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Original article

miR-223 inhibits dengue virus replication by negatively regulating the microtubule-destabilizing protein STMN1 in EAhy926 cells

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Abstract

The pathogenesis of dengue virus (DENV) infection is not completely understood. Endothelial cells may act as a target of the virus and be involved in disease pathogenesis. Therefore, the identification of host cell components involved in DENV replication would provide useful information for better understanding DENV infection. In this study, a significantly decreased level of miR-223 was found in DENV2-infected EAhy926 cells, a human endothelial-like cell line, whereas miR-223 overexpression inhibited DENV2 replication. Furthermore, we identified that miR-223 directly targeted the 3′ untranslated region (3′UTR) of the messenger RNA (mRNA) for microtubule-destabilizing protein stathmin 1 (STMN1), thereby reducing its mRNA and protein levels. The depletion of miR-223 or overexpression of STMN1 enhanced DENV2 replication, whereas the opposite (increased miR-223 or decreased STMN1) suppressed DENV2 replication, indicating that miR-223 down-regulates STMN1 expression by targeting the 3′UTR of the *STMN1* gene to inhibit DENV2 replication. Finally, we demonstrated that two transcription factors, C/EBPα and E2F1, are involved in the regulation of miR-223 levels after DENV2 infection in EAhy926 cells. Collectively, our results suggest that miR-223 may act as a novel antiviral factor, which may open an avenue to limit DENV infection.

Keywords: Dengue virus; miR-223; STMN1; C/EBPα; E2F1

1. Introduction

Dengue virus (DENV), a member of the *Flaviviridae* family, is the etiologic agent of dengue fever (DF), which manifests as a non-specific febrile illness, i.e., dengue, but may advance to a more complicated and life-threatening clinical form, i.e., severe dengue, which includes dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). There are four antigenically distinct serotypes of DENV (DENV1-4). It is estimated that there are 390 million dengue infections per year, of which 96 million are apparently symptomatic. Therefore, dengue infection has reemerged as an important public health concern in endemic areas. However, there are no vaccines or specific anti-viral treatments currently available [1,2].

The pathogenesis of DENV infection is not completely understood. It is known that vascular leakage and hemorrhage, which are typical clinical manifestations, are often observed in severe dengue, suggesting that vascular endothelial cells (VECs) may act as a target for DENV and be involved in the pathogenesis of the disease [3]. However, the mechanism of how the infection alters endothelial function and/or morphology is not yet clear. In fact, the process of DENV infection occurs due to the interaction between DENV and certain components of the host cells. It has been reported that DENV virions are found in the region from the rough endoplasmic reticulum to the Golgi in infected cells [4]. After the nucleocapsids are assembled and acquire their envelopes and

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associated structures, some viral particles are transferred to the Golgi system for maturation and are then released by exocytosis. However, it remains unclear which regulator or component of the host cells is involved in the DENV replicative cycle during this process, and the answer would provide more details of DENV infection and further insight into therapies for dengue.

microRNAs (miRNAs) have recently been identified as key components of development and cellular homeostasis. miRNAs are endogenous, small (18-25 bp) noncoding RNAs that regulate gene expression mostly at the post-transcriptional level. miRNAs can silence genes through either perfect binding to the coding region of messenger RNA (mRNA), which results in degradation, or through imperfect binding to the 3′ untranslated region (3′UTR) of the mRNA, resulting in translational repression [5–7]. The specificity of miRNAs is thought to be primarily mediated by 2–8 nucleotides at the 5′ end of the miRNA, which is also known as the seed region [8,9]. miRNAs have been implicated in the regulation of diverse cellular processes. Recently, a role for cellular miRNAs against viral infection has been demonstrated in plants, insects, vertebrates, and mammals. It has been reported that cellular miR-122 enhances the replication of the hepatitis C virus (HCV) [10]; in contrast, cellular miR-32 effectively restricts the accumulation of the primate foamy virus type 1 retrovirus in human cells [11]. The Epstein–Barr virus (EBV) has been shown to trigger the expression of miR-21, miR-155, and miR-146a, which may be involved in the development of EBV-associated Burkett's lymphoma [12–14]. Cellular miR-17-5p, miR-20a, and miR-132 are important for regulating HIV-1 replication [15]. More recently, 11 upregulated and 4 downregulated miRNAs were identified in DENV2 infected peripheral blood mononuclear cells, and it was suggested that cyto kines and epigenetic regulators may be putative target genes of these miRNAs [16]. However, little is known about the detailed roles of cellular miRNAs in DENV infection.

In our preliminary experiments, a miRNA microarray and qRT-PCR revealed that miR-223 levels were significantly reduced in DENV2-infected EAhy926 cells. In this study, we further demonstrated that miR-223 inhibits DENV2 replication by negatively regulating the microtubule (MT)-destabilizing protein stathmin 1 (STMN1). In addition, two transcription factors, CCAAT/enhancer-binding protein α (C/EBPα) and E2F1, were found to be likely associated with the down-regulation of miR-223 levels induced by DENV2 infection. These results may contribute to increased understanding of the pathogenesis of the disease and provide a possible approach for limiting DENV infection.

2. Materials and methods

2.1. Cells and virus

The EAhy926 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) containing 15% fetal bovine serum (FBS; Gibco, USA). African green monkey kidney (Vero) cells were grown in modified Eagle's medium (MEM; Gibco, USA) with 5% FBS. *Aedes albopictus* mosquito cells (C6/36) were cultured in RPMI 1640 (Gibco, USA) containing 12% FBS at 28 °C.

DENV2 (strain TR1751), which was isolated from a patient with DF, was propagated in C6/36 cells and stored at −80 °C until use. The titer was determined by plaque assay.

2.2. miRNA microarray preparation after DENV2 infection in EAhy926 cells

EAhy926 cells were infected with DENV2 at a multiplicity of infection (MOI) of 1 for 1 h at 37 °C; the lysate was collected at 24 h, and total RNA was extracted using the TRIzol reagent (Invitrogen, USA). The RNA samples were labeled using Hy3™ and hybridized to the miRCURY™ LNA Array (v.16.0) (Exiqon, Denmark) to prepare the miRNA microarray. The RNA processing, microarray fabrication, array hybridization, and data acquisition were performed by KangChen Bio-tech (Shanghai, China).

2.3. Construction of plasmids and cell screening

The miR-223 inhibition plasmid (pmiR-223-down) and the miR-223 expression plasmid (pmiR-223-up) were constructed using the eukaryotic expression vector pGV249 or pGV251 to express the miR-223 complementary sequence (TGGGGTATTTGACAAACTGACA) or the pre-miR-223 sequence (CCTGGCCTCCTGCAGTGCCACGCTCCGTGTATTTGACAAGCTGAGTTGGACACTCCATGTGGTAGAGTGTCAGTTTGTCAAATACCCCAAGTGCGGCACATGCTTACCAG). The RNAi plasmid against STMN1 (pSTMN1-down), which contained a target sequence for STMN1 (GAGAGAAGGATAAGCACAT) was constructed in the EASY-shRNA eukaryotic expression vector pGV248, and the overexpression plasmid for STMN1 (pSTMN1-up), which encodes the STMN1 sequence (NM_203401.1), was constructed in the eukaryotic expression vector pGV230. All vectors were constructed by Genechem Company (Shanghai, China). The empty vectors pmiR-223-cont1, pmiR-223-cont2, pSTMN1-cont1, and pSTMN1-cont2 were used as the control plasmids in this study.

EAhy926 cells were transfected with 1 μg pmiR-223-down, pmiR-223-up, pSTMN1-down, pSTMN1-up, or control plasmids using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. The cells were selected with G418 (200 μg/ml in DMEM with 15% FBS). The cell lines derived from the EAhy926 cells were identified by qRT-PCR and/or Western blotting and were named EAhy-223-down (expressing the miR-223 inhibitor), EAhy-223-up (overexpressing miR-223), EAhy-223-cont1 (miR-223-down-control), EAhy-223-cont2 (miR-223-up-control), EAhy-STMN1-down (expressing the STMN1 inhibitor), EAhy-STMN1-up (overexpressing STMN1), EAhy-STMN1-cont1 (EAhy-STMN1-down-control), and EAhy-STMN1-cont2 (EAhy-STMN1-up-control).

The pGL3 luciferase reporter vector (Promega, USA) was used as the cloning vector for the reporter gene assay to

analyze the potential target region of miR-223. A 120-bp fragment containing the predicted miR-223 binding sites of the *STMN1* 3′UTR was amplified by PCR using cDNA and the primers STMN1 3′UTR-forward and STMN1 3′UTR-reverse (Table S1). The product was subcloned into the KpnI and XhoI sites of the pGL3-basic vector to generate the reporter plasmid pGLuc-*STMN1* 3′UTR. Another five primers, STMN1 3′UTR-forward2, STMN1 3′UTR-mutant1-forward, STMN1 3′UTR-mutant1-reverse, STMN1 3′UTR-mutant2-forward, and STMN1 3′UTR-mutant2-reverse (Table S1), were used to generate plasmids expressing mutant sequences. The corresponding mutant constructs, pGLuc-*STMN1* 3′UTR-mutant1 and pGLuc-*STMN1* 3′UTR-mutant2, were created by deleting or mutating the binding sites for the seed region of miR-223 (Fig. 3A). All constructs were confirmed by DNA sequencing.

2.4. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) for miRNA or mRNA quantification

DENV2-infected cells (MOI = 1) were collected for miRNA or mRNA quantification at 24 h. Total RNA was extracted using the TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol. After eliminating DNA contamination, 10 μg of total RNA was polyadenylated with ATP using *Escherichia coli* poly(A) polymerase (New England Biolabs, USA). The polyadenylated total RNA was reverse transcribed using EasyScript™ First-Strand cDNA Synthesis SuperMix (Transgen, China) with specific RT primers according to the manufacturer's protocol. Total RNA was also reversely transcribed with oligo(dT) for the mRNA levels. qRT-PCR was performed according to the protocol of the Maxima SYBR Green/ROX qRT-PCR Master Mix (Fermentas, USA) using an ABI 7500 (Applied Biosystems, USA). The level of the U6 small nuclear RNA was used as an endogenous control for miRNAs, and β-actin was used for mRNAs. The qRT-PCR procedure consisted of predenaturation at 95 °C for 5 min and 40 cycles of 95 °C for 30 s, 55 °C for 20 s, and 72 °C for 35 s. The relative expression level was calculated using the $2^{-\Delta\Delta Ct}$ method [17]. Three independent experiments were performed. The primer sequences are listed in Table S1.

2.5. Luciferase reporter gene assay

EAhy-223-cont2 and EAhy-223-up cells were co-transfected with 200 ng pGL3-basic containing the wild-type or mutated *STMN1* 3′UTR and 50 ng pRL-TK using Lipofectamine 2000 (Invitrogen, USA). After culturing for 48 h, the cells were lysed, and the luciferin reagent was added according to the manufacturer's protocol (Promega, USA). The relative firefly luciferase activity against the internal control of firefly luciferase was measured by the Dual-Luciferase Reporter Assay System (Promega, USA) with a GlOMAX™ 20/20 LUMINOMETER (Promega, USA). All experiments were performed independently at least three times.

2.6. Western blotting

Lysates from EAhy926, EAhy-223-down, EAhy-223-up, EAhy-STMN1-down, EAhy-STMN1-up, and control cells with or without DENV2 infection were obtained using RIPA lysis buffer. Cell lysates were separated by SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride (PVDF) membrane (GE Healthcare, USA). Subsequently, anti-STMN1 (1:1000 dilution; BD,

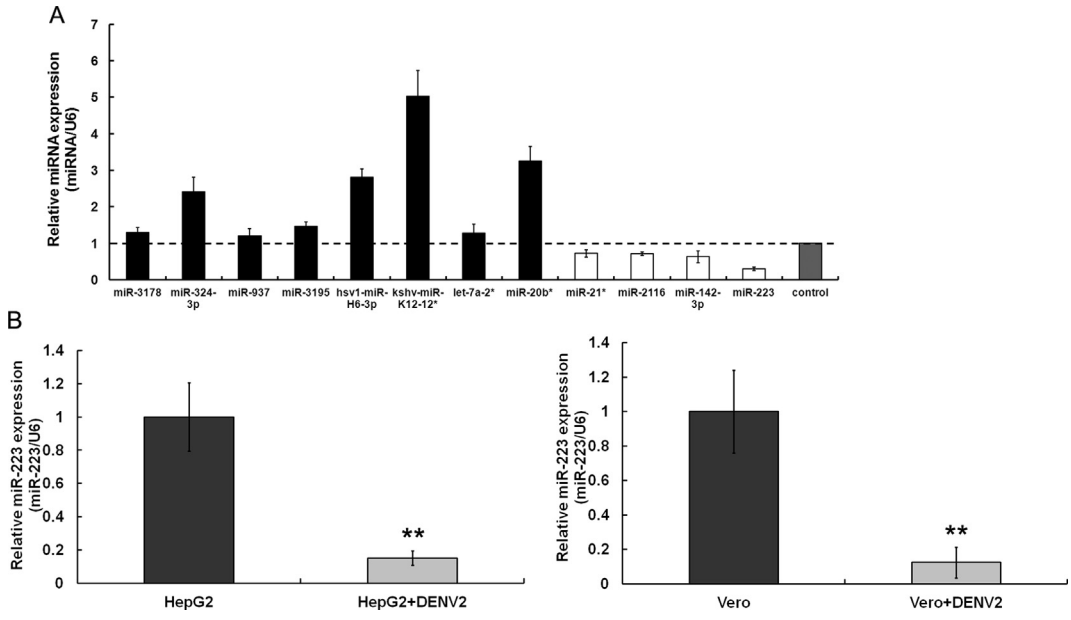

Fig. 1. Identification of miRNA expression at 24 h after DENV2 infection (MOI = 1). (A) miRNA expression levels were analyzed by qRT-PCR in EAhy926 cells with or without DENV2 infection. (B) Expression levels of miR-223 were determined by qRT-PCR in HepG2 and Vero cells with or without DENV2 infection. $^{**}P < 0.01$ vs. HepG2 or Vero cells. The data represent the mean ± SD from three independent experiments relative to mock-infected cells.

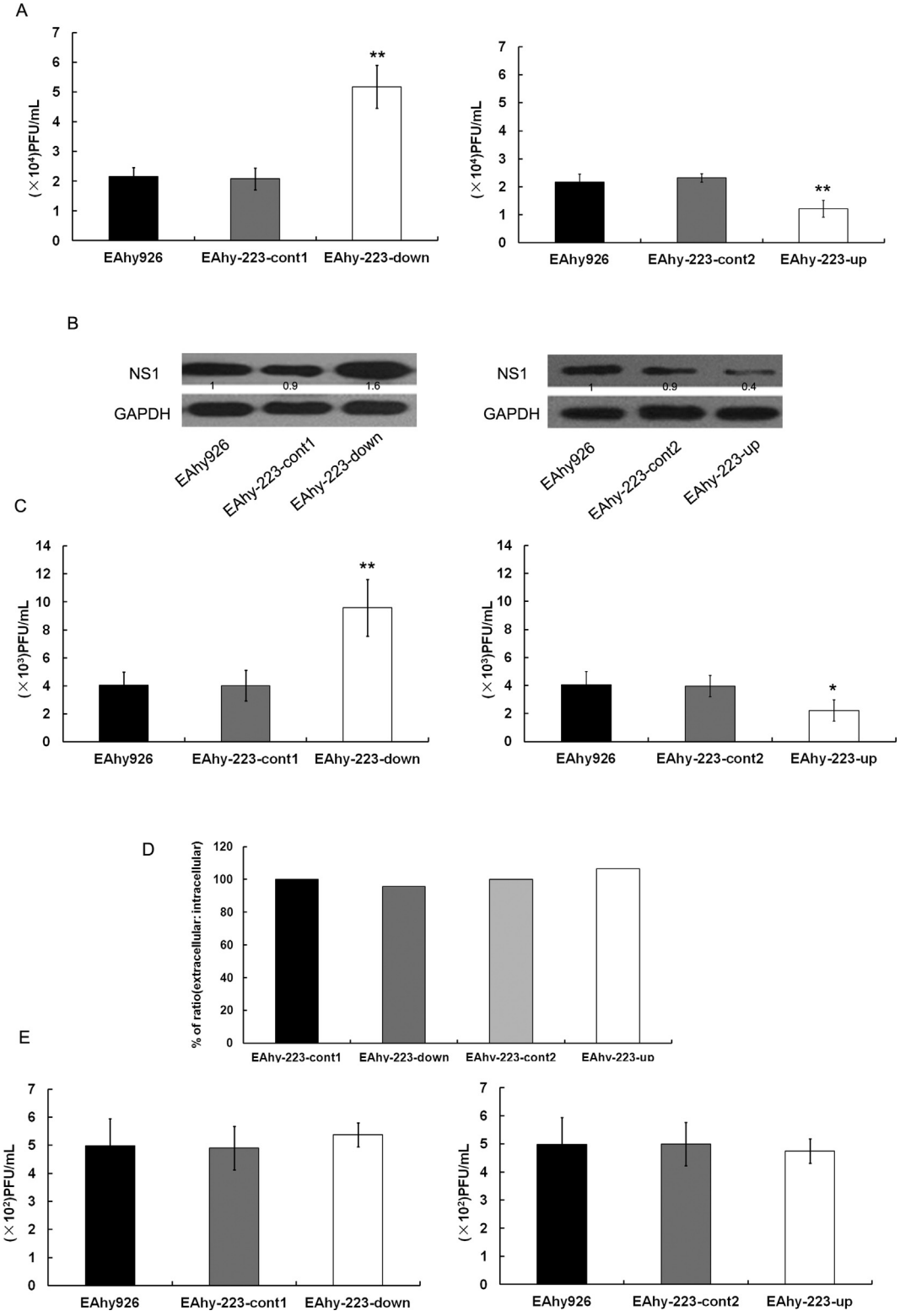

Fig. 2. The effect of miR-223 on DENV2 infection. (A) The lysates of EAhy-223-down and EAhy-223-up cells were collected at 24 h after infection and intracellular DENV2 titers were determined by plaque assay ($n = 3$). (B) Expression of NS1 protein in EAhy-223-down and EAhy-223-up cells was detected by Western blotting. GAPDH was used as loading control. (C) DENV2 viral titer in supernatants of EAhy-223-down and EAhy-223-up cells was determined by plaque assay ($n = 3$). (D) The ratios of extracellular to intracellular viral titer are shown as percentages. (E) Entry of DENV2 into EAhy-223-down and EAhy-223-up cells was determined by plaque assay ($n = 3$). The data represent the mean ± SD from three independent experiments. *$P < 0.05$ vs. control cells. **$P < 0.01$ vs. control cells.

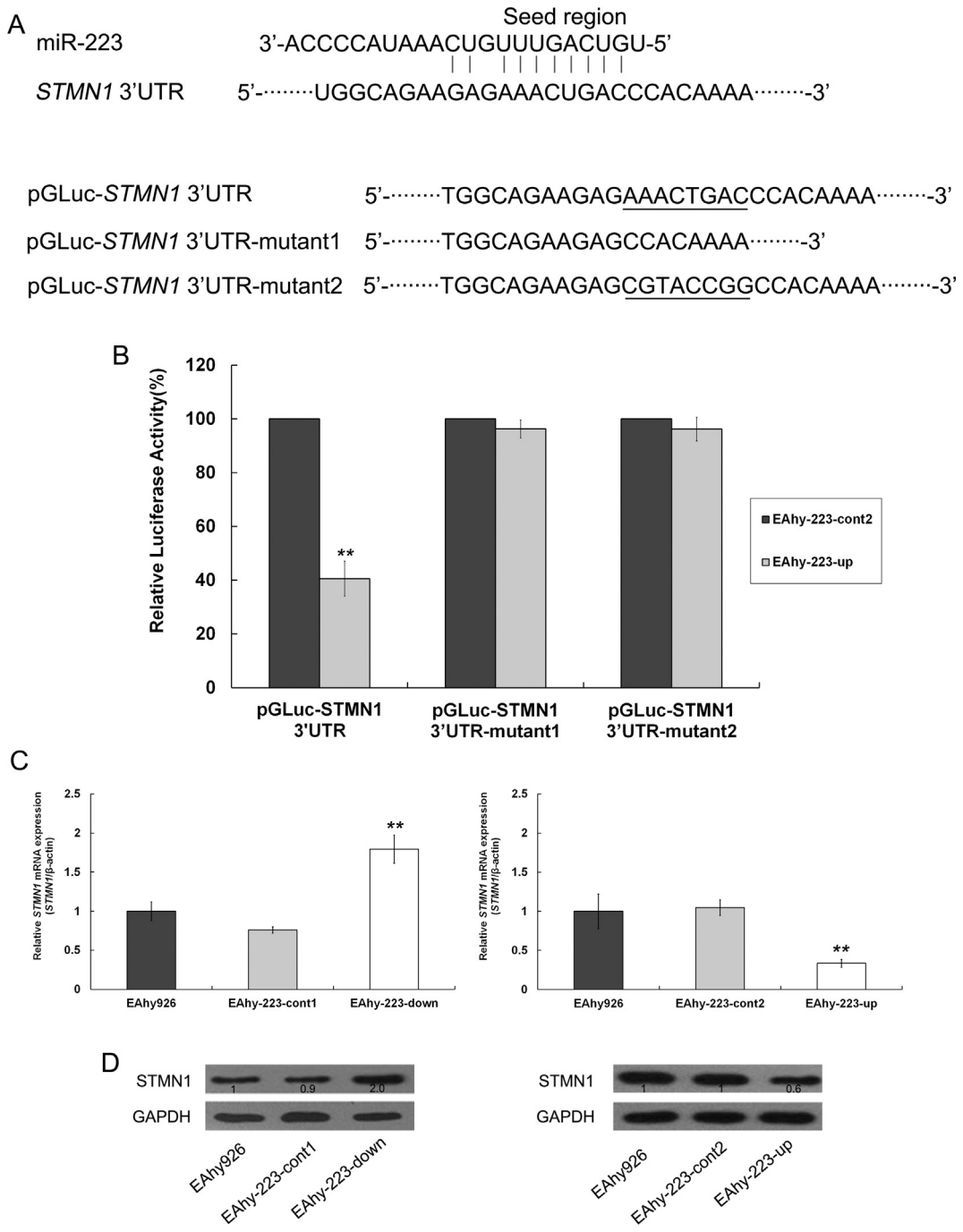

Fig. 3. STMN1 is down-regulated by miR-223. (A) Putative miR-223 binding site in the *STMN1* 3′UTR. A *STMN1* 3′UTR plasmid (pGLuc-*STMN1* 3′UTR) and two mutant plasmids (pGLuc-*STMN1* 3′UTR-mutant1 and pGLuc-*STMN1* 3′UTR-mutant2) were constructed with mutations in the underlined positions that pair with the sequence of the miR-223 seed region. (B) Luciferase activity of pGLuc-*STMN1* 3′UTR, pGLuc-*STMN1* 3′UTR-mutant1, or pGLuc-*STMN1* 3′UTR-mutant2 in EAhy-223-cont2 and EAhy-223-up cells was determined at 48 h after transfection. Renilla luciferase activities normalized for firefly luciferase are presented. (C) The mRNA expression levels of *STMN1* in EAhy-223-down, EAhy-223-up and control cells were detected by qRT-PCR. (D) The expression levels of STMN1 in EAhy926, EAhy-223-down, EAhy-223-up and control cells were detected by Western blotting. GAPDH was used as loading control. The values of the mean ± SD are from three independent experiments. $^{**}P < 0.01$ vs. control cells.

USA), anti-E2F1 (1:400 dilution; Santa Cruz Biotechnology, Inc., USA), anti-C/EBPα (1:1000 dilution; Cell Signaling Technology, USA) or anti-NS1 (1:1000 dilution; gift of Professor X.Y. Che, China) was used as the primary antibody. GAPDH was detected with an anti-GAPDH polyclonal antibody (1:1000; Beijing Protein Innovation, China) to demonstrate equal loading of protein samples. After incubation with a secondary antibody conjugated to horseradish peroxidase, the protein bands were incubated with ECL™ Western Blot Detection Reagents (GE Healthcare, USA), captured on film, and analyzed with the Quantity one software.

2.7. Methylation analysis of the miR-223 sequence

Genomic DNA was isolated and purified from the EAhy926 cells with or without DENV2 infection using the Biomed DNA Kit (Biomed, Beijing, China) according to the manufacturer's protocol. A total of 2 μg of genomic DNA per sample was bisulfite treated using the EpiTect Fast DNA Bisulfite kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The methylation-specific PCR (MSP) for the promoter of pri-miR-223 and the coding region of pre-miR-223 was performed using specific primers (Table S1). The products were sequenced by the SinoGenoMax Company (Beijing, China) and then analyzed with DNAMAN software.

2.8. Statistical analysis

Statistical analysis was performed with SPSS 16.0. The quantitative data between two groups were compared using a *t*-test. Differences among the groups were considered to be significant at $P < 0.05$.

3. Results

3.1. miR-223 is down-regulated in DENV2-infected EAhy926, HepG2 and Vero cells

By miRNA microarray and qRT-PCR, 12 miRNAs showed notable changes in expression level in DENV2-infected EAhy926 cells, including 8 up-regulated miRNAs and 4 down-regulated miRNAs when compared with mock-infected cells (Fig. 1A). Among these miRNAs, the level of miR-223 significantly decreased, with a reduction to 30% of that of mock-infected cells. Moreover, similar results were observed in HepG2 and Vero cells at 24 h after infection. As shown in Fig. 1B, miR-223 levels in HepG2 and Vero cells were reduced to 15% and 12%, respectively, of control cells. Our data indicated that DENV2 infection down-regulated the expression of miR-223.

3.2. Involvement of miR-223 in DENV2 replication

To investigate whether miR-223 is involved in the replication cycle of DENV2, EAhy926 cells were transfected with the pmiR-223-down, pmiR-223-cont1, pmiR-223-up, or pmiR-223-cont2 plasmids. After screening, the derived cells were named EAhy-223-down, EAhy-223-cont1, EAhy-223-up, and EAhy-223-cont2, respectively. qRT-PCR revealed that miR-223 expression was significantly decreased to one-third of that of the control cells in the EAhy-223-down cells and increased by 3.5-fold in the EAhy-223-up cells compared with the control cells (Supplemantary 1). These results indicated that these cells were appropriate for the subsequent experiments.

At 24 h after infection, the viral titers in EAhy-223-down, EAhy-223-cont1, EAhy-223-up, and EAhy-223-cont2 cell lysates were collected to determine the effect of miR-223 on viral replication. Significantly increased DENV2 titers of 2.6-fold were observed in the EAhy-223-down cells compared with the EAhy-223-cont1 titers (Fig. 2A). Accordingly, a markedly elevated level of the viral protein NS1 (1.6-fold), an indicator of DENV replication, was also observed in the EAhy-223-down cells by Western blotting (Fig. 2B). In contrast, the viral titer in the EAhy-223-up cells was reduced to 50% of that in the EAhy-223-cont2 cells (Fig. 2A), and the NS1 protein level was correspondingly decreased (0.4-fold) (Fig. 2B). These results indicated that miR-223 inhibited DENV2 replication in EAhy926 cells.

To confirm the effect of miR-223 on viral release, supernatants of the EAhy-223-down, EAhy-223-cont1, EAhy-223-up, and EAhy-223-cont2 cells were also collected at 24 h after infection to determine viral titer. Consistent with the change in viral titer in the cell lysates, a 2.4-fold increase in viral titer was observed in the supernatant of EAhy-223-down cells compared with the EAhy-223-cont1 cells, whereas a 40% reduction in viral titer was observed in EAhy-223-up cells compared with EAhy-223-cont2 cells (Fig. 2C). The ratios of extracellular to intracellular viral titers were calculated to evaluate the role of miR-223 in viral release. However, there were no significant differences in the ratios between EAhy-223-down and EAhy-223-cont1 cells or between EAhy-223-up and EAhy-223-cont2 cells (Fig. 2D). These results implied that the decreased viral quantities in the supernatant were due to reduced viral replication, indicating that altered miR-223 expression did not influence viral release from EAhy926 cells.

Additionally, lysates of EAhy-223-down, EAhy-223-cont1, EAhy-223-up, and EAhy-223-cont2 cells were collected at 1 h after infection to investigate the effect of miR-223 on viral entry. The results showed that there were no differences in the viral titer among all the cell lines (Fig. 2E), indicating that the entry of DENV2 into EAhy926 cells was not affected by miR-223.

3.3. Identification of the target genes for miR-223

We used three miRNA target prediction software programs, miRanda (http://www.microrna.org), PicTar (http://pictar.mdc-berlin.de/), and TargetScan (http://www.targetscan.org/), to predict the target genes for miR-223. Among the many detected targets, we focused on STMN1, a prominent microtubule destabilizer that preferentially acts on microtubule minus ends. The target sequence from nucleotides 662 to 669 in the 3′UTR of the *STMN1* gene was predicted to have complete complementarity to the miR-223 seed region (Fig. 3A). To verify whether *STMN1* mRNA is a direct target of miR-223, the 3′UTR and two mutated 3′UTRs of the *STMN1* gene were cloned into the pGL3-basic vector, and the plasmids pGLuc-STMN1 3′UTR, pGLuc-STMN1 3′UTR-mutant1 and pGLuc-STMN1 3′UTR-mutant2 were constructed (Fig. 3A). The EAhy-223-up and EAhy-223-cont2 cells were transfected with the plasmids to determine the interaction of the target sequences with the miR-223 seed region. Compared with EAhy-223-cont2 cells, the expression of the *STMN1* 3′UTR was repressed by approximately 60% after transfection of pGLuc-STMN1 3′UTR (the wild-type

construct) in the EAhy-223-up cells, indicating that the exogenous expression of miR-223 substantially repressed the expression of the *STMN1* 3′UTR. However, this inhibitory effect was not observed in the EAhy-223-up cells transfected with the pGLuc-STMN1 3′UTR-mutant1 and pGLuc-STMN1 3′UTR-mutant2 plasmids (Fig. 3B), indicating that the deletion or mutation of the miR-223 binding site completely abrogated the miR-223-dependent repressive effect on the *STMN1* 3′UTR. These data demonstrated that miR-223 directly interacts with the *STMN1* 3′UTR.

To assess whether miR-223 negatively regulates the activation of STMN1 in EAhy926 cells, the levels of STMN1 mRNA and protein were measured in EAhy926, EAhy-223-down, EAhy-223-up, and control cells by qRT-PCR and Western blotting, respectively. The expression level of *STMN1* mRNA in EAhy-223-down cells was 1.8 times higher than that in EAhy-223-cont1 cells (Fig. 3C), and the protein level of STMN1 also increased (2.0-fold) (Fig. 3D). In contrast, the expression level of *STMN1* mRNA was reduced to 40% of that in EAhy-223-cont2 cells in the EAhy-223-up cells (Fig. 3C), and the protein level of STMN1 was correspondingly changed (0.6-fold) (Fig. 3D). These results showed that miR-223 down-regulation elevated the mRNA and protein levels of STMN1, whereas miR-223 overexpression reduced the mRNA and protein levels of STMN1, indicating that miR-223 is critical for controlling the activation of STMN1 in EAhy926 cells via negative regulation.

3.4. STMN1 functions in DENV2 replication

To investigate the relationship between STMN1 and DENV2 infection, the expression level of STMN1 in EAhy926 cells was determined at 24 h after infection. As shown in Fig. 4A, the mRNA level of *STMN1* in infected cells was 63-fold higher than that of mock-infected cells. Accordingly, the expression of STMN1 protein also increased

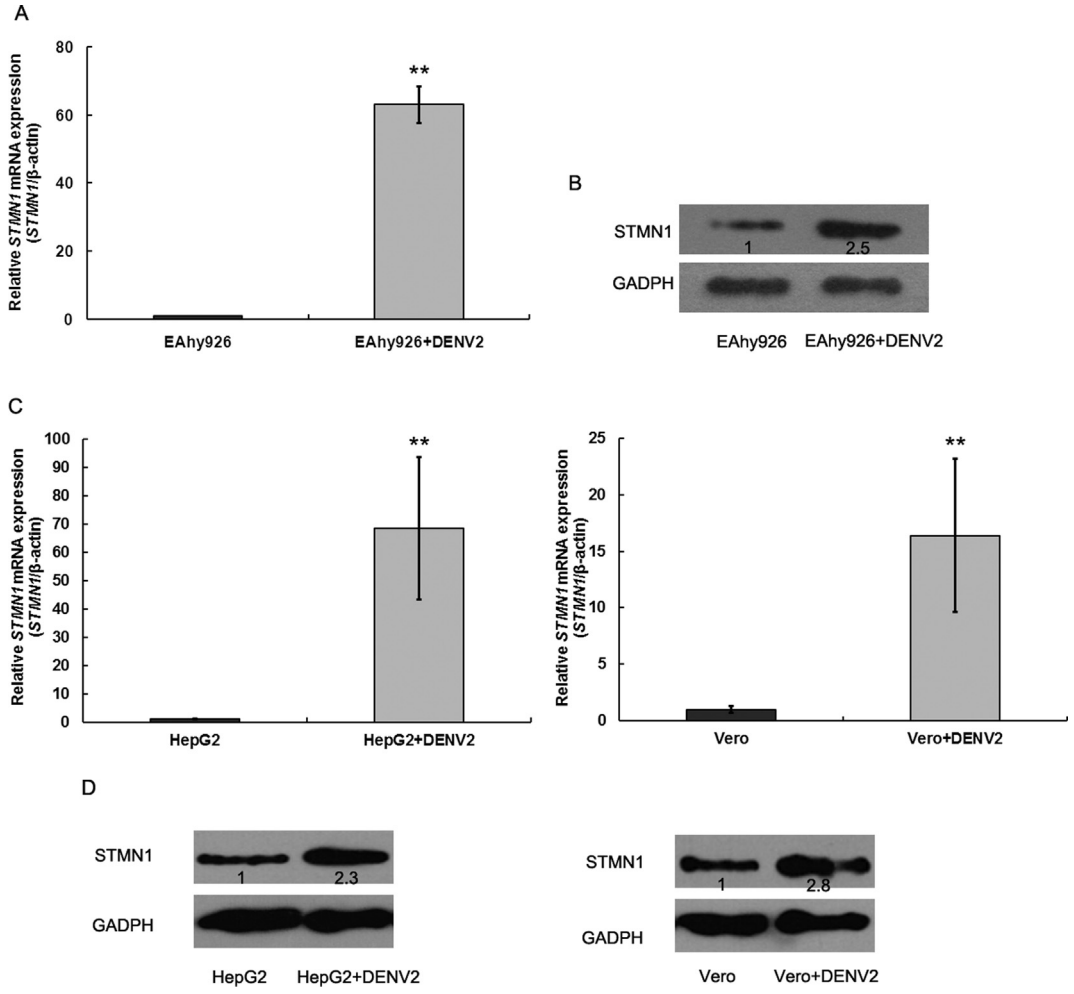

Fig. 4. Determination of STMN1 expression level in cells at 24 h after DENV2 infection (MOI = 1). (A) The mRNA levels of *STMN1* were analyzed by qRT-PCR in EAhy926 cells with or without DENV2 infection. The data represent the mean ± SD from three independent experiments relative to mock-infected cells. $^{**}P < 0.01$ vs. EAhy926 cells. (B) The expression levels of STMN1 were determined by Western blotting in EAhy926 cells with or without DENV2 infection. GAPDH was used as loading control. (C) The mRNA levels of *STMN1* in HepG2 and Vero cells with or without DENV2 infection were analyzed by qRT-PCR. The data represent the mean ± SD from three independent experiments relative to mock-infected cells. $^{**}P < 0.01$ vs. HepG2 or Vero cells. (D) The expression level of STMN1 in HepG2 and Vero cells with or without DENV2 infection was determined by Western blotting. GAPDH was used as loading control.

(2.5-fold) (Fig. 4B). Furthermore, in HepG2 and Vero cells at 24 h after DENV2 infection, the mRNA level of STMN1 increased by 65-fold and 17-fold, respectively, and levels of STMN1 protein were also correspondingly enhanced (2.3-fold and 2.8-fold, respectively) in both cell lines (Fig. 4C &D). This indicated that DENV2 infection induced STMN1 expression.

To investigate the effect of STMN1 on DENV2 replication in EAhy926 cells, EAhy-STMN1-down, EAhy-STMN1-cont1, EAhy-STMN1-up, and EAhy-STMN1-cont2 cells were established by transfection with the pSTMN1-down, pSTMN1-cont1, pSTMN1-up, and pSTMN1-cont2 plasmids, respectively. By qRT-PCR, the mRNA levels of *STMN1* were found to be significantly reduced to 20% in the EAhy-STMN1-down cells compared to the EAhy-STMN1-cont1 (Supplemantary 2A). In contrast, the *STMN1* mRNA levels were markedly increased in the EAhy-STMN1-up cells and were 6.5 times higher than that in the EAhy-STMN1-cont2 cells (Supplemantary 2A). Western blotting demonstrated that the expression levels of the STMN1 protein were consistent with mRNA levels (Supplemantary 2B): expression of the STMN1 protein was reduced (0.1-fold) in the EAhy-STMN1-down cells and increased (2.1-fold) in the EAhy-STMN1-up cells. These results indicated that the cells with down-regulated or up-regulated STMN1 were appropriate for the subsequent experiments.

Furthermore, the EAhy-STMN1-down, EAhy-STMN1-cont1, EAhy-STMN1-up, and EAhy-STMN1-cont2 cells were infected with DENV2 to analyze the effect of STMN1 on DENV2 replication. An inhibitory effect on DENV2 replication was observed in the EAhy-STMN1-down cells, where the viral titer reduced to 40% of that in the EAhy-STMN1-cont1 cells (Fig. 5A); Western blotting also showed decreased NS1 protein level (0.5-fold) in the EAhy-STMN1-down cells (Fig. 5B). A significantly increased DENV2 titer of approximately 2.2-fold was observed in the EAhy-STMN1-up cells compared with the EAhy-STMN1-cont2 cells (Fig. 5A), and an elevated DENV2 NS1 protein level (1.7-fold) was also observed in the EAhy-STMN1-up cells (Fig. 5B). Meanwhile, the supernatants were also collected at 24 h after DENV2 infection to determine extracellular viral titers. A 50% decreased viral titer was observed in the supernatant of EAhy-STMN1-down cells compared with EAhy-STMN1-cont1 cells; as expected, a 2-fold increase in viral titer was detected in the supernatant of EAhy-STMN1-up cells compared with EAhy-STMN1-cont2 cells (Fig. 5C). The ratio of extracellular to intracellular infectious particles was then calculated to evaluate the effect of STMN1 on viral release. However, no significant differences in the ratios of viral titers between EAhy-STMN1-down and EAhy-STMN1-cont1 cells or between EAhy-STMN1-up and EAhy-STMN1-cont2 cells were found (Fig. 5D). Our data indicated that STMN1 did not affect DENV2 release from EAhy926 cells. We also investigated the effect of STMN1 on the entry of DENV2 into EAhy926 cells and found no differences in intracellular viral titer among all the cell lines (Fig. 5E), indicating that the entry of DENV2 was not influenced by STMN1.

3.5. Possible mechanisms of miR-223 down-regulation induced by DENV2 infection

To explore the possible mechanism of miR-223 down-regulation in DENV2-infected EAhy926 cells, we detected and analyzed hypermethylation of the promoter sequences of miR-223. Although there are several CpG dinucleotides in the promoter region of miR-223 [18], no hypermethylation was detected in the promoter sequences of miR-223 in DENV2-infected cells compared with uninfected cells. These results indicated that hypermethylation of miR-223 sequences might be not involved in DENV2-induced miR-223 down-regulation. Other factors likely play an important role in this process.

Previous studies demonstrated that miR-223 expression is mediated by several regulators [18–22]. Among many factors, two transcription factors, C/EBPα and E2F1, were found to be association with down-regulation of miR-223 in this study. In mRNA levels, C/EBPα decreased to 35% of that in control cells, and E2F1 increased to 12.5-fold compared with control cells at 24 h after DENV2 infection (Fig. 6A). Meanwhile, Western blotting demonstrated that the levels of the C/EBPα and E2F1 proteins were consistent with the changes in mRNA levels: C/EBPα was reduced (0.5-fold), whereas E2F1 was increased (1.6-fold) at the same time point after infection (Fig. 6B). It has been reported that C/EBPα and E2F1 bind to the miR-223 promoter to elevate or inhibit the transcription of miR-223, whereas miR-223 targets the E2F1 3′UTR to reduce E2F1 translation [20]. The results suggested that the decreased levels of C/EBPα and increased levels of E2F1 likely contribute to the down-regulation of miR-223 induced by DENV2 infection.

4. Discussion

Although almost half of the world's population is at risk for dengue infection, there is a lack of licensed vaccines and specific anti-viral therapies against dengue disease [1,2]. Thus, clarifying the pathogenesis of dengue infection and identifying new therapeutic approaches are necessary to treat dengue infection. It is known that miRNAs inhibit viral infection in addition to playing a general role in cell differentiation, proliferation, apoptosis, and immunity [23–25]. However, there are few reports on the altered expression level of miRNAs in dengue infection, and their precise roles are not clear [16]. In this study, we focused on the changes in miRNA expression during DENV infection to determine the involvement of miRNAs in the DENV replication cycle and pathogenesis and to provide insight into therapies for DENV disease.

We found that DENV2 infection significantly decreased the expression of miR-223 in EAhy926, HepG2 and Vero cells, indicating that the down-regulation of miR-223 may be a common event in DENV infection. The overexpression of miR-223 suppressed DENV2 replication in EAhy926 cells, implying that miR-223 may be an antiviral miRNA against DENV2 replication. Although a functional viral small RNA-5 (vsRNA-5) encoded by DENV has been newly reported and inhibits the replication of DENV1, 2, and 4 by targeting the

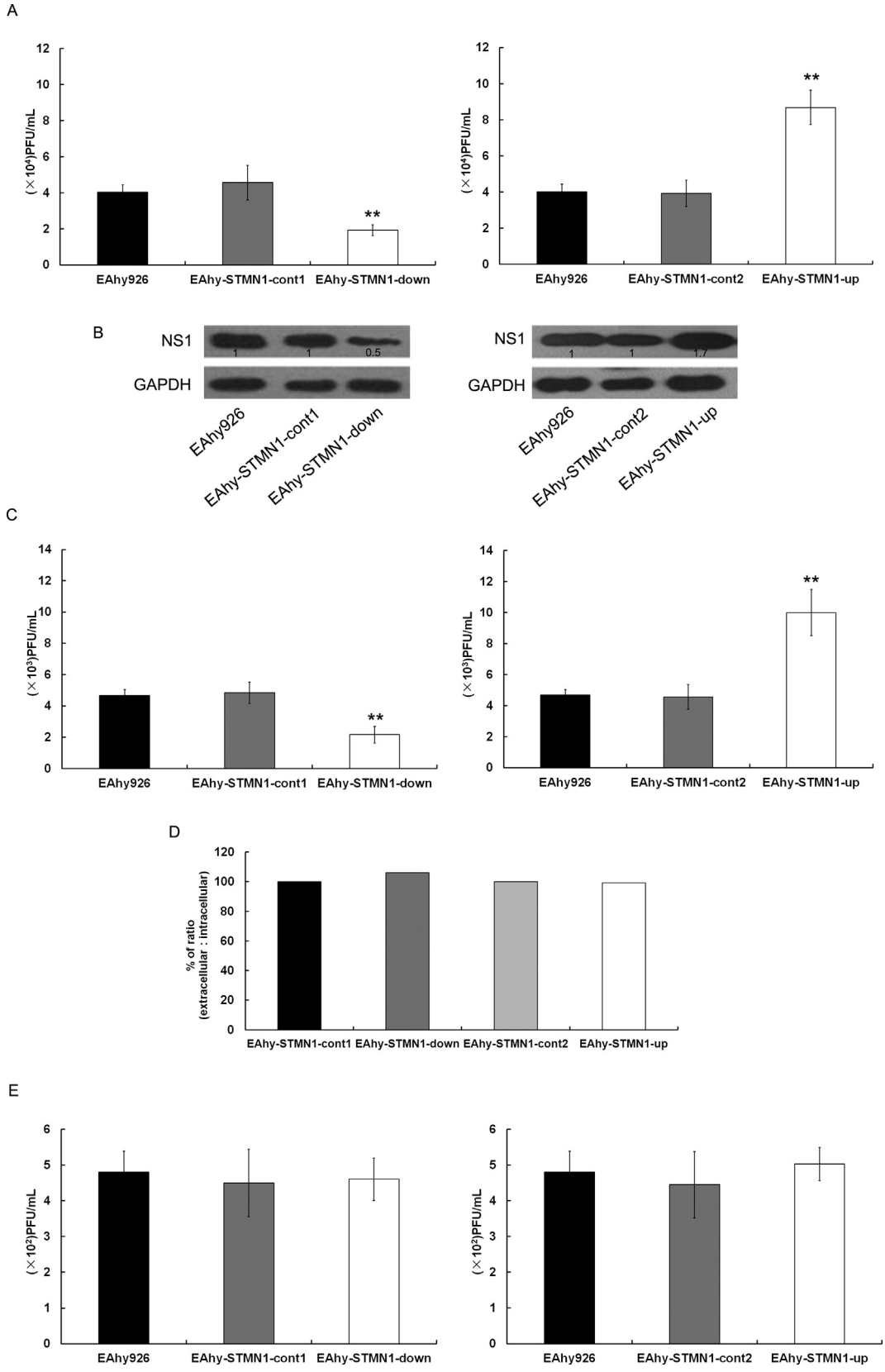

Fig. 5. Identification of STMN1 functions in DENV2 infection. (A) The lysates of EAhy-STMN1-up and EAhy-STMN1-down cells were collected at 24 h after infection and intracellular DENV2 titers was determined by plaque assay ($n = 3$). (B) DENV2 replication as indicated by NS1 levels in EAhy-STMN1-up and EAhy-STMN1-down cells was determined by Western blotting. GAPDH was used as loading control. (C) DENV2 viral titers in supernatants of EAhy-STMN1-up and EAhy-STMN1-down cells were determined by plaque assay ($n = 3$). (D) The ratios of extracellular to intracellular viral titer are shown as percentages. (E) Entry of DENV2 into EAhy-STMN1-up and EAhy-STMN1-down cells was determined by plaque assay ($n = 3$). The values of the mean ± SD are from three independent experiments. $^{**}P < 0.01$ vs. control cells.

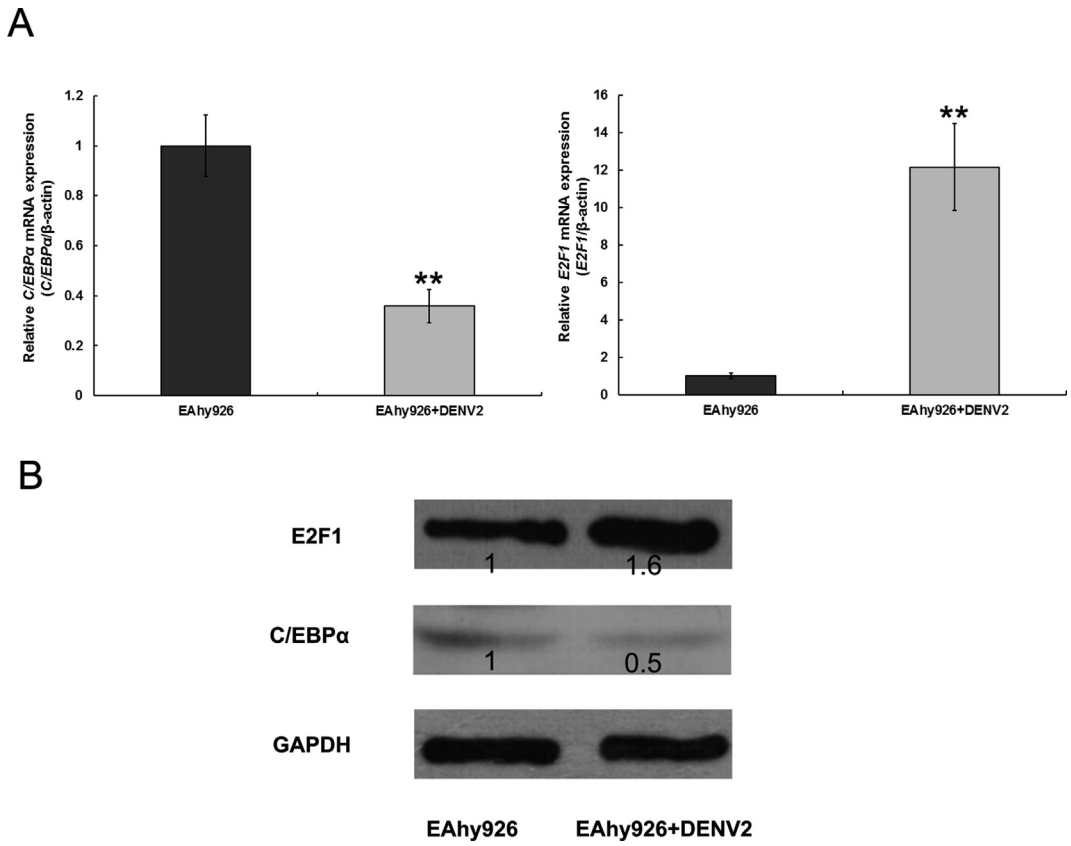

Fig. 6. Identification of possible mechanisms of miR-223 down-regulation induced by DENV-2 infection. (A) The mRNA levels of *C/EBPα* and *E2F1* in EAhy926 cells with or without DENV2 infection were determined by qRT-PCR. (B) The expression levels of C/EBPα and E2F1 in EAhy926 cells with or without DENV2 infection were determined by Western blotting. GAPDH was used as a loading control. The values of the mean ± SD are from three independent experiments. $^{**}P < 0.01$ vs. control cells.

virus nonstructural protein 1 gene [26], the development of antiviral drugs from host factors has the advantage of limiting the emergence of drug-resistant viral variants. The identification of miR-223 as an inhibitor of DENV2 replication may provide a possible approach to limit DENV infection.

Recently, a large amount of evidence has indicated that cellular miRNAs serve as critical effectors in the intricate networks of cellular physiology and in host–pathogen interactions. It was reported that miR-223 functions as a modulator in the differentiation of human cells. For example, miR-223 is markedly down-regulated during erythroid and granulocytic differentiation and maturation and is necessary for erythropoiesis [27,28]. It was also found that the down-regulation of miR-223 is involved in the pathogenesis of hepatocellular carcinoma (HCC) [19,29]. Specifically, it has been reported that miR-223 plays a role in the pathogenesis of certain infectious diseases, such as influenza and chronic hepatitis B virus infection [23,25]. Moreover, an anti-viral effect for miR-223 was observed in SARS coronavirus-infected bronchoalveolar stem cells and in freshly isolated HIV-1-infected monocytes [24,30]. However, less is known about the roles of miRNAs, especially miR-223, in DENV infection. Interestingly, miRNA expression profiling has been described in DENV2-infected peripheral blood mononuclear cells [16]. However, further investigation into the effect of miRNAs on DENV infection is lacking. Here, we demonstrate that miR-223 inhibits DENV2 replication and may act as a novel antiviral host factor against DENV2. Further studies will be required to investigate miR-223 as a possible therapeutic target for all DENV serotypes.

We also identified the specific target acted upon by miR-223, which consisted of 8 nucleotides in the 3′UTR of the mRNA for STMN1. miR-223 negatively modulated STMN1 by reducing its mRNA and protein levels in EAhy926 cells. STMN1 overexpression induced by DENV2 infection was also observed in HepG2 and Vero cells in addition to EAhy926 cells, indicating a common relationship between DENV infection and STMN1. Moreover, STMN1 expression promoted DENV2 replication in EAhy926 cells. Taken together, it is plausible that the reduced expression of miR-223 may result in an increase in DENV2 replication via the induction of STMN1 expression, suggesting that miR-223 modulates DENV2 replication by negatively regulating STMN1. However, because each miRNA likely targets multiple mRNAs, the involvement of additional targets of miR-223 in DENV2 infection cannot yet be ruled out.

STMN1 is a ubiquitous cytosolic phosphoprotein and key microtubule regulatory protein that controls MT dynamics by directly interacting with the α/β-tubulin dimer [31]. This intracellular protein is abundantly expressed in the central

nervous system, reproductive system, and VECs and is highly evolutionarily conserved among vertebrates. Previous reports that investigated the relationship between STMN1 and disease have mainly focused on the impact of STMN1 on the occurrence and development of cancers, and most of these reports indicated that increased levels of STMN1 were frequently observed in several types of cancers, including nasopharyngeal carcinoma, gastric cancer and cholangiocarcinoma, and were closely associated with poor disease prognosis [32–34]. Recently, it has been demonstrated that STMN1, as a downstream molecule of the STAT3 (signal transducers and activators of transcription 3) pathway, markedly increased HCV replication [35]. However, there has been no report on the role of STMN1 in DENV infection thus far. In this study, we found that DENV2 induced the overexpression of STMN1, which promoted viral replication, indicating that STMN1 is necessary for DENV2 infection.

Currently, the precise mechanisms of STMN1 involvement in DENV2 infection are not clear. However, there are some possible explanations: 1) it was reported that STAT3 positively regulates MT dynamics by the direct sequestration of STMN1 and that this, in turn, leads to an increase in HCV replication [35]. Furthermore, STAT3 activation was observed in DSS patients during the early stages of infection [36]. Accordingly, the increased levels of STMN1 observed in this study may promote DENV infection by a similar mechanism. Alternatively, 2) many viruses utilize components of the cellular machinery, such as actin and MTs, to promote intracellular transport and to reach specific sites of viral replication [37] and there is cooperation between actin and MTs in host cells. Our previous studies demonstrated that the Rho GTPase Rac1 plays an important role in DENV2 infection through the regulation of actin cytoskeleton rearrangements [38]. It is known that the actin cytoskeleton is a key factor involved in the endocytosis and phagocytosis of several pathogens. Thus, there may be a possibility that increased STMN1 affects actin function by changing MT dynamics, which may provide advantageous conditions, including energy and viral transportation microenvironment, that facilitate DENV2 replication. Further related studies are ongoing in our laboratory to examine and confirm these possible mechanisms of STMN1 action in DENV infection.

Taking our results together, we propose that DENV2 infection down-regulates the expression of miR-223, which enhances STMN1 expression and affects the function of STMN1. Then STMN1 promotes DENV2 replication, possibly by regulating MT dynamics in an indirect fashion. Our study revealed that a cellular miRNA modulates host gene expression to control DENV2 propagation, and these results may open an avenue for limiting DENV infection.

To investigate the mechanism of miR-223 down-regulation in DENV2-infected EAhy926 cells, we examined the expression levels of several transcription factors, such as C/EBPα, E2F1, NFI-A, and acute myelocytic leukemia (AML) 1/ETO, which likely bind to the promoter region of miR-223 [18–20]. Of them, the expression levels of two transcription factors, C/EBPα and E2F1, were substantially altered. C/EBPα is a key transcriptional regulator of granulocyte differentiation [39], and E2F1 is a master regulator for cell-cycle progression [40]. The function of miR-223 in granulopoiesis and AML has been connected to C/EBPα-mediated inhibition of E2F1 [20]. It was reported that C/EBPα binds to the miR-223 promoter to elevate miR-223 transcription and that miR-223 then targets the 3′UTR of E2F1, resulting in translational inhibition. However, E2F1 also binds to the miR-223 promoter and inhibits miR-223 transcription, generating a negative feedback loop. In this study, down-regulated miR-223 levels, decreased C/EBPα, and markedly increased E2F1 were observed after DENV2 infection. Taken together, the data from the previous study and our current results lead us to hypothesize that DENV-induced decreased expression of C/EBPα inhibits miR-223 expression, which then increases E2F1 levels, further promoting the inhibition of miR-223 by the binding of E2F1 to its promoter region. Therefore, our results suggest that these two transcription factors (C/EBPα and E2F1) are likely to be major contributors in DENV2-induced miR-223 down-regulation.

Generally, hypermethylation of promoter sequences is considered to be a key mechanism for the suppression of miRNAs [41,42]. However, we found that there were no sites of hypermethylation in the promoter region of miR-223. Thus, we hypothesize that methylation is not a mechanism of DENV2-induced miR-223 down-regulation.

In summary, we report here that the expression and function of a cellular miRNA, miR-223, are involved in DENV2 replication by negatively regulating STMN1. Moreover, we suggest that decreased C/EBPα and increased E2F1 played a major role in the DENV2-induced down-regulation of miR-223 via a negative feedback loop. Overall, this study reveals a mode of exploitation of the cellular miRNA machinery by viruses. Clarifying the role of miRNAs in viral replication will provide new insights into and further understanding of the pathogenesis of the disease and the control of viral infection.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.micinf.2014.08.011.

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