu04722	5.875e-03	KRAS IRS1 CSK MAPK14 TRP53	3.79
Chemokine signaling pathway	mmu04062	2.115e-03	GRK1 KRAS CSK VAV2 VAV3 JAK2 ROCK2	3.70
Focal adhesion	mmu04510	2.993e-03	RELN VAV2 VAV3 CCND1 PTEN ROCK2 ITGA11	3.50

Gap junction	mmu04540	2.762e-02	KRAS PRKG1 MAP3K2	3.41
Cytokine-cytokine receptor interaction	mmu04060	2.935e-03	TNFRSF1B IL10 LTA IFNG ACVR1 IL17RB IL5 TNFSF4	3.25
Natural killer cell mediated cytotoxicity	mmu04650	2.094e-02	KRAS VAV2 VAV3 IFNG	3.20
Type I diabetes mellitus	mmu04940	5.579e-02	LTA IFNG	3.17
Toxoplasmosis	mmu05145	2.297e-02	IL10 IFNG JAK2 MAPK14	3.12
GnRH signaling pathway	mmu04912	4.042e-02	KRAS MAP3K2 MAPK14	3.03
Chagas disease (American trypanosomiasis)	mmu05142	4.308e-02	IL10 IFNG MAPK14	2.97
Autoimmune thyroid disease	mmu05320	7.777e-02	IL10 IL5	2.78
Long-term depression	mmu04730	7.777e-02	KRAS PRKG1	2.78
Arrhythmogenic right ventricular cardiomyopathy (ARVC)	mmu05412	8.317e-02	TCF7L2 ITGA11	2.70
Complement and coagulation cascades	mmu04610	8.875e-02	F2R THBD	2.63
VEGF signaling pathway	mmu04370	8.875e-02	KRAS MAPK14	2.63
Salivary secretion	mmu04970	9.160e-02	PRKG1 ATP1A2	2.60
Pathways in cancer	mmu05200	1.715e-02	KRAS TCF7L2 FGFR2 CCND1 WNT4 PTEN RET TRP53	2.45
Rheumatoid arthritis	mmu05323	1.160e-01	ANGPT1 IFNG	2.35
Cell cycle	mmu04110	8.930e-02	CDKN1C CCND1 TRP53	2.34
ECM-receptor interaction	mmu04512	1.193e-01	RELN ITGA11	2.32
Apoptosis	mmu04210	1.193e-01	APAF1 TRP53	2.32
Progesterone-mediated oocyte maturation	mmu04914	1.259e-01	KRAS MAPK14	2.27
Axon guidance	mmu04360	9.780e-02	KRAS ARHGEF12 ROCK2	2.27
Endocytosis	mmu04144	6.082e-02	GRK1 F2R FGFR2 RET NEDD4L	2.24

Tight junction	mmu04530	1.090e-01	KRAS HCLS1 PTEN	2.19
Hepatitis C	mmu05160	1.090e-01	KRAS MAPK14 TRP53	2.19
Fc gamma R-mediated phagocytosis	mmu04666	1.396e-01	VAV2 VAV3	2.17
Neuroactive ligand-receptor interaction	mmu04080	5.524e-02	EDNRA ADORA2B ADRA2A F2R ADRA2B NR3C1	2.17
MAPK signaling pathway	mmu04010	1.259e-01	KRAS FGFR2 MAP3K2 MAPK14 TRP53	1.86
Amoebiasis	mmu05146	2.344e-01	IL10 IFNG	1.72
Osteoclast differentiation	mmu04380	2.432e-01	IFNG MAPK14	1.69
Calcium signaling pathway	mmu04020	2.247e-01	EDNRA ADORA2B F2R	1.68
Insulin signaling pathway	mmu04910	3.315e-01	KRAS IRS1	1.46
Systemic lupus erythematosus	mmu05322	4.067e-01	IL10 IFNG	1.32
Alzheimer's disease	mmu05010	6.033e-01	APAF1 ERN1	1.06
Staphylococcus aureus infection	mmu05150	1	IL10	1.00
Parkinson's disease	mmu05012	1	APAF1	1.00
Nicotinate and nicotinamide metabolism	mmu00760	1	NMNAT2	1.00
Proximal tubule bicarbonate reclamation	mmu04964	1	ATP1A2	1.00
Biosynthesis of unsaturated fatty acids	mmu01040	1	ELOVL6	1.00
Dilated cardiomyopathy	mmu05414	1	ITGA11	1.00
Renal cell carcinoma	mmu05211	1	KRAS	1.00
Proteasome	mmu03050	1	IFNG	1.00
Arachidonic acid metabolism	mmu00590	1	PTGS1	1.00
Cardiac muscle contraction	mmu04260	1	ATP1A2	1.00
Histidine metabolism	mmu00340	1	DDC	1.00
Hematopoietic cell lineage	mmu04640	1	IL5	1.00
NOD-like receptor	mmu04621	1	MAPK14	1.00

signaling pathway				
Alanine, aspartate and glutamate metabolism	mmu00250	1	GPT2	1.00
Arginine and proline metabolism	mmu00330	1	OTC	1.00
Toll-like receptor signaling pathway	mmu04620	1	MAPK14	1.00
Tryptophan metabolism	mmu00380	1	DDC	1.00
Phenylalanine metabolism	mmu00360	1	DDC	1.00
Graft-versus-host disease	mmu05332	1	IFNG	1.00
Hedgehog signaling pathway	mmu04340	1	WNT4	1.00
Protein processing in endoplasmic reticulum	mmu04141	1	ERN1	1.00
Adherens junction	mmu04520	1	TCF7L2	1.00
Gastric acid secretion	mmu04971	1	ATP1A2	1.00
Huntington's disease	mmu05016	6.574e-01	APAF1 TRP53	1.00
Selenocompound metabolism	mmu00450	1	CTH	1.00
Viral myocarditis	mmu05416	1	CCND1	1.00
ErbB signaling pathway	mmu04012	1	KRAS	1.00
Ubiquitin mediated proteolysis	mmu04120	1	NEDD4L	1.00
Cell adhesion molecules (CAMs)	mmu04514	1	ICOSL	1.00
Type II diabetes mellitus	mmu04930	1	IRS1	1.00
Regulation of autophagy	mmu04140	1	IFNG	1.00
Notch signaling pathway	mmu04330	1	NOTCH2	1.00
Pancreatic secretion	mmu04972	1	ATP1A2	1.00
RNA degradation	mmu03018	1	SKI	1.00
Antigen processing and presentation	mmu04612	1	IFNG	1.00
Glycine, serine and threonine metabolism	mmu00260	1	CTH	1.00
Nitrogen metabolism	mmu00910	1	CTH	1.00
Fat digestion and absorption	mmu04975	1	MTTP	1.00

Phosphatidylinositol signaling system	mmu04070	1	PTEN	1.00
Long-term potentiation	mmu04720	1	KRAS	1.00
Protein digestion and absorption	mmu04974	1	ATP1A2	1.00
Bile secretion	mmu04976	1	ATP1A2	1.00
Carbohydrate digestion and absorption	mmu04973	1	ATP1A2	1.00
Hypertrophic cardiomyopathy (HCM)	mmu05410	1	ITGA11	1.00
Bacterial invasion of epithelial cells	mmu05100	1	HCLS1	1.00
Inositol phosphate metabolism	mmu00562	1	PTEN	1.00
Steroid hormone biosynthesis	mmu00140	1	COMT	1.00
Cysteine and methionine metabolism	mmu00270	1	CTH	1.00
Circadian rhythm	mmu04710	1	ARNTL	1.00
ABC transporters	mmu02010	1	ABCC9	1.00
RIG-I-like receptor signaling pathway	mmu04622	1	MAPK14	1.00
Metabolic pathways	mmu01100	1	CTH DDC COMT GPT2 PTGS1	0.59
Olfactory transduction	mmu04740	1	NMNAT2 OTC CNGB1 PDC PRKG1	0.30

Supplementary Table 7: miRNA-mRNA interaction analysis and regulatory network construction

miRNA	Target gene
mmu-let-7a-5p	Edn1, Pctp, Map3k2
mmu-let-7f-5p	Edn1, Hmga1, Pctp, Map3k2
mmu-let-7i-5p	Edn1, Hmga1, Pctp
mmu-miR-1306-5p	Apaf1, Arhgef12
mmu-miR-133a-3p	Agt, Otc, Ptpro, Myrf
mmu-miR-133b-3p	Agt, Otc, Ptpro, Myrf
mmu-miR-143-3p	Clcn3, Kras, Asb4, Tardbp
mmu-miR-145a-5p	Ddc, Irs1, Mttp, Il17rb, Kcnk6, Nedd4l, Myrf, Adam1a
mmu-miR-148a-3p	Kcnj8, Pten, Cth, Itga11
mmu-miR-182-5p	Acvr1, Adra2a, Otc, Phb, Rgs2, Wnt4
mmu-miR-183-5p	Clcn3, Irs1, Nrp2, Tcf7l2, Spry2
mmu-miR-1a-3p	Clcn3, Edn1, Epcam, Rgs2, Smarca4, Sox17, Corin
mmu-miR-200a-3p	Nr3c1, Pten, Sox17, Tfap2b, Thbd, Corin
mmu-miR-200b-3p	F2r, Nr3c1, Il10, Ntf3, Pkd1, Reln
mmu-miR-203-3p	Adra2b, Arntl, Fkbp1b, Nr3c1, Prkg1, Nedd4l, Atp1a2, Tardbp
mmu-miR-204-5p	Comt, Nr3c1, Il10, Ddr2, Prkg1, Ski, Wnt4, Nedd4l, Elovl6, Dicer1
mmu-miR-206-3p	Clcn3, Edn1, Epcam, Rgs2, Sox17, Corin
mmu-miR-211-5p	Comt, Nr3c1, Il10, Ddr2, Prkg1, Ski, Wnt4, Elovl6, Dicer1
mmu-miR-328-3p	Nr3c1, Tcf7l2, Trp53
mmu-miR-3473b	Pten, Vav3
mmu-miR-3473c	Irs1, Lta, Ret, Rock2, Abcc9, Nol3, Slc43a2
mmu-miR-382-5p	Ifng, Kras, Ptgs1, Arhgef12, Rhoh
mmu-miR-429-3p	F2r, Nr3c1, Il10, Ntf3, Pkd1, Reln
mmu-miR-9-5p	Cdkn1c, Erg, Notch2, Tnfsf4, Vav3
mmu-miR-124-3p	Angpt1, Rock2, Map3k2, Mapk14, Fstl3, Gpt2, Myrf, Nr3c1, Arhgef1, Ern1
mmu-miR-145a-3p	Acvrl1, Ccnd1, Fgfr2, Mnat1, Slc43a2, Il5, Irs1
mmu-miR-1895	Pdc
mmu-miR-1929-3p	Ednra, Htt, Smad9
mmu-miR-216a-5p	Csk, F2r, Ddr2, Tdrd7, Gpt2, Jak2
mmu-miR-217-5p	Kras, Tdrd7
mmu-miR-3087-5p	Ednra, Htt, Stim1, Slc2a9, Rho, Slc24a1, Sh3pxd2b, Add2, Wt1
mmu-miR-3102-3p	Nmnat2, Nyx, Pcdh15, F2r, Nol3
mmu-miR-379-5p	Edn1
mmu-miR-411-5p	Icosl, Myrf, Angpt1
mmu-miR-709	Hcls1, Grk1, Nr5a1, Corin, Slc43a2, Cngb1, Adora2b
mmu-miR-486b-3p	Hmga1, Vav2, Tnfrsf1b, Pkd1, Itga11
mmu-miR-486a-3p	Hmga1, Vav2, Tnfrsf1b, Pkd1

Supplementary Table 8: Differential miRNAs regulate target genes' GO enrichment results (Top 20)

GO_ID	Term	GO_namespace	gene_num
GO:0005623	cell	CC	1146
GO:0044464	cell part	CC	1145
GO:0009987	cellular process	BP	1071
GO:0005488	binding	MF	979
GO:0043226	organelle	CC	951
GO:0044699	single-organism process	BP	923
GO:0065007	biological regulation	BP	837
GO:0008152	metabolic process	BP	820
GO:0050789	regulation of biological process	BP	793
GO:0016020	membrane	CC	643
GO:0044422	organelle part	CC	629
GO:0050896	response to stimulus	BP	581
GO:0032501	multicellular organismal process	BP	510
GO:0071840	cellular component organization or biogenesis	BP	484
GO:0044425	membrane part	CC	471
GO:0048518	positive regulation of biological process	BP	461
GO:0032502	developmental process	BP	461
GO:0051179	localization	BP	453
GO:0023052	signaling	BP	444
GO:0048519	negative regulation of biological process	BP	406

Supplementary Table 9: KEGG Pathway enrichment results of differential miRNAs regulating target genes (Top 20)

ID	Description	GeneRatio	*p* value	*p*.adjust
mmu04010	MAPK signaling pathway	34/521	<0.001	0.1180
mmu04330	Notch signaling pathway	10/521	0.001	0.1188
mmu04721	Synaptic vesicle cycle	11/521	0.001	0.1497
mmu04668	TNF signaling pathway	15/521	0.004	0.2528
mmu04210	Apoptosis	17/521	0.005	0.2528
mmu04724	Glutamatergic synapse	15/521	0.005	0.2528
mmu04120	Ubiquitin mediated proteolysis	17/521	0.006	0.2676
mmu05031	Amphetamine addiction	10/521	0.010	0.3195
mmu05221	Acute myeloid leukemia	10/521	0.011	0.3195
mmu04014	Ras signaling pathway	24/521	0.012	0.3195
mmu04024	cAMP signaling pathway	21/521	0.012	0.3195
mmu04215	Apoptosis - multiple species	6/521	0.013	0.3195
mmu04360	Axon guidance	19/521	0.014	0.3195
mmu05231	Choline metabolism in cancer	12/521	0.021	0.4242
mmu04392	Hippo signaling pathway - multiple species	5/521	0.021	0.4242
mmu04211	Longevity regulating pathway	11/521	0.025	0.4573
mmu00250	Alanine, aspartate and glutamate metabolism	6/521	0.027	0.4573
mmu00220	Arginine biosynthesis	4/521	0.028	0.4573
mmu05030	Cocaine addiction	7/521	0.029	0.4573
mmu00310	Lysine degradation	8/521	0.030	0.4573