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Article

Enterovirus-Induced miR-141 Contributes to Shutoff of Host Protein Translation by Targeting the Translation Initiation Factor eIF4E

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SUMMARY

Viruses rely on the host translation machinery to complete their life cycles. Picornaviruses use an internal ribosome entry site to initiate cap-independent protein translation and in parallel host cap-dependent translation is shut off. This process is thought to occur primarily via cleavage of host translation initiation factors eIF4GI and eIF4GII by viral proteases. Here we describe another mechanism whereby miR-141 induced upon enterovirus infection targets the cap-dependent translation initiation factor, eIF4E, for shutoff of host protein synthesis. Knockdown of miR-141 reduces viral propagation, and silencing of eIF4E can completely reverse the inhibitory effect of the miR-141 antagomiR on viral propagation. Ectopic expression of miR-141 promotes the switch from cap-dependent to cap-independent translation. Moreover, we identified a transcription factor, EGR1, which is partly responsible for miR-141 induction in response to enterovirus infection. Our results suggest that upregulation of miR-141 upon enterovirus infection can facilitate viral propagation by expediting the translational switch.

INTRODUCTION

In general, viruses are predisposed to evolve new antigenic variations and drug resistances that impede the development of effective antiviral therapies. Nearly all viruses rely on the host translation machinery to complete their life cycles. Virus infection may induce shutoff of host protein synthesis, particularly in picornavirus, the protein translation of which is cap independent (Belsham and Sonenberg, 2000; Schneider and Mohr, 2003). Enteroviruses in the *Picornaviridae* family are important human pathogens that can cause fatal diseases including cardiopulmonary failure, aseptic meningitis, paralysis, myocarditis, and encephalomyelitis (Chang et al., 2007; Whitton et al., 2005). Recently, enterovirus 71 (EV71) has become a newly emerging life-threatening pathogen, particularly in the Asia-Pacific region (Chang et al., 2007; Wang et al., 2004).

The genome of picornaviruses is a single-stranded plus sense RNA molecule and uses internal ribosome entry site (IRES) to initiate protein translation, independent of cap structure. The IRES-mediated translation requires both canonical initiation factors and IRES transactivating factors (ITAFs), many of which have been proven to only facilitate cap-independent translation (Lin et al., 2009; Martinez-Salas et al., 2008). The ITAFs enhance IRES-mediated translation by acting as RNA chaperons of high-order structural organization or activators of IRES activity. The canonical initiation factors, eIF4G, eIF4A, eIF3, and eIF2, but not eIF4E, are required for IRES-mediated picornaviral translation. The picornavirus infection induced a dramatic inhibition of host protein synthesis (Goldstaub et al., 2000). Although eIF4GI and eIF4GII have different kinetics cleavages in certain picornavirus infections, it is well known that both eIF4GI and eIF4GII cleavages contributed to virus-mediated host protein synthesis shutoff (Gradi et al., 1998; Svitkin et al., 1999). In addition, the cleavage of poly(A)-binding protein (PABP) and dephosphorylation of eIF4E binding protein 1 (4E-BP1) as well as the eIF4E

relocalization have been reported to contribute to the shutoff of host protein synthesis (Gingras et al., 1996; Kuyumcu-Martinez et al., 2004; Sukarieh et al., 2010). Therefore, the host factors that are involved in the blockage of host protein synthesis during enterovirus infection might not be completely identified. eIF4E is a cap-binding protein guiding cap-dependent or cap-independent translation (Figure 1A) (Fraser and Doudna, 2007). Whether eIF4E is involved in this protein synthesis switch is still unclear and remains to be investigated.

MicroRNAs (miRNAs) are a recently discovered class of small non-protein-coding RNAs that may act via endogenous RNA interference (Hammond, 2006). By posttranscriptional regulation of target gene expression, miRNAs govern a wide range of biological functions including host-virus interaction (Umbach and Cullen, 2009). Nevertheless, our understanding of their role in the reciprocal interaction between virus and host is quite limited. Recent evidence indicates that certain DNA viruses encode miRNAs that are able to regulate viral or cellular gene expression (Gottwein et al., 2007; Pfeffer et al., 2004; Umbach et al., 2008). On the contrary, it is also known that certain cellular miRNAs could modulate human hepatitis C virus (HCV) and human immunodeficiency virus type 1 (HIV-1) replication (Jopling et al., 2005; Triboulet et al., 2007). However, few studies have explored the role of miRNAs in virus infection, and whether miRNAs contribute to the blockage of host protein synthesis is totally unknown. To address this issue, the miRNA profile of enterovirus-infected cells was generated by real-time reverse transcriptase-polymerase chain reaction (real-time RT-PCR). Our results suggest that upregulation of miR-141 upon enterovirus infection could facilitate viral propagation by expediting a shift from cap-dependent to cap-independent translation (this process termed as ''translational switch'' hereafter).

RESULTS

The Expression of MicroRNA-141 Is Induced by Enterovirus 71 Infection

To investigate the role of miRNAs in EV71 infection, we determined the replication cycle of EV71 in rhadomyosarcoma (RD) cells. The replication cycle of EV71 was approximately 8 hr at a multiplicity of infection (moi) of 10 (see Figure S1A available online). Next, we optimized the time points for the assay and determined the best moi for EV71 infection. The time-lapse microscopy data indicated that RD cells infected with EV71 at an moi of 10 showed an almost 50% cytopathic effect (CPE) at 8 hr postinfection (h.p.i.) (Figure S1B). To ensure the majority of the cells were infected simultaneously and to avoid the unexpected cellular responses caused by massive infection at a high viral input, the cells were infected with EV71 at an moi of either 5 or 10. The infected cells were stained for viral protein 1 (VP1) (Figure S1C), and the staining results showed that approximately 85% and 100% of the cells were infected at 8 and 12 h.p.i., respectively, when moi of 10 was used. However, less than 50% cells were infected when lower viral input was used. Hence, RNAs from EV71-infected cells at an moi of 10 were extracted for the miRNA profiling analysis at 4 and 8 h.p.i.

The expression profiles of 250 miRNAs in EV71-infected cells were analyzed by quantitative real-time RT-PCR. The expression of 16 miRNAs had a greater than 2-fold changes upon EV71 infection. The expression levels of two miRNAs (miR-141 and miR-146a) were increased, but the expression levels of the other 14 miRNAs were decreased (Figure 1B). It makes sense because a lot of studies demonstrated that the synthesis of host RNAs was decreased to 10%–30% during poliovirus infection (Fenwick, 1963; Holland, 1963; Zimmerman et al., 1963). We asked whether the miRNAs induced by EV71 infection could play a role in facilitating viral proliferation and the induction of CPE via miRNA-mediated negative gene regulation. It has been reported that miR-146a is induced upon infection with Epstein-Barr virus (EBV) and vesicular stomatitis virus (VSV) (Cameron et al., 2008; Hou et al., 2009; Motsch et al., 2007). MiR-141 was chosen for further analysis since it showed the greatest upregulation, with a greater than 16-fold change, and has never been studied as part of host-pathogen interactions. To investigate whether the upregulation of miR-141 is a common characteristic in enterovirus infection, the expression of miR-141 was determined in cells infected with poliovirus 3 (PV3) and coxsackievirus B3 (CVB3). As shown in Figure 1C, CVB3 and PV3 infections induced miR-141 expression by up to 14- and 31-fold, respectively, at 8 h.p.i., compared with mock infection. To understand the kinetics of miR-141 induction in EV71 infection, we measured the expression of mature miR-141 and primary miR-141 (pri-miR-141). The induction of mature miR-141 was coincided with that of pri-miR-141 and occurred as early as 2 h.p.i. (Figures S1D and S1E). Next we used a thiouridine incorporation assay to measure the de novo-synthesized miR-141. The data indicated that the thiouridine-labeled miR-141 was gradually increased in a time-dependent manner (Figure S1F).

eIF4E Is a Target of miR-141

Twenty-seven potential target genes of miR-141 were predicted by the target prediction program TargetCombo using the option involving intersection of the generation programs PicTar, TargetScanS, and miRanda (Sethupathy et al., 2006). One of the targets is eIF4E, which is a key element in the cap-dependent translation of host proteins (Figure 1A). Out of the 27 predicted targets for miR-141, eIF4E is the only one involved in the host translational machinery. We further used the PicTar (http://pictar.org/) and RNA22 (http://cbcsrv.watson.ibm.com/rna22.html) to predict the potential miR-141-binding sites within the 3′ untranslated region (3′UTR) of eIF4E, and only one miR-141-binding site was predicted (Figure 1D and Table S1) (Krek et al., 2005; Miranda et al., 2006; Sethupathy et al., 2006). To determine the effect of miR-141 on the expression of eIF4E, miR-141 was overexpressed in RD cells and the expression levels of miR-141 were measured by real-time RT-PCR (Figure 1F). The results showed that ectopic expression of miR-141, but not negative control, was able to specifically reduce the protein level of endogenous eIF4E (Figure 1E). We cannot rule out the possibility that the decreased eIF4E induced by ectopic expression of miR-141 was due to nonspecific effects. To assess this possibility, RD cells were transfected with non-eIF4E-targeting miRs and analyzed for eIF4E expression. We constructed five random selected miRs (miR-137, miR-372, miR-146a, miR-10a, and miR-27a) that did not target eIF4E predicted by the miRNA target prediction programs, and one

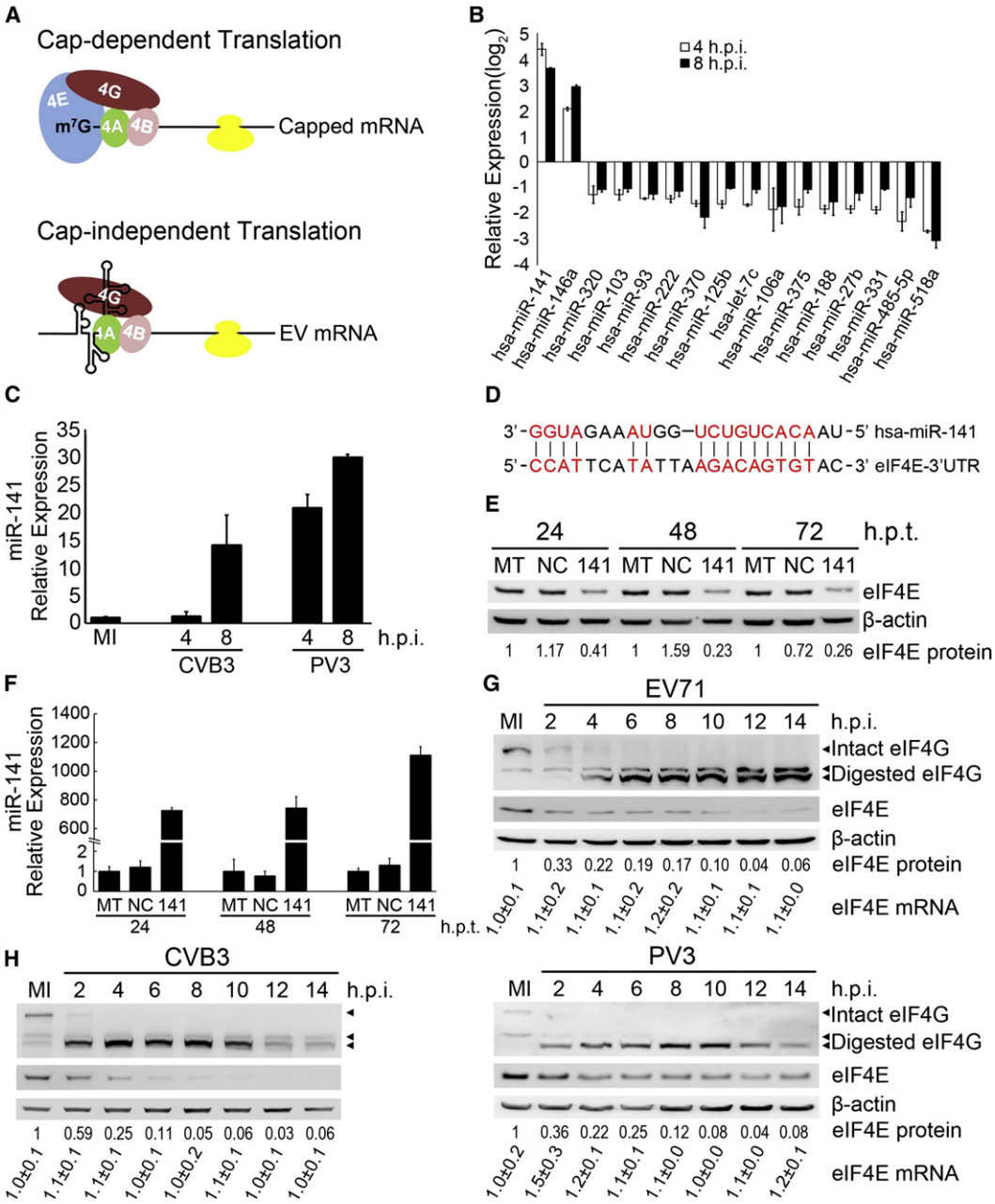

Figure 1. Expression of eIF4E Is Suppressed by miR-141

(A) Cap-dependent and cap-independent translation initiation.

(B) The differentially expressed miRNAs induced by EV71 infection. The open bar and the closed bar represented 4 and 8 hr postinfection (h.p.i.), respectively. All the data are normalized against mock infection.

(C) miR-141 was induced by CVB3 or PV3 infection. The expression of miR-141 was measured by real-time RT-PCR in the RD cells infected with CVB3 or PV3. MI, mock infection (without virus infection).

(D) Predicted miR-141 binding site within the eIF4E 3′UTR. A potential miR-141 binding site located at nucleotides 905–926 of eIF4E downstream (the first nucleotide following the stop codon was designated as +1).

(E and F) The effect of miR-141 on endogenous eIF4E. RD cells were transfected with pSilencer vector harboring negative control miRNA (NC) or miR-141 (141). The expression of endogenous eIF4E and miR-141 was analyzed by western blot (E) and real-time RT-PCR (F). MT, mock transfection (transfection reagent control); h.p.t., hours posttransfection.

(G and H) The effect of EV71 (G), CVB3, or PV3 (H) infection on endogenous eIF4E. The protein expression of eIF4E and eIF4G was detected by western blot, and the RNA expression of eIF4E was measured by real-time RT-PCR. The relative expression of eIF4E protein and of eIF4E mRNA are indicated at the bottom. The intact and digested eIF4G were indicated by arrowheads.

All the data are normalized against mock infection or mock transfection and error bars present as means ± SD (n = 3).

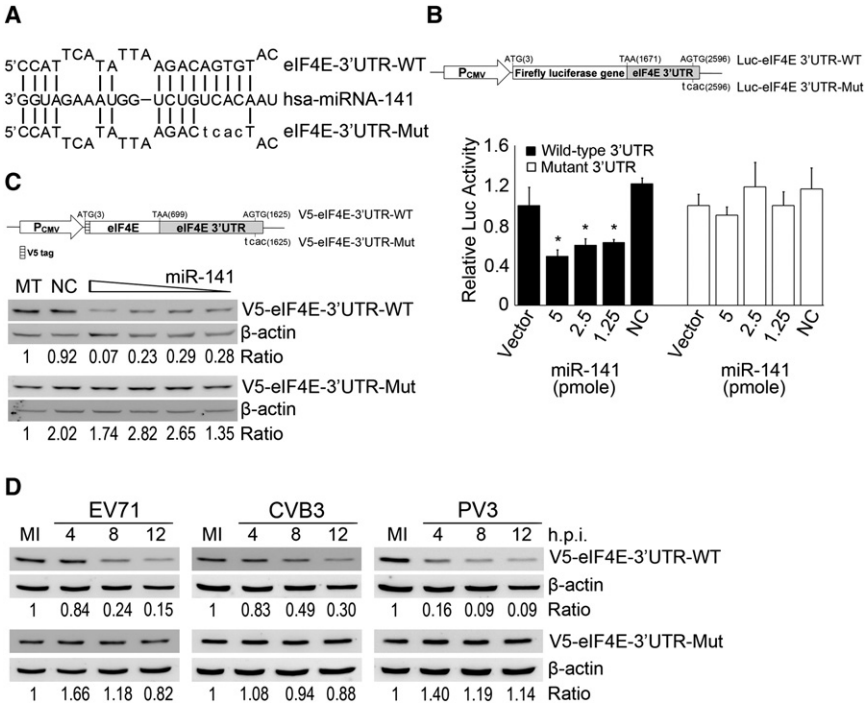

Figure 2. eIF4E Is a Target of Virus-Induced miR-141

(A) Predicted sequence of the miR-141 binding site within the eIF4E 3′UTR.

(B) The effect of miR-141 on the luciferase activity of reporter vectors with wild-type (close bar) or mutant (open bar) eIF4E 3′UTR. HEK293T cells were transfected with the reporter vector and miRNAs, as indicated, and firefly luciferase activity measured 48 hr later. NC, 5 pmole of negative control miRNA. Error bars, mean ± SD; *$p < 0.05$ (two-tailed Student's t test).

(C) The effect of miR-141 on the expression of eIF4E with wild-type or mutant 3′UTR. Various amounts of miR-141 (25, 12.5, 6.25, and 3.125 pmol) were transfected into RD cells stably expressing V5-eIF4E.

(D) The effect of virus infections on the expression of eIF4E with wild-type or mutant 3′UTR in RD cells infected with EV71, CVB3, or PV3.

mutant miR-141, named miR-141M (six point mutations in miR-141 seed region). The result showed that neither five miRs nor miR-141M could decrease the expression of endogenous eIF4E at 72 hr posttransfection (Figure S1G).

Next, the effect of different enterovirus infections on endogenous eIF4E expression was examined. The protein level of eIF4E was gradually reduced in a time-dependent manner from 2 h.p.i. onward, while the mRNAs of eIF4E remained in a steady state during the conditions analyzed (Figures 1G and 1H). To clarify the major reason causing eIF4E decrease, we performed pulse-labeling assays. RD cells were mock infected (MI) or infected with EV71 and pulse labeled with [^{35}S] methionine. The data showed that the de novo-synthesized eIF4E decreased from 1 h.p.i. onward and remained 7% compared with mock infection at 2 h.p.i. (Figure S1H). These findings imply that the reduction in eIF4E might be regulated by miRNAs and that miR-141 might play a crucial role. To clarify whether eIF4E is a direct target of miR-141, a luciferase assay was performed. A four base pair mutation (mutant type) was introduced into the miR-141 binding site within the 3′UTR of eIF4E cDNA (Figure 2A). As shown in Figure 2B, miR-141 significantly suppressed the luciferase activity of the reporter vector harboring the wild-type 3′UTR at all the three doses assayed, but mutant type was not suppressed by miR-141. To further explore the posttranscriptionally regulatory effect of miR-141 on eIF4E, two expression vectors encoding V5-eIF4E fusion proteins, in which the open reading frame of eIF4E was followed by either the wild-type or mutant type 3′UTR, were constructed and are designated as V5-eIF4E-3′UTR-WT and V5-eIF4E-3′UTR-Mut, respectively. These two vectors were individually used to generate V5-eIF4E stable expression cell lines. These stable clones were then transfected with miR-141 and assayed for the expression of V5-eIF4E and miR-141 (Figure 2C and Figure S2). Paralleling the previous results, miR-141 negatively regulated V5-eIF4E with a wild-type 3′UTR in a dose-dependent manner, but not with the mutant one. Interestingly, the expressions of miR-141 were increased to 20.3 and 9.1 times after pre-miR-141 transfection compared with MT control (column 5 and 6 in left panel of Figure S2). The upregulations of miR-141 were 25.0 and 12.3 times compared with MI control at 4 and 8 h.p.i., respectively, in Figure 1B. In the case of Figure 2C, the overexpression level of miR-141 is comparable to that of EV71 infection. To elucidate whether miR-141 does directly mediate the suppression of eIF4E during virus infection, the V5-eIF4E-3′UTR-WT and V5-eIF4E-3′UTR-Mut stable expression cells were infected with EV71, CVB3, or PV3 at an moi of 10, and their V5-eIF4E protein expression levels were measured at the indicated time points by western blot. The expression of V5-eIF4E with the wild-type 3′UTR was markedly suppressed when the RD cells were infected with EV71, CVB3, or PV3 virus at all time points assayed (Figure 2D). However, there was no significant effect on the expression of V5-eIF4E with the mutant 3′UTR. To validate the effect of miR-141 on endogenous eIF4E,the antagomiR-141, an anti-sense RNA molecule complementary to miR-141, was used to block the induction of miR-141 in EV71-infected cells. RD cells were transfected with antagomiR-141 or an antagomiR-negative control before EV71 infection. The protein expression of eIF4E and eIF4G was determined by western blot. The suppression of eIF4E caused by EV71 infection was almost eliminated in antagomiR-141 transfected cells but not in antagomiR-negative control transfected cells or the mock transfected cells at 4 h.p.i. (Figure 3A). These results clearly demonstrated that miR-141 has an important role in the suppression of eIF4E during virus infection.

MiR-141 Involved in Virus-Induced Translational Switch

We investigated whether virus-infection-induced miR-141, which targets eIF4E, did contribute to translational switch.

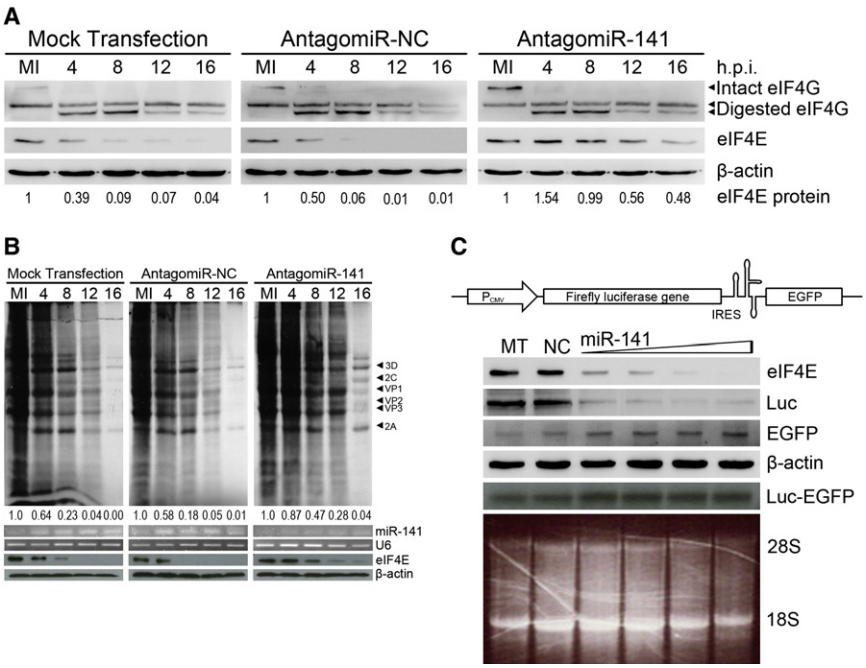

Figure 3. The Protein Translation Switch Induced by Virus Infection Is Attenuated by AntagomiR-141

(A) The reduction in eIF4E was restored by antagomiR-141. RD cells were transfected with antagomiR-141 followed by EV71 infection.

(B) Shutoff of host protein synthesis was delayed by antagomiR-141. RD cells were infected with EV71, and de novo protein synthesis was detected at the indicated time points using metabolic labeling. The [^{35}S]methionine incorporation assay was quantified by densitometry and showed at the bottoms. The viral proteins were indicated by arrowheads. The expressions of miR-141 and eIF4E were assayed by semiquantitative RT-PCR and western blot assay, respectively.

(C) miR-141 specifically attenuated cap-dependent translation. Luciferase is expressed through cap-dependent translation and EGFP is expressed through cap-independent translation. The expression of Luc-EGFP was measured by northern blot assay. 18S and 28S acted as RNA loading control.

Isotope metabolic labeling was performed to characterize the role of miR-141 in the shutoff of host protein synthesis during virus infection. We performed semiquantitative RT-PCR instead of real-time RT-PCR to check the expression of miR-141 due to isotope issue and performed western blot to determine the eIF4E expression. As the above results showed, antagomiR-141 could attenuate the decrease of eIF4E companied with suppression of miR-141. The antagomiR-141, but not the antagomiR-negative, slowed down EV71-infection-induced shutoff of host protein synthesis compared with the mock transfection control (13% versus 42% reduction of protein synthesis at 4 h.p.i.) (Figure 3B). Even at 12 h.p.i., there was still 28%, compared with 5% in the antagomiR-negative control, of host protein synthesis remained in antagomiR-141 transfected cells. The drastic decrease of host protein synthesis at 16 h.p.i. might be due to detachment of infected cells. The blockage of miR-141 expression with antagomiR-141 could manifestly delay the process of translation switching, with host protein synthesis remained relatively stable at 4 h.p.i. and partially decreased at 12 h.p.i. To investigate whether miR141 can directly cause protein synthesis switch, we construct a plasmid, which transcribes a transcript containing fire luciferase gene and enhanced green fluorescence protein (EGFP) driven by cap-dependent CMV promoter and type II cap-independent IRES structures, respectively. The plasmid was cotransfected with pSilencer-miR-141 or pSilencer-miR-NC into RD cells, and the relative expression of Luc and GFP was determined by western blot. We found that miR-141 could reduce the expression of luciferase and increase the expression of GFP (Figure 3C). On the other hand, we performed northern blot to check the intactness of Luc-IRES-EGFP transcript in all transfectants. The data showed that the transcripts in miR-141 transfectants kept in a steady state compared with mock transfection and negative control transfectants (Figure 3C).

Silencing of miR-141 Delays CPE Occurrence and Reduces Virus Production

To directly evaluate the effect of antagomiR-141 on the synthesis of viral proteins and viral RNA replication, the antagomiR transfected RD cells were infected with EV71, and the viral capsid proteins (VPs) and genomic RNAs were detected by western blot assay and real-time RT-PCR at indicated time points, respectively. The VPs and genomic RNAs became detectable from 8 h.p.i. onward and the expression of viral proteins and genomic RNAs were moderately inhibited at 8, 12, and 16 h.p.i. in the presence of antagomiR-141 as compared with the absence of antagomiR-141 (Figure 4A and Figure S3A). Furthermore, the onset of cytopathic effects induced by EV71 infection was delayed in the presence of antagomiR-141 as compared with antagomiR-negative controls (Figure 4B). As shown in Figure S3B, CPE was imaged with 50× magnification and quantified at indicated time points. To address whether antagomiR-141 can reduce viral replication, the virus titers were determined in the presence or absence of antagomiR-141. A 1000-fold reduction in viral propagation, accompanied by the complete digestion of eIF4G, was observed when the virus-induced miR-141 was neutralized by antagomiR-141 (Figures 3A and 4C). The IFN-alpha levels were measured at all of time points assayed to understand whether the reduction of virus production is due to off-target effect of siRNA transfection. The results showed that RD cells transfected with antagomiR-141 did not increase the IFN-alpha level compared with antagomiR-NC transfectants and mock transfection (Table S3B). The roles of eIF4E in enterovirus replication were further validated by an RNAi strategy. Three eIF4E-pecific siRNAs were individually introduced into RD cells in the presence of antagomiR-141 followed by virus infection. The results indicated that the silencing of eIF4E was able to entirely rescue the decrease in viral propagation caused by

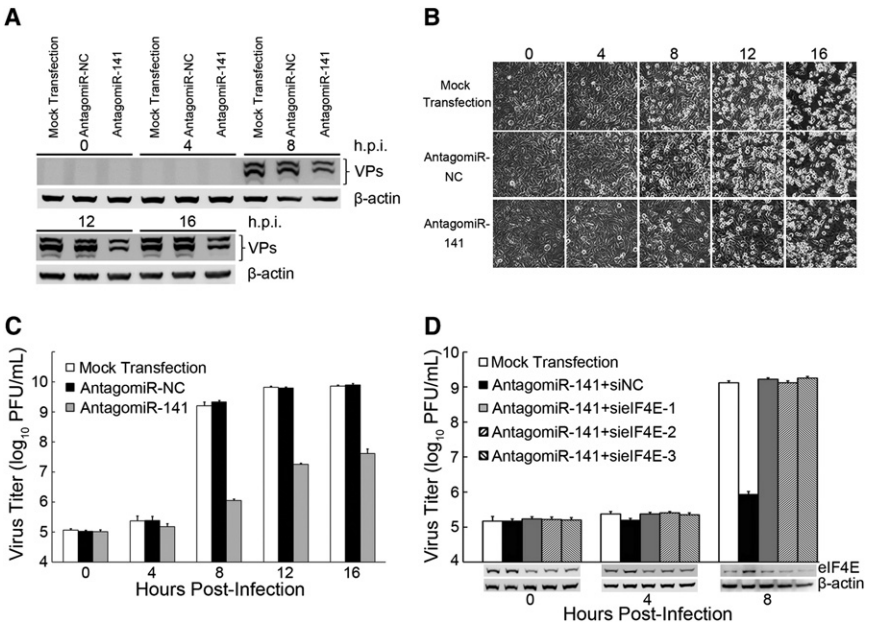

Figure 4. The Virus Virulence Is Attenuated by AntagomiR-141

(A) Expression of viral proteins was suppressed by antagomiR-141. The antagomiRs transfected RD cells were infected with EV71, and the viral proteins (VPs) were immunoblotted with anti-EV71 antibody at the indicated time points in triplicate.

(B) CPE was attenuated by antagomiR-141. RD cells were infected with EV71, and CPE was monitored at the indicated time points. Magnification, 50×.

(C) Virus production was attenuated by antagomiR-141. Virus yield was measured by plaque assay. Error bars, mean ± SD.

(D) Reduction of virus production, which was induced by antagomiR-141, was rescued by eIF4E siRNAs. The expression of eIF4E and virus yield in antagomiR-141 transfected cells with/without eIF4E siRNAs was measured by western blot and plaque assay, respectively. Error bars, mean ± SD.

antagomiR-141 (Figure 4D). The results clearly demonstrated that the effect of antagomiR-141 on virus production was mainly, if not totally, via regulation of eIF4E. Additionally, EV71 infection is known to cause acute neurological disorders and severe sequelae (Chang et al., 2007; Huang et al., 1999). To ascertain whether the miR-141-mediated eIF4E suppression also occurs in neural cells, the eIF4E expression in EV71-infected SF-268 cells in the presence or absence of antagomiR-141 was measured. Consistent with the findings in RD cells, EV71 infection induced eIF4E suppression, which can be rescued, accompanied with decrease EV71 replication, by antagomiR-141 (Figures S3A–S3C). These data suggest that the miR-141-mediated regulatory mechanism might be a general phenomenon for EV71-infected cells and that miRNA-mediated eIF4E suppression might not only play a role in viral pathogenesis but also in viral replication.

miR-141 Is Partly Regulated by EGR1

Recently, a database of miRNA promoters has been generated by silica genomic analysis (Saini et al., 2007), and one of the regulatory mechanisms of the miR-200 family (miR-200a, miR-200b, and miR-429) has thus been elucidated (Bracken et al., 2008). Although the transcription start site of miR-141 has been predicted to be 800 bp upstream from miR-141, the regulation of miR-141 expression has not been well studied. To explore the regulatory mechanisms adopted by EV71 to induce miR-141 expression, the regulatory element responsible for miR-141 induction was determined by luciferase reporter assays using pGL reporter vectors harboring a series of truncated upstream fragments of miR-141. Individual construct was transfected into RD cells seeded onto 96-well culture plates the day before infection. After 16 hr, all transfectants were infected with EV71 at an moi of 10 and the luciferase activities of cell lysates were measured at 8 h.p.i. (Figures 5A and 5B). A fragment (−1309 to −2382 relative to the first nucleotide of the miR-200c precursor) was found to increase the luciferase activity up to 27-fold compared with the pGL vector control (Figure 5B). Using TRANSFAC software, 105 potential transcription factor binding sites within the miR-141 upstream region (−1 to −2382) were predicted. Next, the expression profile of transcription factors after EV71 infection was determined by microarrays, and the expression of 20 transcription factors had a greater than 2-fold change (FDR < 0.05) as compared with mock infection at 4 or 8 h.p.i. (Table S2). Further matching the results from prediction of binding sites and the expression profile of transcription factors identified the early growth response 1 (EGR1). Hence, the mRNA and protein expression levels of EGR1 in EV71-infected cells were determined. Consistent with the microarray results, EGR1 expression was dramatically induced by up to 100-fold at 4 and 8 h.p.i. compared with mock infection control both at the RNA and protein levels (Figure 5C). To further understand the kinetic response of EGR1 signaling, we determined the EGR1 expression in the early stage of EV71 infection. Surprisingly, the increase of EGR1 was occurred at 0.25 h.p.i. (Figure S4F). Two potential EGR1 binding sites were predicted in the putative regulatory element of miR-141 (Figure 5A). Next, a chromatin immunoprecipitation (ChIP) assay was performed to pinpoint whether EGR1 could directly bind onto the two predicted binding sites. The ChIP assay was performed using exogenous EGR1 with a V5 tag because commercial anti-EGR1 antibody yielded nonspecific bands. RD cells were transfected with an expression vector expressing V5 tag EGR1, and anti-V5 antibody was used to perform ChIP assays. The results indicated that both EGR1 binding sites were able to bind EGR1 (Figure 5D), and both sites were essential for maximal expression of miR-141 (Figure 5B). To evaluate whether the EGR1 binding sites are also important to miR-141 regulation in nature context, the luciferase activity of reporter vector containing full-length regulatory fragment (−1 to −2382) with or without mutant EGR1 binding site was assayed. EV71 infection could activate the transcriptional

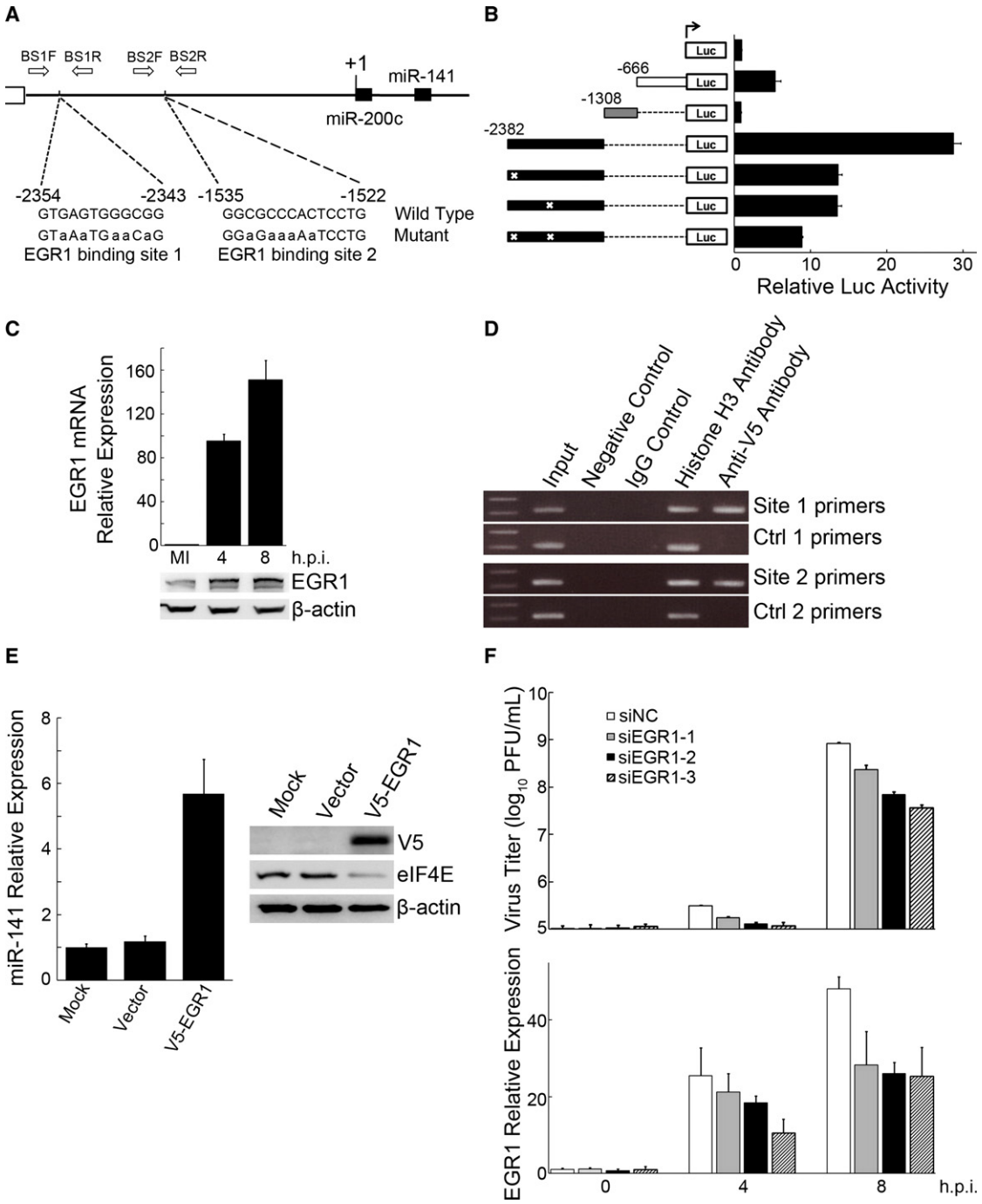

Figure 5. Regulation of miR-141

(A) Schematic organization of miR-141. EGR1 binding sites were predicted by TRANSFAC software.

(B) Identification of the regulatory elements of miR-141. Transcriptional activity of miR141-truncated upstream sequences with or without EGR1 binding site mutations was determined by luciferase activity assays. Data are normalized against the vector control and error bars present as means ± SD (n = 3).

(C) EGR1 induced by EV71 infection. The mRNA and protein expression levels of EGR1 were determined by real-time RT-PCR and western blot, respectively. Data are normalized against mock infection and error bars present as means ± SD (n = 3).

(D) EGR1 binding sites were determined by ChIP assays. Ctrl-1 and ctrl-2 primers located at the 3 kb downstream of EGR1 binding site 1 and site 2 act as PCR primer controls.

(E) EGR1 activated miR-141 expression. RD cells were transfected with the V5-ERG1-expressing plasmid and assayed for miR-141 and eIF4E. Data are normalized against mock infection and error bars present as means ± SD (n = 3).

(F) EGR1 siRNAs attenuated virus propagation. Upper panel, virus yield was measured by plaque assay in the presence of a negative control siRNA (siNC) or three kinds of EGR1 siRNAs (siEGR1-1, siEGR1-2, and siEGR1-3). Error bars present as means ± SD. Lower panel, EGR1 expression was measured by real-time RT-PCR and normalized against TBP compared with mock transfection. Error bars present as means ± SD (n = 3).

activity of vector containing wild-type regulatory element. The mutations at site 1 and site 2 attenuated the luciferase activity to 50% approximately after EV71 infection (Figure S4A). Moreover, EGR1, just like miR-141, was also induced after CVB3 or PV3 infection (Figure S4B). It has been predicted that miR-200c and miR-141 form a cluster and are located within a same pri-miRNA (Saini et al., 2007). EGR1 should activate miR-141 expression as well as miR-200c if miR-141 shares a promoter with miR-200c. Thus, we examined whether miR-141 and miR-200c were located within the same pri-miRNA. We designed two sets of specific primers to amplify the pri-miRNA containing miR-141 and miR-200c. We observed that both pairs of primers could specifically amplify the expected products using RT-PCR. The results revealed that the expression of both miR-141 and miR-200c was induced in the V5-EGR1 transfectants but not in the mock transfection or vector control transfectants (Figures S4C and S4D). These data imply that miR-141 is clustered with miR-200c and that miR-200c expression is also regulated by EGR1.

To directly demonstrate the importance of EGR1 in miR-141 regulation, the expression levels of miR-141 and its target, eIF4E, were determined in the presence or absence of exogenous EGR1. As shown in Figure 5E, EGR1 induced the expression of miR-141 and the suppression of eIF4E. Next, the role of EGR1 in EV71 replication was elucidated by knocking down the expression of EGR1 with three EGR1 siRNAs. When treated with each of these EGR1-specific siRNAs, EV71 production was attenuated ranging from 3- to 20-fold (Figure 5F). Subsequently, experiments were performed to rule out the possibility that the suppressive effect of EGR1 siRNAs on the virus production was due to a nonspecific interferon response induced by EGR1 siRNAs or an off-target effect of the EGR1 siRNAs. The levels of interferon α in the EGR1 siRNA and negative control transfectants were measured by interferon α multisubtype ELISA; the interferon α expression levels were then compared with the cell controls. The results showed that there was no induction of interferon α by the EGR1 siRNAs or the negative control compared with the cell controls (Table S3B). We also evaluated the potential off-target effects of EGR1 siRNAs using sequence alignment to calculate whether the viral genome might be targeted by these siRNAs. The alignment revealed that sequences with no three or fewer mismatches could be identified (Table S3C), suggesting that the reduction of virus production caused by the EGR1 siRNAs was not due to a nonspecific off-target effect. To experimentally demonstrate that the suppressive effect of EGR1 siRNAs was not due to off-target effect, we evaluate the inhibitory activity of EGR1 siRNAs on the production of viral proteins with or without eIF4E siRNAs. In Figure S4E, siEGR1 (mixture of siEGR1-1, siEGR1-2, and siEGR1-3) could inhibit the expression of viral proteins. siEV71 2C (against EV71 protease 2C) has been successfully used to inhibit EV71 replication through protease 2C suppression (Tan et al., 2007). siEV71 2C acts herein as a control that targets on the genome of EV71. sieIF4E (mixture of sieIF4E-1, sieIF4E-2, and sieIF4E-3) could block the suppressive activity of siEGR1 but not siEV71 2C on viral protein expression. These data provide direct evidence that the suppressive activity of siEGR1 is not due to off-target effect and both EGR1 and eIF4E seem to belong to a same regulatory pathway. If the effect of siEGR1 results from off-target effect, the suppressive activity of siEGR1 should be not able to rescue by sieIF4E.

DISCUSSION

In this study we investigated the role of miRNAs in viral replication. Up to now, only a few reports have shown that cellular or viral miRNAs are involved in reciprocal interactions between virus and host cells (Gottwein et al., 2007; Jopling et al., 2005; Pfeffer et al., 2004; Triboulet et al., 2007; Umbach et al., 2008). Picornavirus infection is known to induce a dramatic shutoff of host protein synthesis that subsequently contributes to the pathogenesis of virus infection. However, it does not completely understand how viruses, particularly the picornaviruses, can specifically block host protein synthesis, although poliovirus 2A protease-mediated cleavage of eIF4G, which shuts down host protein synthesis, has been reported (Belsham and Sonenberg, 2000; Goldstaub et al., 2000; Schneider and Mohr, 2003). We found that enterovirus infection may disturb the expression of host miRNAs; specifically, miR-141 is upregulated and then inhibits host protein synthesis by posttranscriptional repression of its target gene, eIF4E, which is a key element involved in the cap-dependent translation. The availability of eIF4E is regulated at least by eIF4E repressors, 4E-BPs (Gingras et al., 1996; Kuyumcu-Martinez et al., 2004), and by nuclear assortment. To date, 4E-BPs and digested eIF4G could induce the relocalization of eIF4E to nucleus (Sukarieh et al., 2009, 2010). Sukarieh and coworkers found that the nuclear relocalization of eIF4E corresponds with shutoff of host protein synthesis in response to poliovirus infection (Sukarieh et al., 2010). However, no study investigates whether picornavirus infection can directly regulate the expression of eIF4E itself. In this study, we demonstrated that picornavirus infection could repress eIF4E expression and that the knockdown of miR-141 by a specific inhibitor, antagomiR-141, is able to restore host eIF4E expression, delay the occurrence of CPE, and reduce viral propagation. Consistently, silencing of eIF4E was able to completely block the effect of antagomiR-141 on viral propagation. We have also demonstrated that the induction of miR-141 is at least partially due to increased expression of EGR1 upon EV71 infection; furthermore, the silencing of EGR1 attenuated virus production. These evidences imply that enterovirus infection promotes the expression of the host miRNA, miR-141, which, by suppression of host cap-dependent translation, switches the host machinery to support viral replication through cap-independent translation. However, we noted that antagomiR-141 moderately suppressed the accumulation of VPs and replication of viral genomic RNAs, but it dramatically reduced the virus production. A possible explanation, we reasoned, is that the formation of infectious viral particles requires multiple steps, including viral genomic RNA replication, incorporation, virus assembly, and so on. Therefore, any disruption between the viral protein synthesis and release of infectious virus particles may contribute to a significant decrease of virus yield. It implied that the reduction of virus production might be the sum of multiple effects. Suppression of viral proteins, relief of host translational shutoff, and reduction of viral genomic RNA as well as certain unidentified mechanisms might contribute to the virus titer reduction.

The fact that EV71 supports its own replication through regulation of cellular miRNAs may post a potential target for

antiviral intervention. Recent reports have identified several cellular miRNAs that contribute to viral replication. HIV-1 was shown to suppress the expression of the host miR-17/92 cluster that targets the cellular cofactor of HIV-1 tat, and such suppression is necessary for efficient HIV-1 replication (Triboulet et al., 2007). Another example is miR-122, which is specifically expressed in the liver and is able to stabilize HCV genomic RNA (Jopling et al., 2005). Consequently, the suppression of miR-122 by a locked nucleic acid-modified oligonucleotide was found to attenuate HCV infection in the chimpanzee model (Lanford et al., 2010). A phase I clinical trial has been conducted to assess the safety and efficacy of such a miRNA-based antiviral strategy. This growing number of examples suggests that miRNAs may serve as targets for antiviral therapy in the future.

MiR-141 suppressed the luciferase activity and the expression of recombinant eIF4E with the wild-type eIF4E 3′UTR but not with the mutant eIF4E 3′UTR. In addition, antagomiR-141 rescued the expression of eIF4E and inhibited viral replication during EV71 infection. Importantly, the metabolic labeling data provided two additional pieces of evidence to support our model. First, at 4 hr after EV71 infection, eIF4G was near completely degraded, but the protein switch did not begin in presence of antagomiR-141. The downregulation of eIF4E is likely associated with the initiation of host protein synthesis switch. Second, in presence of eIF4E we can determine how much of the observed repression of host protein synthesis is due to the contribution of other translational factors including eIF4G depletion, 4E-BP phosphorylation, and so on. The treatment of EV71-infected cells with eIF4E siRNAs completely abolished the suppressive effect of antagomiR-141 on viral replication. These results ruled out the possibility that the suppression of eIF4E was caused by a mechanism(s) other than miR-141. Hence, the seed region of the miR-141-binding sites within eIF4E 3′UTR is the critical determinant for the suppressive effect of virus infection on eIF4E expression. Taken together, these findings suggest that virus-infection-induced miR-141 might play a major role in controlling the suppression of eIF4E and the success of viral replication.

Our results showed that EGR1 is, at least partially, responsible for the upregulation of miR-141. Although we have demonstrated that miR-141 and miR-200c were located within the same transcript and induced by EGR1, the increase of miR-200c (1.54-fold) is much less than that of miR-141 after EV71 infection. Recently Buck and coworkers demonstrated that CMV infection specifically suppresses miR-27a expression but not miR-23a and miR-24 located in the same genomic cluster through posttranscriptional regulation (Buck et al., 2010). Hence it is possible that the posttranscriptional processing and/or decay of miRNAs may also contribute the unequal upregulation between miR-141 and miR-200c during EV71 infection. EGR1 is a cellular transcription factor involved in several cellular functions such as cell proliferation, apoptosis, and differentiation (O'Donovan et al., 1999; Thiel and Cibelli, 2002). It has recently been reported that EGR1 is induced during various virus infections, including JC virus, hepatitis B virus, EBV, and herpes simplex virus type 1 (HSV-1), and that this induction promotes viral replication (Chang et al., 2006; Chen et al., 2008). However, we noticed that the suppression of virus-infection-induced miR-141 by antagomiR-141 led to a decrease in virus production of up to 1000-fold but that the neutralization of virus-infection-induced EGR-1 by siRNAs only caused a 20-fold decrease. This suggests that, in addition to the EGR1-mediated pathway, other unidentified mechanisms also contribute to the upregulation of miR-141 on virus infection.

In conclusion, we have identified a virus-host interaction pathway wherein virus infection results in EGR1-mediated miR-141 induction and that this leads to the silencing of eIF4E, a switch from cap-dependent to cap-independent translation in the host cells, augmentation of CPE, and increased virus production (Figure 6).

EXPERIMENTAL PROCEDURES

Cell Cultures and Virus Infection

RD and SF-268 were cultured in MEM medium or RPMI-1640. RD cells were used in propagation and plaque titration of PV3, CVB3, and EV71. The virus infection was performed in serum-free condition. The cytopathic effect induced by virus infection was photographed by ZEISS Axiovert 200M (Zeiss) with 50× magnification. Details are given in the Supplemental Information.

RNA Extraction and miRNA Profiling

RNAs were extracted from virus-infected or mock-infected RD cells by Trizol reagent (Invitrogen). The expression levels of 250 human miRNAs were measured using the TaqMan MicroRNA Assays (Applied Biosystems) as described previously (Yu et al., 2008). Details are given in the Supplemental Information.

Oligonucleotide Microarray Analysis

cRNA preparation and array hybridization were performed according to the Affymetrix GeneChip Expression Analysis Technical Manual by the NTU Microarray Core Facility of National Research Program for Genomic Medicine of National Science Council in Taiwan. Details are given in the Supplemental Information.

Individual Real-Time RT-PCR

Quantification of miR-141, Pri-miR-141, and eIF4E was performed using TaqMan MicroRNA individual assay and TaqMan gene expression assay (000463, Hs03303157_Pri, and Hs00913390_m1; Applied Biosystems) according to the manufacturer's instructions as described previously (Yu et al., 2008).

Western Blot

Cells were harvested in RIPA lysis buffer. Proteins were resolved by 12.5% sodium dodecyl sulfate polyacrylmide gel electrophoresis and reacted with primary antibodies. β-actin acts as an internal control. Details are given in the Supplemental Information.

Thiouridine Incorporation Assay

The thiouridine incorporation assay was performed as described previously (Norman and Sarnow, 2010). RD cells were infected with EV71 and the culture medium contained 4-thiouridine at 100 μM. Total RNAs were collected at indicated time points and further conjugated with EZ-Link Biotin HPDP (Pierce). The biotinylated RNAs were captured with streptavidin beads (Invitrogen). The captured RNAs were quantified by real-time RT PCR for miR-141 and U6 snRNA. Total RNA input was normalized based on the Ct values of the TaqMan U6 snRNA assay as an endogenous control.

Luciferase Assay

HEK293T cells (1×10^4 per well/96-well plate) were seeded 24 hr prior to transfection. The luciferase reporter constructs along with the control plasmid (pRL-TK Vector; Promega) were cotransfected into cells by RNAi fect reagent (QIAGEN). After 48 hr incubation the Dual-Glo luciferase substrate (Promega) was added and the luminescent signals were measured by Victor^3 multilabel counter (PerkinElmer) The activity of *Renilla* luciferase was used as an internal control to normalize transfection efficiency. Details are given in the Supplemental Information.

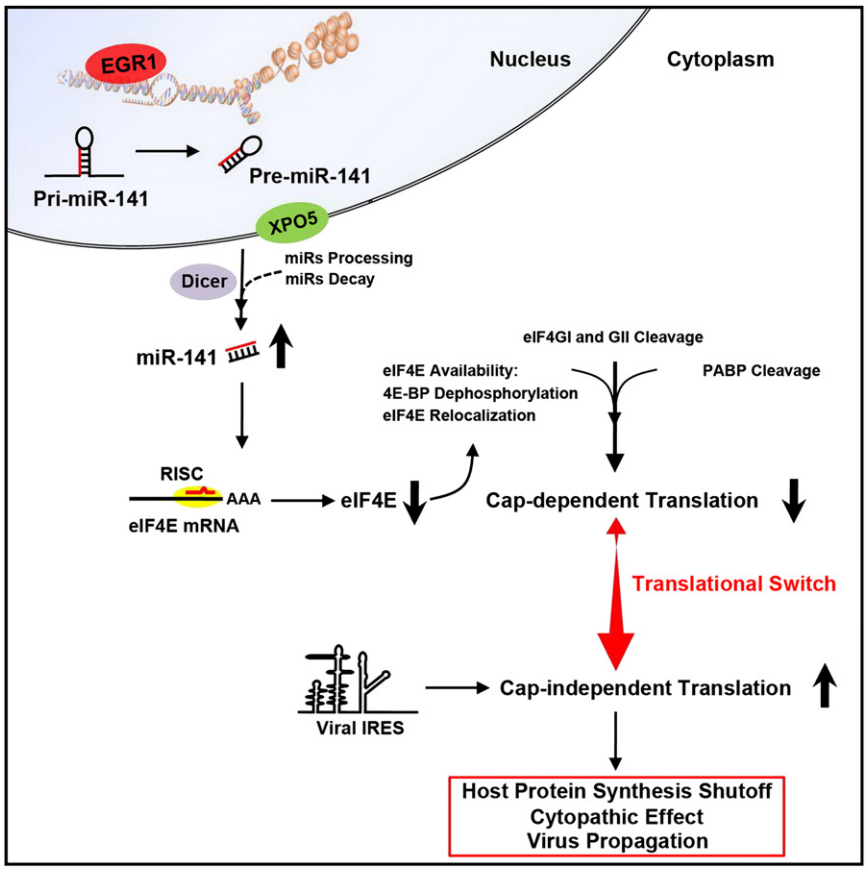

Figure 6. Model for the Regulatory Role of miR-141 in Enterovirus Infection

The upregulation of EGR1 induced by virus infection increases miR-141 expression. Consequently, miR-141 represses the expression of eIF4E via imperfect base pairing between miR-141 and the 3′UTR of eIF4E. The reduction in eIF4E causes a protein synthesis switch from cap-dependent to cap-independent translation that might contribute viral pathogenesis and virus propagation. EGR1, early growth response 1; XPO5, exportin 5; RISC, RNA-induced silencing complex; eIF, eukaryotic translation initiation factor; 4E-BP, eIF4E binding protein; PABP, poly(A)-binding protein; IRES, internal ribosome entry site.

Plasmid Constructions

The full-length eIF4E 3′UTR was amplified from genomic DNA of RD cells using forward primer F1 (Table S2) and the reverse primer R1. Two paired primers (F1 plus mutR1 and mutF1 plus R1) were used to generate mutant-type eIF4E 3′UTR in which the four mutated nucleotides were underlined within the seed region of miR-141-binding site by PCR-based mutagenesis method. Both PCR fragments were cloned into pMIR-reporter luciferase vector (Ambion). The full-length eIF4E fragments were amplified from cDNA of RD cells and cloned into pcDNA 3.1 expression vector (Invitrogen) along with V5 tag and eIF4E 3′UTR. The miR-141 precursor fragment was amplified by PCR-based ligation and constructed into psilencer vector (Ambion). Three upstream fragments of the miR-200c precursor were constructed into pGL reporter vectors. Regulatory element (RE) primers were used for amplification of wild-type regions of EGR1, and EGR1 MutF1, EGR1 MutR1, EGR1 MutF2, and EGR1 MutR2 primers were for mutant EGR1-binding site constructs. EGR1F and EGR1R primers were used for EGR1 ectopic expression construct. EGR1 PCR fragments were cloned into pcDNA3.1 (Invitrogen). For P_{CMV}-Luc-IRES-GFP construct, EMCV-derived type II IRES and EGFP fragments were amplified from pLKO AS2 and pEGFP C3 (Clontech) plasmids, respectively, and cloned into pMIR reporter vector (Applied Biosystems). Details are given in the Supplemental Information.

Stable eIF4E Transfection of RD Cells and AntagomiR and siEGR1 Transfections

To generate the stably eIF4E-expressing cell lines, RD cells were transfected with 2 μg of plasmid DNA encoding V5-eIF4E fusion protein with wild-type or mutant 3′UTR by Lipofectamine 2000 reagent (Invitrogen) and treated with G418 (1 mg/mL; Invitrogen). For antagomiR transient transfection trypsinized RD cells at 3×10^5/mL were transfected with antagomiRs (Ambion) by siPORT *NeoFX* transfection reagent (Ambion) according to the manufacturer's instructions. For the EGR1 transient silencing experiments, three siRNAs against EGR1 (s4537, s4538, and s4539; Applied Biosystems) were transfected into RD cells by Lipofectamine 2000 reagent (Invitrogen) and the transfected RD cells were infected with virus after 24 hr. RNAs and culture supernatants were collected and assayed for SYBR Green real-time PCR (Applied Biosystems) and plaque assays, respectively.

Metabolic Labeling and Immunoprecipitation

For characterizing the role of miR-141 in the shutoff of host protein synthesis during virus infection, RD cells were transfected with antagomiRs 24 hr before metabolic labeling experiments. Cells were infected with EV71 and then incubated in methionine-free DMEM (Invitrogen) for 20 min. De novo-synthesized proteins were labeled by incubation in pulse medium supplemented with 20 Ci/mL [^{35}S] methionine (NEN) for 15 min. Cells were harvested and analyzed by 12% SDS-PAGE. To evaluate the newly synthesized eIF4E RD cells were MI or infected with EV71 and pulse labeled with [^{35}S] methionine. Cells were infected with EV71 for the indicated periods of time and then incubated in methionine-free DMEM (Invitrogen) for 30 min. De novo-synthesized proteins were labeled by incubation in pulse medium supplemented with 20 Ci/mL [^{35}S] methionine (NEN) for 15 min. Cells were harvested in protein lysis buffer and total proteins were incubated with protein A/G beads (Santa Cruz) for immunocleaning. The supernatants were reacted with anti-eIF4E antibody (Upstate) for 16 hr at 4°C and then incubated with protein A/G beads for 1 hr at 4°C. The beads were resolved by 12.5% SDS-PAGE. A tenth of total proteins were analyzed by western blot. Details are given in the Supplemental Information.

Northern Blot

Of total RNAs, 20 μg was mixed with equal volume of glyoxal dye (Applied biosystems) and incubated at 50°C for 30 min. The RNAs were then resolved on a 0.8% agarose gel and transferred to membrane (Immobilon-NY$^+$, Millipore). The membrane was reacted with DNA probes labeled with P^{32}-dCTP. Details are given in the Supplemental Information.

Plaque Assay

EV71 plaque assays were carried out in triplicate in 6-well plates. RD cells were infected with 100 μl per well of diluted viral stocks. After 1 hr incubation the infected cells were washed and incubated for 3 days in 0.3% agar medium overlay. Cells were fixed with formaldehyde and stained with crystal violet. The plaques were counted.

Chromatin Immunoprecipitation Assay

In vivo binding of EGR1 to the regulatory element of miR-141 was investigated using the ChIP assay according to the protocol of Upstate Biotechnology. Details are given in the Supplemental Information.

Statistical Analysis

Student's t test was used to compare the miRNA expression at different time points during EV71 infection. The p value < 0.05 for significance and two-tailed tests were used in this study. The significant miRNAs with greater than 2-fold change of expressions at both 4 and 8 h.p.i. compared with mock were selected for further study.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, three tables, and Supplemental Experimental Procedures and can be found with this article at doi:10.1016/j.chom.2010.12.001.

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