Cellular & Molecular Immunology (2012) 9, 497–502

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RESEARCH ARTICLE

MicroRNA-466l inhibits antiviral innate immune response by targeting interferon-alpha

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Effective recognition of viral infections and subsequent triggering of antiviral innate immune responses are essential for the host antiviral defense, which is tightly regulated by multiple regulators, including microRNAs (miRNAs). A previous study showed that miR-466l upregulates IL-10 expression in macrophages by antagonizing RNA-binding protein tristetraprolin-mediated IL-10 mRNA degradation. However, the ability of miR-466l to regulate antiviral immune responses remains unknown. Here, we found that interferon-alpha (IFN-α) expression was repressed in Sendai virus (SeV)- and vesicular stomatitis virus (VSV)-infected macrophages and in dendritic cells transfected with miR-466l expression. Moreover, multiple IFN-α species can be directly targeted by miR-466l through their 3′ untranslated region (3′UTR). This study has demonstrated that miR-466l could directly target IFN-α expression to inhibit host antiviral innate immune response.
Cellular & Molecular Immunology (2012) **9,** 497–502; doi:10.1038/cmi.2012.35; published online 8 October 2012

Keywords: innate immunity; interferon-alpha; macrophages; microRNA; miR-466l

INTRODUCTION

Effective recognition of viral infections and subsequent triggering of antiviral innate and adaptive immune responses are essential for the survival of the host. Currently, several new intracellular viral sensors and their corresponding downstream signals have been suggested, and the mechanisms of their induction and regulation have attracted much attention.[1] Upon recognition of a viral infection, host intracellular viral sensors and downstream signals are activated to produce proinflammatory cytokines and type I interferon (IFN).[1–7] Type I IFNs, such as IFN-β and many IFN-α species, play pivotal roles in antiviral immune responses mainly through induction of cellular resistance to viral infection and apoptosis of virus-infected cells.[8,9] Type I IFN expression is tightly controlled by multiple intracellular regulators to prevent the development of immunopathological conditions.[10–12] Viruses have also developed several strategies to subvert the host immune response to ensure their survival in infected cells. Disruption of host recognition and subsequent impairment of type I IFN production is one of the well-known evading strategies used by viruses.[13,14] Hence, identification of the regulators modulating type I IFN production and elucidation of the detailed mechanisms of regulation have attracted much attention and are currently being actively pursued.

microRNAs (miRNAs) are an abundant class of highly conserved, small, non-coding RNAs involved in post-transcriptional regulation of gene expression. They function mainly by binding to the 3′ untranslated region (UTR) of target mRNAs to either induce degradation or suppress translation. miRNAs have been shown to play critical roles in the regulation of diverse biological processes, including development, differentiation, carcinogenesis, infection and immunity.[15–21] Since their initial observation, approximately 1000 miRNA sequences have been identified in mammals, but the detailed biological functions of a large number of miRNAs remain elusive. Previous reports have shown that some miRNAs, such as miR-146a and miR-155, are induced during virus infection and participate in the regulation of the innate antiviral response.[22–24] Whether the type I IFN response is regulated by other miRNAs is still unknown.

miR-466l was first identified in embryonic stem (ES) cells *via* high-throughput sequencing.[25] The seed sequence of miR-466l is AUAAAUA, and it is complementary to the typical AU-rich elements (ARE) located in the 3′UTR of multiple cytokines, chemokines and growth factors. A previous report has suggested that miR-466l could bind to the ARE of IL-10 3′UTR, thereby upregulating IL-10 expression by antagonizing RNA-binding protein tristetraprolin-mediated IL-10 mRNA degradation.[26] Whether miR-466l can regulate host antiviral innate immune response, specifically type I IFN production, remains unknown. In this study, we focused on the role of miR-466l in this process.

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Received 11 August 2012; revised 16 August 2012; accepted 16 August 2012

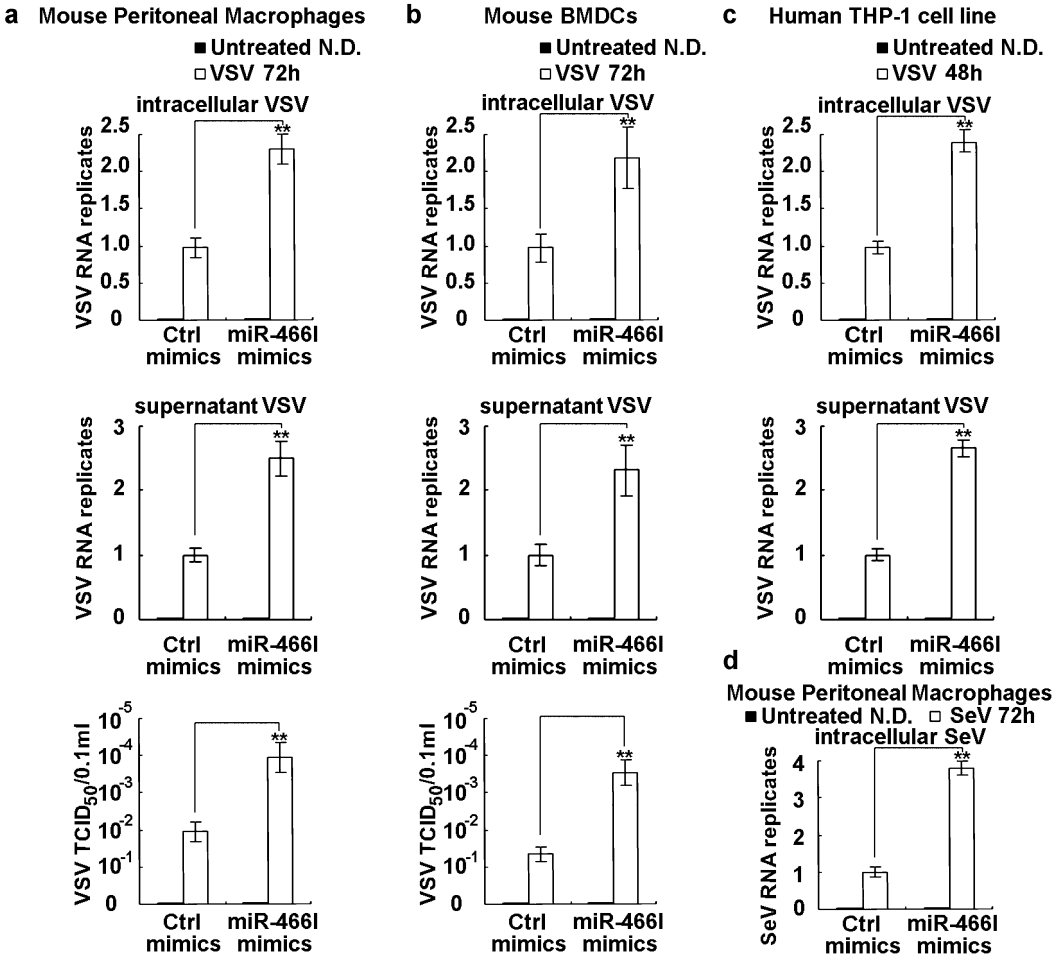

Figure 1 miR-466l enhances viral replication in APCs. (**a**, **b**) Mouse peritoneal macrophages (**a**) and BMDCs (**b**) were transfected with control mimics or miR-466l mimics at a final concentration of 10 nM. After transfection for 24 h, cells were infected with VSV at MOI 10 for 1 h and washed with fresh medium. After 72 h, intracellular (upper panel) VSV RNA replicates were quantified using qRT-PCR and normalized to the expression of β-actin in each sample. Supernatant (middle panel) VSV replicates were measured by extracting RNA from equal volumes of cultural supernatants and quantified using qRT-PCR. The viruses in the supernatants were serially diluted on the monolayer of BHK21 cells, and $TCID_{50}$ was measured (lower panel) (ND). (**c**) The human THP-1 cell line was transfected and treated as described in (**a**), and intracellular and supernatant VSV RNA replicates were quantified using qRT-PCR as indicated. (**d**) The mouse peritoneal macrophages were transfected as in (**a**), infected with SeV at MOI 10 for 1 h and washed with fresh medium. After 72 h, intracellular SeV RNA replicates were quantified using qRT-PCR as in (**a**). Data are shown as the mean±s.d. (n=4) of one representative experiment. Similar results were obtained in three independent experiments. $^{**}P<0.01$. APC, antigen-presenting cell; BMDC, bone marrow-derived dendritic cell; ND, not detected; qRT-PCR, quantitative RT-PCR; SeV, Sendai virus; VSV, vesicular stomatitis virus.

MATERIALS AND METHODS

Mice and reagents

Six- to eight-week-old C57BL/6 mice (Joint Ventures Sipper BK Experimental Animal Co., Shanghai, China) were used in the animal experiments. All of the animal experiments were undertaken in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Scientific Investigation Board of the Second Military Medical University, Shanghai. Vesicular stomatitis virus (VSV) was a gift from Professor Wei Pan. Sendai virus (SeV) was kindly provided by Profrssor Bing Sun.

Cell culture and transfection

THP-1 and HEK293 cell lines (ATCC, Manassas, VA, USA) were cultured as previously reported.[22] The HEK293 cells (1×10^4) were seeded into 96-well plates and incubated overnight. JetSI-ENDO transfection reagents (Polyplus Transfection, Illkirch, France) were used for the cotransfection of plasmids and miRNAs as previously reported.[27] Thioglycolate-elicited mouse peritoneal macrophages were prepared and cultured as reported.[28] The macrophages (0.5 ml of 4×10^5 cells) were seeded into each well of 24-well plates and incubated overnight; then they were transfected with miRNAs using INTERFERin (Polyplus Transfection) as previously reported.[29] Bone marrow-derived dendritic cells (BMDCs) were also generated as previously reported.[30]

miRNA mimics

miRNA mimics, which were double-stranded RNA oligonucleotides (GenePharma, Shanghai, China), were tranfected into macrophages or BMDCs at final concentrations of 10 nM. Negative control mimics were transfected as matched controls.

Luciferase reporter assays

IRF3 and IRF7 activities were measured using the IFN-α promoter luciferase reporter plasmid as reported previously.[31] The 3′UTR

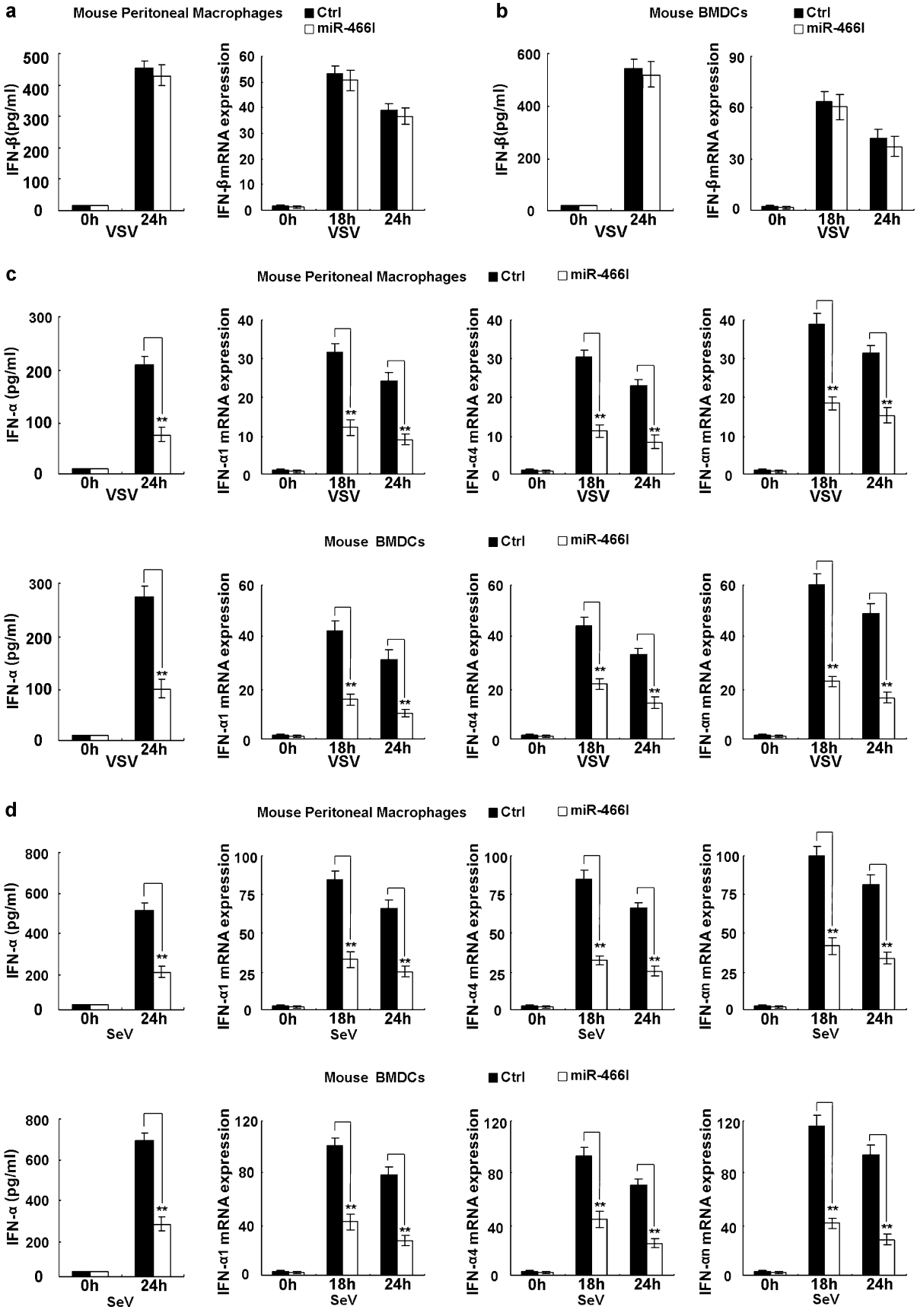
a
Mouse Peritoneal Macrophages
Ctrl
miR-466l
IFN-β(pg/ml)
IFN-β mRNA expression
0h
18h
24h
VSV
b
Mouse BMDCs
c
Mouse Peritoneal Macrophages
Mouse BMDCs
IFN-α (pg/ml)
IFN-α1 mRNA expression
IFN-α4 mRNA expression
IFN-αn mRNA expression
**
d
SeV

Figure 2 miR-466l suppresses RNA virus-triggered IFN-α production in APCs. The mouse peritoneal macrophages or BMDCs (0.5 ml of 4×10^5) were transfected and infected as in Figure 1. The supernatant IFN-β concentration and intracellular IFN-β mRNA were measured using ELISA and qRT-PCR, respectively (**a**, **b**). The supernatant IFN-α concentration and intracellular IFN-α mRNA species were measured using ELISA and qRT-PCR, respectively (**c**, **d**). Data are shown as the mean±s.d. (n=4) of one representative experiment. Similar results were obtained in three independent experiments. $^{**}P<0.01$. APC, antigen-presenting cell; BMDC, bone marrow-derived dendritic cell; IFN, interferon; qRT-PCR, quantitative RT-PCR.

luciferase reporter construct of IFN-α species was created by amplifying the relevant 3′UTR sequence using PCR and cloning it into the 3′UTR region of a PMIR-Report construct (Ambion, Austin, TX, USA). The HEK293 cells described above were cotransfected with 80 ng luciferase reporter plasmid, 40 ng pRL-TK-Renilla-luciferase plasmid and the indicated RNAs (final concentration: 20 nM). After 24 h, the luciferase activities were measured using a Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. The data were normalized for transfection efficiency by dividing firefly luciferase activity with that of Renilla luciferase.

RNA quantification

Total RNA, including miRNA, was extracted with TRIzol reagent (Invitrogen) following the manufacturer's instructions. Real-time quantitative RT-PCR (qRT-PCR) analysis was performed using a LightCycler (Roche, Indianapolis, IN, USA.) and a SYBR RT-PCR kit (Takara, Dalian, China). For mRNA analysis of mouse β-actin, IFN-β, IFN-α1, IFN-α4 and IFN-αn (including IFN-a1, -a2, -a7, -a11 and -a12 subtypes), the primers were designed as previously reported.[22,32,33] For VSV Indiana serotype analysis, the primers were as follows: 5′-ACG GCG TAC TTC CAG ATG G-3′ (forward) and 5′-CTC GGT TCA AGA TCC AGG T-3′ (reverse). For SeV analysis, the primers were as follows: 5′-TGC CCT GGA AGA TGA GTT AG-3′ and 5′-GCC TGT TGG TTT GTG GTA AG-3′. The relative expression levels of the mRNAs were normalized to β-actin, an internal control, by using the $2^{-\Delta\Delta Ct}$ cycle threshold method.[34]

ELISA

The cells (0.5 ml of 4×10^5 cell) were seeded into each well of 24-well plates and incubated overnight; they were then transfected with miRNAs as described above. After 48 h, the cells were infected with VSV or SeV for the indicated time periods. The concentrations of IFN-β or IFN-α in the cultural supernatants were measured using an ELISA Kit (PBL, New Brunswick, NJ, USA).

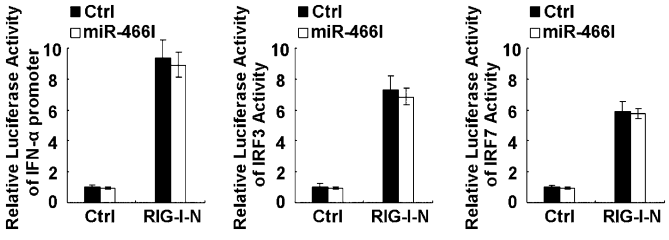

Figure 3 miR-466l does not influence IFN-α mRNA transcription. Luciferase assay for the activation of the IFN-α promoter (left), IRF3 (middle) and IRF7 (right) in lysates of HEK293 cells transfected with 80 ng IFN-α promoter reporter plasmid (left), 40 ng Gal4-IRF3 plasmid plus 40 ng Gal4 reporter plasmid (middle), 40 ng Gal4-IRF7 plasmid plus 40 ng Gal4 reporter plasmid (right), 20 ng pTK-renilla-luciferase, or 20 ng constructively active RIG-I N-terminus CARD domain expressing the plasmid or its empty vector, together with control mimics or miR-466l mimics as indicated. The luciferase activity was normalized to the renilla luciferase activity and is presented relative to basal luciferase activity. Data are shown as the mean±s.d. (n=4) of one representative experiment. Similar results were obtained in three independent experiments. IFN, interferon.

VSV yield qualification

The macrophages were transfected and then infected with VSV as indicated. The cultural supernatants (0.1 ml) were serially diluted on the monolayer of BHK21 cells, which were obtained from ATCC, and 1×10^4 cells were seeded into 96-well plates 1 day prior to measurement. $TCID_{50}$ was measured after 3 days. The viral RNA in the supernatant was extracted using a Body Fluid Viral DNA/RNA Miniprep Kit (Axygen, Union, CA, USA), and the VSV RNA replicates were qualified as described above.

Statistical analysis

Statistical significance was determined using Student's t-test, and P values of <0.05 were considered to be statistically significant.

RESULTS

miR-466l enhances RNA virus replication in macrophages and dendritic cells

As miR-466l was reported to upregulate IL-10 production in macrophages, we focused on the roles of miR-466l in host innate immune response against RNA virus infection. Using transfection of miR-466l mimics, we found that miR-466l could enhance the viral replication in different VSV-infected antigen-presenting cells (APCs), such as mouse peritoneal macrophages (Figure 1a and Supplementary Figure 1a), mouse BMDCs (Figure 1b and Supplementary Figure 1b) and human monocytic THP-1 cells (Figure 1c and Supplementary Figure 1c). Enhanced virus replication was also observed during SeV infection of APCs tranfected with miR (Figure 1d). These results demonstrate that miR-466l enhances RNA virus replication in APCs.

miR-466l inhibits IFN-α production in RNA virus-infected macrophages and dendritic cells

As type I IFN plays pivotal roles in the host antiviral innate immune response, we questioned whether repressed IFN production was responsible for the elevated virus replication in miR-466l-overexpressing APCs. miR-466l significantly suppressed mRNA and protein expression of IFN-α (Figure 2c and d) but did not suppress IFN-β (Figure 2a and b). These results suggest that miR-466l enhances viral infection by inhibiting IFN-α production in APCs.

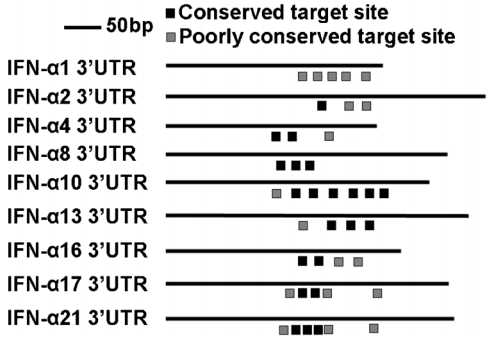

Figure 4 3′UTR sequences of IFN-α species may be targeted by miR-466l. The sequence alignment of miR-466l and its predicted target sites in the 3′UTR of the indicated IFN-α mRNA species is shown. TargetScan 5.0 software was used to predict target sites. IFN, interferon; UTR, untranslated region.

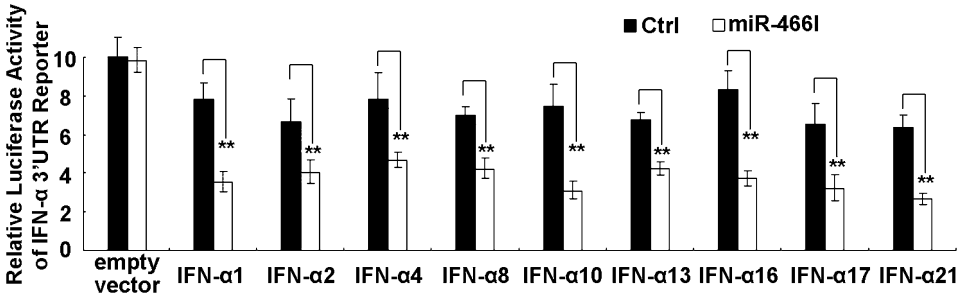

Figure 5 miR-466l targets the 3′UTR sequences of IFN-α species. The HEK293 cells were cotransfected with 80 ng of the indicated IFN-α species 3′UTR firefly luciferase reporter plasmids and 40 ng of pTK-Renilla-luciferase plasmids, together with the control or miR-146a mimics as indicated. After 24 h, firefly luciferase activity was measured and normalized to Renilla luciferase activity. Data are shown as the mean±s.d. (n=4) of one representative experiment. Similar results were obtained in three independent experiments. $^{**}P<0.01$. IFN, interferon; UTR, untranslated region.

miR-466l directly targets the 3′UTRs of IFN-α mRNA species

To further investigate the underlying mechanisms responsible for the repressed IFN-α production by miR-466l, we investigated whether miR-466l could inhibit IFN-α production by modulating its transcription. Using a luciferase reporter assay, it was determined that miR-466l cotransfection did not influence the activation of IRF3, IRF7 or the construct containing the IFN-α promoter subsequent to the overexpression of a constitutively active RIG-I N-terminus CARD domain[32] (Figure 3). These results suggest that miR-466l has little effect on IFN-α mRNA transcription and may regulate IFN-α production post-transcriptionally.

As miRNAs function mainly through targeting the 3′UTRs of mRNAs, we investigated whether miR-466l could directly target the 3′UTR sequences of IFN-α mRNA species. Using TargetScan 5.0 (www.targetscan.org) prediction, we found that the 3′UTR sequences of several IFN-α mRNA species contained both conserved and poorly conserved multiple miR-466l target sites (Figure 4). We then constructed the luciferase reporter plasmids containing the 3′UTR sequences of these IFN-α mRNA species, including IFN-α1, -α2, -α4, -α8, -α10, -α13, -α16, -α17 and -α21, and determined whether miR-466l could directly target these sequences. Cotransfection of miR-466l was able to inhibit the expression of all these 3′UTR luciferase reporter plasmids (Figure 5). This result suggests that miR-466l can directly target the 3′UTR sequences of IFN-α mRNA species, thus inhibiting IFN-α production and enhancing viral replication.

DISCUSSION

Type I IFNs play critical roles in the host antiviral immune response. The production of type I IFNs is tightly regulated by multiple mechanisms so that a proper response against virus infection is induced. The excessive production of type I IFNs may promote the development of immuno-pathological conditions or immune disorders. Additionally, viruses have also developed several strategies to antagonize IFN signaling to ensure their survival in host cells. Here, we demonstrate that miR-466l targets the ARE sequences located in the 3′UTRs of many IFN-α species, including IFN-α1, -α2, -α4, -α8, -α10, -α13, -α16, -α17 and -α21, thus inhibiting IFN-α production and enhancing viral replication. Our data suggest a new direct negative regulator of IFN-α production, which may be applied in modulating IFN-α production against autoimmune diseases or viral infection.

The induction of miRNAs that regulate the host antiviral immune response in APCs has been previously reported.[22] However, we did not observe endogenous miR-466l levels influenced by viral infection (data not shown). This suggests that there are threshold levels of endogenous miR-466l required to prevent the expression of IFN-α species in resting APCs. Additionally, the varying expression levels of miR-466l observed in APCs may reflect different IFN-α-producing abilities of different cell types. A viral miRNA could also function as an orthologue of cellular miR-466l, suggesting a new approach in viral evasion of the IFN-α antiviral response.[13,14,35] Conversely, inhibiting the expression or activity of miR-466l may enhance host IFN-α production against viral infection. This strategy may be exploited to treat viral diseases.

Excessive production of type I IFNs may be one of the important mechanisms in the development of autoimmune diseases, such as systemic lupus erythematosus.[36,37] Here, miR-466l is suggested to be a negative regulator of multiple, proinflammatory IFN-α species. A previous study has also shown that miR-466l enhances the production of immune-suppressive IL-10.[26] Thus, enhancing the expression or activity of miR-466l may be a new approach for both the prevention of virus-induced cytokine storms and the treatment of autoimmune diseases.

The seed region sequence of miR-466l (AUAAAUA) is complementary to the typical ARE, which is located in the 3′UTRs of several cytokines, chemokines, growth factors and IFN-α species. Because miRNAs target multiple mRNAs to regulate gene expression, a single miRNA might regulate the protein synthesis of thousands of genes either directly or indirectly.[38] It is likely that miR-466l targets other cytokines and regulates multiple physiological or pathological processes. Consequently, further studies to reveal additional targets of miR-466l are warranted.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

ACKNOWLEDGEMENTS

This work was supported by grants from the National Natural Science Foundation of China (no. 81070880), and the China Postdoctoral Science Foundation funded project number 42201. The authors thank Ms Panpan Ma and Tingting Fang for their excellent technical assistance.

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