# Replication of Many Human Viruses Is Refractory to Inhibition by Endogenous Cellular MicroRNAs

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## ABSTRACT

The issue of whether viruses are subject to restriction by endogenous microRNAs (miRNAs) and/or by virus-induced small interfering RNAs (siRNAs) in infected human somatic cells has been controversial. Here, we address this question in two ways. First, using deep sequencing, we demonstrate that infection of human cells by the RNA virus dengue virus (DENV) or West Nile virus (WNV) does not result in the production of any virus-derived siRNAs or viral miRNAs. Second, to more globally assess the potential of small regulatory RNAs to inhibit virus replication, we used gene editing to derive human cell lines that lack a functional Dicer enzyme and that therefore are unable to produce miRNAs or siRNAs. Infection of these cells with a wide range of viruses, including DENV, WNV, yellow fever virus, Sindbis virus, Venezuelan equine encephalitis virus, measles virus, influenza A virus, reovirus, vesicular stomatitis virus, human immunodeficiency virus type 1, or herpes simplex virus 1 (HSV-1), failed to reveal any enhancement in the replication of any of these viruses, although HSV-1, which encodes at least eight Dicer-dependent viral miRNAs, did replicate somewhat more slowly in the absence of Dicer. We conclude that most, and perhaps all, human viruses have evolved to be resistant to inhibition by endogenous human miRNAs during productive replication and that dependence on a cellular miRNA, as seen with hepatitis C virus, is rare. How viruses have evolved to avoid inhibition by endogenous cellular miRNAs, which are generally highly conserved during metazoan evolution, remains to be determined.

## IMPORTANCE

Eukaryotic cells express a wide range of small regulatory RNAs, including miRNAs, that have the potential to inhibit the expression of mRNAs that show sequence complementarity. Indeed, previous work has suggested that endogenous miRNAs have the potential to inhibit viral gene expression and replication. Here, we demonstrate that the replication of a wide range of pathogenic viruses is not enhanced in human cells engineered to be unable to produce miRNAs, indicating that viruses have evolved to be resistant to inhibition by miRNAs. This result is important, as it implies that manipulation of miRNA levels is not likely to prove useful in inhibiting virus replication. It also focuses attention on the question of how viruses have evolved to resist inhibition by miRNAs and whether virus mutants that have lost this resistance might prove useful, for example, in the development of attenuated virus vaccines.

Two forms of small-RNA-mediated RNA interference (RNAi) in somatic eukaryotic cells have been described. One form of RNAi, mediated by small interfering RNAs (siRNAs), was initially discovered in nematodes (1) and involves the sequential exonucleolytic processing of long, perfect, double-stranded RNAs (dsRNAs) by the RNase III enzyme Dicer to yield ~22-bp siRNA duplexes, one strand of which is then incorporated into the RNA-induced silencing complex (RISC) (2, 3). The siRNA guides RISC to RNA molecules that generally bear perfect sequence complementarity to the siRNA, which are then subjected to endonucleolytic cleavage and degradation. While siRNAs can derive from endogenous dsRNAs, they are frequently derived from exogenous dsRNAs introduced by experimental transfection or resulting from viral infection.

A second form of eukaryotic RNAi is mediated by a similar but distinct family of small RNAs called microRNAs (miRNAs). miRNAs are encoded within the genome as part of a long, primary miRNA (pri-miRNA) transcript (4). Within the pri-miRNA, the miRNA forms part of an ~80-nucleotide (nt) stem-loop structure that is recognized and cleaved by the microprocessor, consisting in mammals of the RNase III enzyme Drosha and

its cofactor, DGCR8, to release an ~60-nt-long pre-miRNA hairpin intermediate. After nuclear export by Exportin 5, the pre-miRNA is bound by Dicer, which cleaves the pre-miRNA ~22 bp from the base of the hairpin to release the miRNA duplex intermediate. As in the case of the siRNA duplex, one strand of the miRNA duplex is loaded into RISC, where it serves to guide RISC to targets generally bearing partial homology to the miRNA, in particular to nucleotides 2 to 8, referred to as the miRNA seed sequence (5). Binding of RISC to such partially complementary

mRNA targets can result in inhibition of translation and/or degradation. Of note, while siRNAs therefore generally bind to perfect targets to induce mRNA degradation and miRNAs normally bind to partially complementary targets to attenuate mRNA function, each small-RNA class is able to act equivalently when presented with the same type of mRNA target, at least in mammalian cells; i.e., both miRNAs and siRNAs can induce the degradation of mRNAs bearing perfect targets and inhibit the expression of mRNAs bearing partially complementary targets (6, 7).

While miRNAs are found in all animal cell types, siRNAs are more restricted. In particular, while siRNAs can be readily detected in nematodes or insects infected with RNA viruses and evidence indicates that they play an important role in mediating innate immunity to virus infections in invertebrates (8–12), there have been several reports documenting the lack of detectable virus-specific siRNAs in infected mammalian somatic cells (13, 14). Biochemical evidence suggests that the full-length Dicer protein found in mammalian somatic cells is not able to effectively use long, perfect dsRNAs as a substrate for cleavage yet is able to cleave short, imperfect, pre-miRNA stem-loops effectively (15). This specificity has been mapped to the amino-terminal RNA helicase domain of Dicer, which appears to inhibit long dsRNA cleavage. Of interest, it has recently been demonstrated that mice express an amino-terminally-truncated, oocyte-specific Dicer isoform that is fully capable of processing long dsRNAs into siRNAs in oocytes (16) and likely also in mouse embryonic stem (ES) cells, which have been reported to generate protective siRNAs upon viral infection (17). However, in somatic cells expression of this shorter Dicer isoform is not detectable, and murine somatic cells, like human somatic cells, therefore are unable to generate significant levels of siRNAs from viral or other forms of long dsRNAs (14). While the consensus view of the field is therefore that virus-induced RNAi in mammals, unlike virus-induced RNAi in invertebrates and also in plants, does not represent a major intrinsic immune response, there have nevertheless been a number of reports suggesting that RNAi can occur in some settings, especially in stem cells, and confer some degree of antiviral protection (17, 18).

There is no doubt that, in contrast to siRNAs, all somatic cells express a range of different miRNAs. Over a thousand distinct miRNAs are encoded within the human and mouse genomes, and these are frequently expressed in a highly tissue-specific manner (5, 19). Therefore, any virus growing in a mammalian cell will transcribe mRNAs that have the potential to be inhibited by endogenous cellular miRNAs, and there have indeed been a number of studies reporting the inhibition of human immunodeficiency virus type 1 (HIV-1), influenza A virus (IAV), enterovirus, vesicular stomatitis virus (VSV), and primate foamy virus mRNAs, among others, by cellular miRNAs (20–30). However, miRNAs are highly conserved during evolution, and as targeting of an mRNA by an miRNA can potentially be blocked by single-nucleotide changes in the RNA target, it seems probable that viruses would have evolved to be largely refractory to inhibition by endogenous miRNAs (31). Moreover, extensive RNA secondary structure, an attribute of many viral RNAs (32), would also be expected to inhibit binding by miRNA-programmed RISCs (33). Finally, most viral mRNAs contain very short 3′ untranslated regions (3′UTRs), the most effective location for miRNA-mediated inhibition, and instead consist almost entirely of open reading

frames where ribosomal transit would be expected to displace bound RISCs (34).

While miRNAs normally act as inhibitors of mRNA function, there have been several reports documenting instances where cellular miRNAs facilitate viral replication. The most striking example occurs with hepatitis C virus (HCV), which requires the hepatocyte-specific cellular miRNA miR-122 for replication (35). Other examples include Epstein-Barr virus (EBV), which induces the expression of cellular miR-155 in infected B cells and requires high-level miR-155 expression in order to maintain viral latency in B cells. Evidence suggests that miR-155 functions by inhibiting the expression of cellular mRNAs that encode factors able to induce cell cycle arrest and apoptosis (36). In addition, enterovirus has been show to activate cellular miR-141 expression in order to inhibit cellular, cap-dependent mRNA translation (37), while Eastern equine encephalitis virus uses host miR-142-3p to attenuate the host innate immune response (38). Finally, a number of DNA viruses and retroviruses encode viral miRNAs that facilitate immune evasion and viral replication (39, 40).

Given the potential importance of RNAi in limiting viral replication and especially given recent reports arguing that both siRNAs and endogenous miRNAs can inhibit virus replication in mammalian somatic cells (17, 18, 20–30), we decided to rigorously test whether viruses are indeed either dependent on, or restricted by, siRNAs or endogenous miRNAs expressed in human cells. For this purpose, we used genome editing to generate human cell lines that entirely lack Dicer function and that therefore are unable to generate endogenous miRNAs or siRNAs (41), and we analyzed the abilities of a wide range of human viruses to replicate in these cells in culture. We report that virus replication in general is neither inhibited by nor enhanced by the complete loss of endogenous human miRNAs. We conclude that wild-type viruses have evolved mechanisms to avoid inhibition of viral gene expression by endogenous human miRNAs during the lytic replication cycle and that dependence on an endogenous miRNA for successful replication, as is seen with HCV, is rare.

## MATERIALS AND METHODS

**Small-RNA isolation from dengue virus- and West Nile virus-infected cells.** We infected $10^6$ Huh7 cells at a multiplicity of infection (MOI) of 1 with either West Nile virus (WNV) strain TX-IC7 (42) or dengue virus type 4 (DENV) isolate TVP360 (WHO reference strain). Total RNA was then isolated from cells using TRIzol (Invitrogen) at 48 h and 72 h post-WNV infection or at 96 h post-DENV infection. Small-RNA deep-sequencing libraries were generated from size-fractionated, small RNAs (~17 to 29 nt), ligated to Illumina adapter sequences, reverse transcribed, and then PCR amplified, as described previously (43). cDNA libraries were sequenced on an Illumina GA IIx sequence analyzer. Reads were obtained in FASTA format, preprocessed using the FASTX Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/index.html), and aligned concurrently to the human genome (hg19) and the respective viral genomes using Bowtie (44), allowing up to two mismatches, as previously described (45).

**qRT-PCR analysis of DENV and WNV RNA expression.** Reverse-transcribed cDNA was generated from flavivirus-infected Huh7 cells using TRIzol-extracted, DNase-treated RNA and SuperScript III (Invitrogen) in the presence of random primers. A DENV reverse transcription-quantitative PCR (qRT-PCR) standard (403-bp product; nt 10232 to 10626 of DENV GenBank accession numberNC_002640) was PCR generated from DENV-infected cells using the following primers: 5′-CAGT GCTCCTTCAGAGAGTGAAGG-3′ (DV4.F1) and 5′-AACAACACCAA TCCATCTTGCGGC-3′ (DV4.R1). Quantitative PCR to detect the

DENV infection level in Huh7 cells was performed using Power SYBR Green Master Mix (Applied Biosystems) and the following primers (140-bp product): DV4.R1 and 5′-TCCTGGTGGAAGGACTAGAGGTT A-3′ (DV4.F2). Published qRT-PCR primers (46) 5′-CGGTCGGAAAAG TGATTGACC-3′ (WNV.F1) and 5′-GCCCTTTGTGTACCCTCTGACT TC-3′ (WNV.R1), which amplify nt 7898 to 7975 of WNV, and qPCR standards for WNV nonstructural protein 5 (NS5) were used to detect WNV copies in Huh7 cells. qPCRs to detect β-actin (5′-CACACCTTCT ACAATGAGCTGCGTG-3′, Actin.F1; and 5′-ATGATCTGTGTCATCT TCTCGCGGT-3′, Actin.R1) were run in parallel.

**Cell culture.** NoDice(2-20) and NoDice(4-25) cells, which were derived from 293T cells by ablation of all copies of the endogenous human *dcr* gene, have been previously described (41). The Huh7, Vero, BHK-21, 293T, and NoDice cell lines were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics.

**Analysis of dengue virus and yellow fever virus replication in NoDice cells.** We plated $5 \times 10^4$ 293T, NoDice(2-20), or NoDice(4-25) cells per well in 48-well plates coated with collagen (Sigma catalog no. C8919). The following day, the media were removed and the cells were infected at an MOI of 1 with either DENV (strain TVP360) or yellow fever virus (YFV) (strain 17D). Virus-containing media were removed, and cells were rinsed once with phosphate-buffered saline (PBS). Virus was collected from triplicate wells at designated time points, and virus production was assayed by focus formation. Serial dilutions of DENV and YFV were used to infect Vero cell monolayers in 96-well microwell plates. After a 1-h incubation, the Vero cell monolayers were rinsed once with PBS and 100 μl of overlay medium (1% tragacanth gum [Sigma catalog no. G1128] in culture medium) was added to each well. Three days postinfection, the Vero cells were fixed in 4% paraformaldehyde, permeabilized with 35 μl 0.5% Triton X-100 in PBS, rinsed 3 times with 0.1% Tween 20 in PBS (PBS-T), and then blocked (1% normal goat serum in PBS-T) for 1 h at room temperature. The primary monoclonal antibody (J2; English & Scientific Consulting), which targets viral dsRNA, was diluted 1:1,000 in blocking solution and incubated with cells overnight at 4°C. Cells were then rinsed 3 times with 0.1% Tween 20 in PBS. The secondary antibody (goat anti-mouse Alexa Fluor 488; Invitrogen catalog no. A-11017) was diluted 1:2,000 in blocking solution and applied to the cells for 1 h at room temperature. Cells were rinsed with PBS-T, and viral foci were quantitated by immunofluorescence microscopy.

**Analysis of West Nile virus replication in NoDice cells.** For each time point, triplicates of 293T and NoDice monolayers were infected with WNV at an MOI of 0.1 in 24-well plates in 150 μl of modified Eagle's medium supplemented with 1% FBS, 10 mm HEPES, and antibiotics for 1 h. Media (350 μl) were added, and cell culture supernatants were harvested at 0, 24, 48, 72, and 96 h and stored at −80°C until use. Virus-containing supernatants were titrated in serial 10-fold dilutions on Vero cell monolayers in 24-well plates. Forty-eight hours after infection, monolayers were fixed with a 1:1 mixture of acetone and methanol. Fixed monolayers were incubated with a 1:1,000 dilution of the DENV E protein-specific, WNV E protein-cross-reactive antibody D1-4G2 in PBS plus 1% normal horse serum, followed by incubation with a 1:1,000 dilution of horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody. A VIP substrate kit (Vector Laboratories) was used for colorimetric detection of infectious foci.

**Analysis of Sindbis virus, Venezuelan equine encephalitis virus, and measles virus replication in NoDice cells.** Sindbis virus (SINV) strain TE (47), Venezuelan equine encephalitis virus (VEEV) strain TC-83 (48), a gift from Ilya Frolov (University of Alabama, Birmingham AL), and measles virus (MeV) strain IC-B (49) were used. Alphavirus stocks were grown in, and viral titers determined by plaque formation on, BHK-21 cells. MeV stocks were grown in, and viral titers determined by plaque formation on, Vero/hSLAM cells (50). 293T, NoDice(2-20), and NoDice(4-25) monolayers were infected with viruses at an MOI of 5 (SINV and VEEV) or 1 (MeV) for 1 h and washed with PBS (pH 6.2), and media were replaced.

Supernatant fluids were collected at 1, 6, 12, and 24 h (SINV and VEEV) or 1, 24, and 48 h (MeV) for assay of infectious virus.

**Analysis of influenza A virus replication in NoDice cells.** IAV strain A/WSN/1933(H1N1) was a gift from Peter Palese. 293T cells or NoDice cells were challenged with the virus (MOI = 0.1) in a minimal volume of DMEM sufficient to cover the plated cells. After 30 min of incubation at 37°C, the cells were washed with PBS and incubated with fresh DMEM. Total RNA was extracted using TRIzol at 0, 3, 6, and 9 h after the 30-min virus adsorption. RNA levels of the IAV nucleoprotein (NP) gene were compared to that at the 0-h time point and normalized to cellular GAPDH mRNA levels and quantified by qRT-PCR as described previously (51).

**Analysis of reovirus replication in NoDice cells.** Reovirus prototype strains T1L and T3D (52) were plaque purified and amplified in mouse L929 cells, purified on a CsCl gradient, and stored in aliquots at −80°C (53). 293T or NoDice(2-20) cells were plated at $6 \times 10^3$ cells per well in duplicate wells of a 96-well cluster and incubated overnight. Overlying media were removed, and virus was added at an MOI of 3. After incubation for 1 h, inocula were removed and replaced with fresh media. After 22 h of incubation, cultures were frozen at −80°C, supplemented to 0.5% NP-40, and then subjected to two additional freeze/thaw cycles to generate lysates. They were then serially diluted, and titers were determined by plaque assay on L929 cells.

**Analysis of HIV-1 infection and production by NoDice cells.** To analyze HIV-1 production in NoDice cells, we transfected $2 \times 10^6$ 293T, NoDice(2-20), or NoDice(4-25) cells in a 10-cm dish using Fugene6 (Promega) according to the manufacturer's protocol, with 10 μg pNL-GFP-HXB and 500 ng pK-GST as controls for transfection efficiency (54). At 48 h posttransfection, the virus-containing supernatant media were filtered through a 0.45-μm-pore-size filter (Acrodisc filter; Pall) and then used to infect TZM-bl cells (55). Twenty-four hours postinfection, the TZM-bl cells were lysed in Passive lysis buffer (Promega) and analyzed for firefly luciferase (FLuc) activity, using a luciferase assay system (Promega). In parallel, the virus producer cells were lysed, the whole-cell lysate was subjected to gel electrophoresis and transferred to a nitrocellulose membrane, and the level of expression of the cotransfected glutathione S-transferase (GST) internal control was analyzed by Western blotting using a rabbit polyclonal anti-GST antibody (sc-459; Santa Cruz) and anti-rabbit IgG peroxidase (A6514; Sigma). GST levels were visualized by chemiluminescence (WesternBright Sirius kit; Advansta), and the image was captured using G:Box (SynGene) and then quantified with GeneTools (SynGene) software.

To analyze HIV-1 infection efficiency in NoDice cells, we transfected $2 \times 10^6$ 293T cells using Fugene6 with 10 μg of pNL4-Luc-HXB and 500 ng pCMV-VSV-G (56). Forty-eight hours posttransfection, virus-containing supernatants were filtered and used to infect 293T, NoDice(2-20), and NoDice(4-25) cells. Twenty-four hours postinfection, cells were lysed and analyzed for FLuc expression.

For pseudotyped HIV-1 virus production experiments, $2 \times 10^7$ 293T cells were transfected in a 15-cm dish using polyethylenimine with 25 μg of pD3-HIV-GFP (57) and 10 μg of pCMV-VSV-G. At 48 and 72 h posttransfection, the supernatant media were filtered and pooled and equal amounts were used to infect 293T, NoDice(2-20), and NoDice(4-25) cells. At 48 h postinfection, cells were analyzed by flow cytometry for green fluorescent protein (GFP) expression to determine the percentage of 293T and NoDice cells infected by HIV-1.

**Analysis of vesicular stomatitis virus infection and production in NoDice cells.** A viral stock of a VSV Indiana strain derivative engineered to express GFP (VSV-GFP; a gift from Elizabeth Ramsburg) was produced in BHK-21 cells as previously described (58). NoDice and 293T cells were infected with VSV and incubated for 9 h, and supernatants and cells were then harvested for analysis of infectious virus and viral RNA production, respectively. Infectious virus production was assayed by limiting dilution onto BHK-21 cells, which were infected for 16 h prior to analysis by flow cytometry for GFP expression. Total RNAs from VSV-infected 293T and NoDice cells were isolated using TRIzol at 9 h postinfection. Total RNA

(250 ng) was then reverse transcribed using Superscript II (Invitrogen) and 50 ng of random primers (Bioline). The resulting cDNAs were assayed for relative VSV genomic RNA levels using Power SYBR Green PCR Master Mix (Applied Biosystems) on a StepOnePlus real-time PCR system (Applied Biosystems) and normalized via the $\Delta\Delta C_T$ method (59), with cellular glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA serving as the internal control. A region of the genomic polymerase (L) gene specific to the Indiana strain of VSV was amplified with 5 pmol of the following primers based on previously published primer designs (60): F, 5′-GGTGGTTATTCCATTTTTCGTA-3′; and R, 5′-GGTG TTGCAGACTATGTTGGAC-3′. As an internal control, GAPDH cDNA was amplified in parallel.

**Analysis of herpes simplex virus 1 replication in NoDice cells.** A viral stock of herpes simplex virus 1 (HSV-1) isolate 17syn(+) was grown in rabbit skin cells as previously described (61). 293T or NoDice cells (3 × 10⁵) were infected in serum-free media with HSV-1 at an MOI of ~0.1 for 1 h. Cells were then washed twice with PBS and incubated for an additional 18 h in complete media (DMEM plus FBS and antibiotics). Cells were then collected, and total DNA was harvested with DNeasy kits (Qiagen), including proteinase K and RNase treatment. qPCR was performed using a custom TaqMan probe for the HSV-1 polymerase gene (UL30), and results were normalized to those for the 18S rRNA gene (Hs99999901_s1) using universal PCR master mix (catalog no. 4324018; Applied Biosystems).

**Data deposition.** Sequencing data have been submitted to the NCBI Sequence Read Archive (NCBI BioProject PRJNA245819). Processed files will be made available on request.

## RESULTS

**Analysis of small viral RNA expression in flavivirus-infected cells.** Previous studies examining small-RNA production in cells infected with flaviviruses have generated contradictory results. On the one hand, Parameswaran et al. (13) reported that deep sequencing of small RNAs present in Huh7 cells infected with DENV did not result in the detection of any DENV-derived miRNAs or siRNAs, while others have reported that DENV infection of mosquitos or of the mosquito cell line Aag2 induces the expression of DENV-specific siRNAs, as expected, but does not give rise to detectable DENV-derived miRNAs (62, 63). In contrast, Hussain and Asgari recently reported (64) that DENV expresses a virally derived miRNA in infected mosquitos and in Aag2 cells, and Kakumani et al. (65) proposed that DENV infection of Huh7 cells gives rise to virally derived siRNAs that can attenuate DENV replication. Analysis of small RNAs in insect cells infected with WNV, a second flavivirus, has also led to the suggestion that WNV expresses a viral miRNA (66), yet deep sequencing analysis of small viral RNAs in WNV-infected mosquitos failed to detect any viral miRNAs (67).

To examine whether either DENV or WNV expresses either siRNAs or viral miRNAs in infected mammalian cells, we infected Huh7 cells with DENV or with WNV, both at an MOI of 1, and then harvested and deep sequenced small RNAs expressed at 96 h postinfection with DENV or at 48 h or 72 h postinfection with WNV. At this time point, the DENV-infected cells expressed an average of ~1.3 × 10³ copies of viral RNA per cell, while the two WNV-infected cultures expressed ≥10⁵ copies of viral RNA per cell. Deep sequencing of small RNAs derived from the DENV-infected Huh7 cells gave 1,262,884 reads 17 to 29 nt in length that could be assigned to either the human or DENV genome, of which 8,057 (0.64%) reads were of viral origin. Analysis of WNV-infected Huh7 cells yielded 4,301,656 and 981,900 assignable 17- to 29-nt reads at 48 h and 72 h postinfection, respectively, of which

27,793 (0.65%) and 11,621 (1.18%) were of WNV origin. Therefore, while DENV replicates less efficiently in Huh7 cells than does WNV, it gives rise to a comparable number of small RNA reads as measured by deep sequencing.

As previously discussed by ourselves and others (17, 40), siRNAs and miRNAs have a number of key, defining characteristics. In the case of siRNAs, they should be 22 ± 2 nt in length and derived in equal proportions from both the plus strand and the minus strand of the viral dsRNA substrate. MiRNAs are also 22 ± 2 nt in length, due to their shared origin as Dicer cleavage products, but are expected to be derived from one or a small number of specific locations on either the viral RNA plus or minus strand.

Analysis of the small-RNA reads obtained by deep sequencing of RNA obtained from DENV- or WNV-infected Huh7 cells showed that the reads of cellular origin were, as expected, largely derived from cellular miRNAs, with miR-21 as the most highly expressed miRNA. The cellular reads were therefore largely 22 ± 2 nt in length (Fig. 1A). In contrast, the short RNA reads derived from DENV were not concentrated at any particular length (Fig. 1B), which is inconsistent with their origin resulting from Dicer cleavage of viral dsRNAs. Similarly, the small-RNA reads obtained from the WNV-infected Huh7 cells were also not concentrated at the 22- ± 2-nt size predicted for Dicer cleavage products but were instead primarily <20 nt in length (Fig. 1C). Therefore, these viral RNAs do not have the characteristics expected for either viral siRNAs or miRNAs. Importantly, the mechanisms underlying miRNA processing are conserved between insect and mammalian cells (68), and we have recently demonstrated that expression of the *Drosophila* Dicer1 protein, together with its cofactor Loqs-PB, can efficiently rescue pre-miRNA processing in mammalian cells lacking a functional Dicer protein (41). Therefore, if either DENV or WNV indeed expressed a viral miRNA in infected insect cells, we would expect to also recover that same miRNA in infected human cells.

We next analyzed the genomic origin of these small-RNA reads. As shown in Fig. 2, for both DENV and WNV almost all reads were found to derive from the viral RNA plus strand, which is expressed at much higher levels than the viral RNA minus strand in flavivirus-infected cells. This again is consistent with these small RNAs being RNA breakdown products, which are expected to arise at levels that are proportional to the level of expression of the long RNA of origin, rather than that of siRNAs, which are expected to derive equally from both strands of the dsRNA intermediates that arise during DENV and WNV genome replication (17). We note that many of these plus-sense small RNAs did derive from "hot spots" on the viral RNA genome, and they therefore might be viral miRNAs. However, these RNAs are not, as discussed above, the ~22-nt size predicted for miRNAs and they also do not bear a defined 5′ end, a key characteristic of authentic viral miRNAs, as it defines the essential "seed" sequence that controls mRNA target specificity (5; also data not shown). Finally, individual candidate viral miRNAs were expressed at levels that are too low to be physiologically relevant and did not accumulate during the viral life cycle (compare Fig. 2B and C). Therefore, these data strongly argue that DENV and WNV neither express viral miRNAs nor induce the production of antiviral siRNAs in infected Huh7 cells.
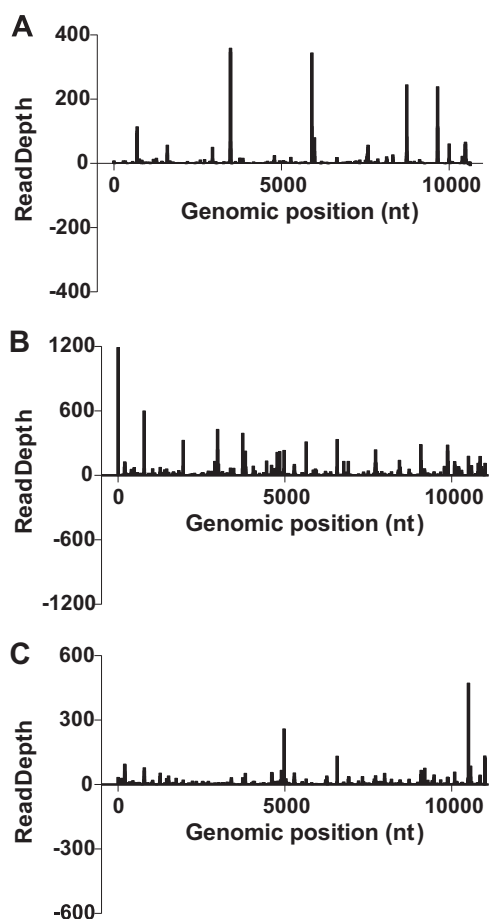
**Analysis of virus replication in Dicer-deficient human cells.** Recently, we described the derivation and analysis of human cells that lack an intact *dcr* gene and, hence, are unable to process pre-
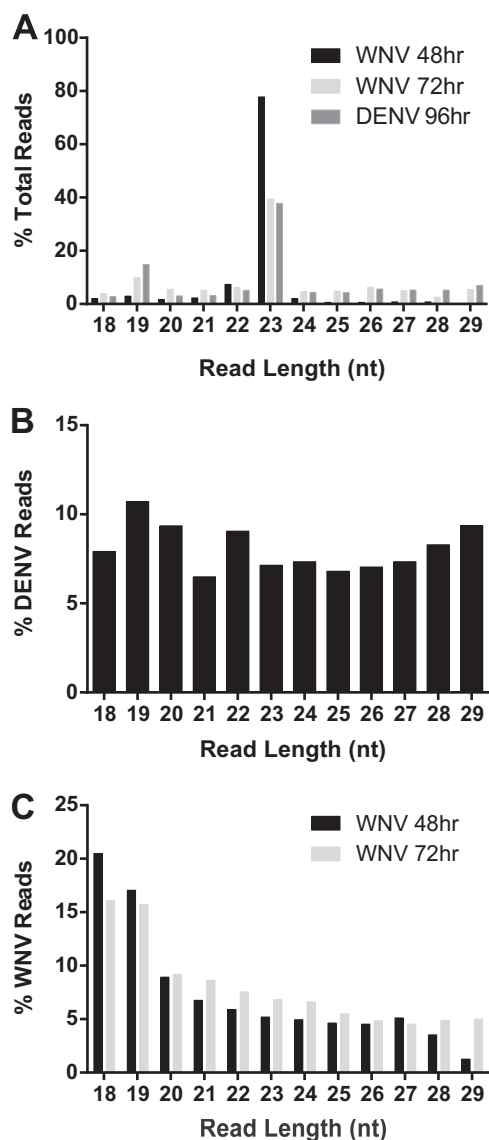
FIG 1 Size distribution of small RNAs expressed in DENV- and WNV-infected Huh7 cells. Human Huh7 cells were infected at an MOI of 1 with either DENV or WNV. Total RNA was harvested at 96 h postinfection for DENV or at both 48 h and 72 h postinfection for WNV. Small RNAs (17 to 29 nt in length) were then subjected to deep sequencing and aligned to the human or relevant viral genome. (A) Size distribution of small RNAs of human origin. (B) Size distribution of small RNAs derived from DENV. (C) Size distribution of small RNAs of WNV origin at 48 h and 72 h postinfection.



FIG 2 Genomic origin of small viral RNAs recovered in DENV- or WNV-infected Huh7 cells. Small viral RNAs recovered by deep sequencing, as described for Fig. 1, were aligned to their genomic location of origin on the DENV genome (A) or the WNV genome (B and C), with RNAs derived from the positive strand shown above the x axis and those originating from the viral negative strand below the x axis. As may be observed, almost all viral reads derived from the viral positive strand.

miRNAs into mature miRNAs or dsRNAs into siRNAs (41). These two clonal cell lines, which we term NoDice(2-20) and NoDice(4-25), were generated by genome editing using transcription activator-like effector nucleases (TALENs) in the context of the human cell line 293T.

While there have been previous articles describing the derivation of murine ES cells or mouse embryo fibroblasts (MEFs) lacking Dicer and miRNA expression (69–71), there has so far been no report examining the ability of viruses to replicate in the absence of Dicer, an enzyme required for both miRNA and siRNA biogenesis, with the exception of a single article demonstrating that the alphavirus SINV replicates with comparable efficiencies in wild-type and Dicer-deficient MEFs (72).
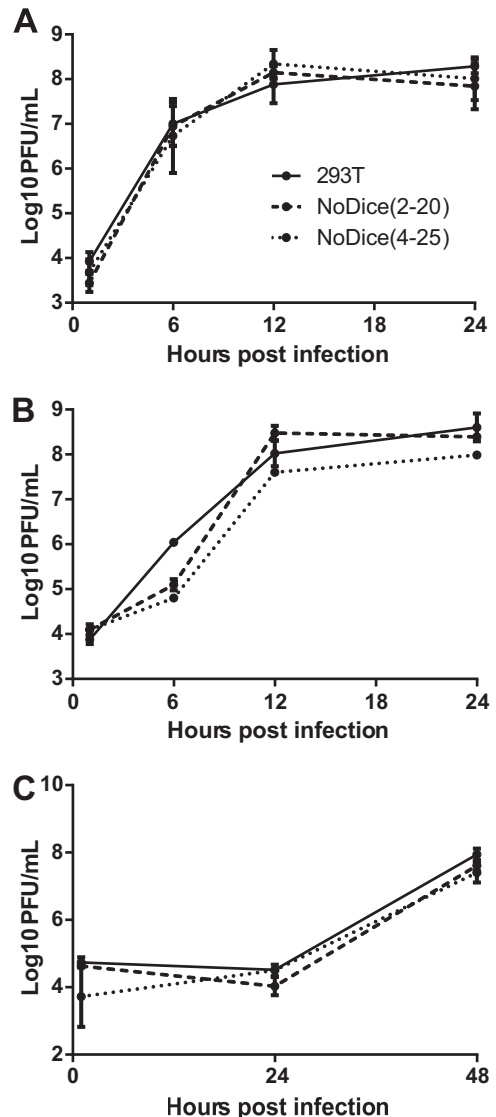
If a given virus was able to induce the production of siRNAs of viral origin, then one would predict that loss of Dicer function would result in enhanced virus replication, as has indeed been reported for invertebrates (9–11, 73). Similarly, if a given virus was subject to inhibition by cellular miRNAs, then loss of Dicer and the concomitant loss of miRNA expression should again result in enhanced virus replication. Conversely, if a virus is dependent on a cellular miRNA for some aspect of its replication cycle, then the total loss of miRNA expression should lead to a reduction in virus replication or possibly even to a complete block, as has been reported in the case of HCV (35). We therefore asked if loss of Dicer expression would affect the replication of DENV, WNV, or YFV, another flavivirus, in human cells. As shown in Fig. 3A, DENV grew slightly more slowly in the two NoDice cell lines than in wild-type 293T cells, while the distantly related YFV replicated essentially indistinguishably in the presence and absence of cellular Dicer activity (Fig. 3C). WNV replicated indistinguishably in 293T cells and in the 2-20 clone of NoDice cells yet replicated to slightly lower titers in the NoDice(4-25) cells (Fig. 3B). Importantly, none of these three flaviviruses showed more rapid repli-
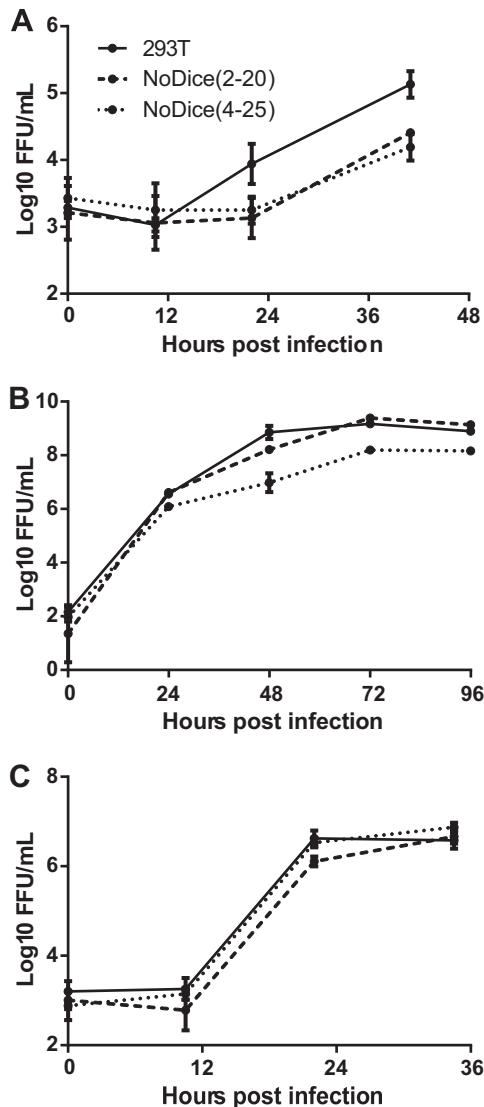
**FIG 3** Analysis of the replication rates of three flavivirus species in cells lacking Dicer function. Either wild-type 293T cells or their Dicer-deficient clonal derivatives, NoDice(2-20) and NoDice(4-25) cells, were infected with DENV (A), WNV (B), or YFV (C) at an MOI of 1 (DENV and YFV) or 0.1 (WNV), and the levels of viral progeny released into the supernatant media were determined by measuring viral focus forming units (FFU) at the indicated time points. Averages of three replicates with standard deviations (SDs) are indicated.

**FIG 4** Analysis of the replication rate of SINV, VEEV, or MeV in the presence or absence of Dicer function. Wild-type 293T cells or their Dicer-deficient derivatives, NoDice(2-20) and NoDice(4-25) cells, were infected with SINV at an MOI of 5 (A), VEEV at an MOI of 5 (B), or MeV at an MOI of 1 (C). Supernatant media were collected at the indicated times and assayed for infectious virus by measurement of PFU on BHK-21 (A and B) or Vero/hSLAM (C) cells. Data from representative duplicate experiments are presented as averages of triplicate samples with SDs indicated.

cation in the absence of Dicer, as would be predicted if viral replication were inhibited by either siRNAs or endogenous miRNAs in the wild-type 293T cells.

We next extended this analysis to two other positive-sense, single-stranded RNA viruses, i.e., SINV and VEEV, both of which are alphaviruses, as well as to MeV, a paramyxovirus belonging to the morbillivirus genus that has a single-stranded, negative-sense RNA genome. As may be observed in Fig. 4, all three of these distinct RNA viruses replicated to approximately equivalent levels in wild-type 293T cells and the two NoDice cell lines. Analysis of IAV, an orthomyxovirus bearing a segmented negative-sense RNA genome, again revealed no difference in the levels of virus replication in the presence and absence of Dicer function (Fig. 5).

Analogous data, showing little or no difference in virus replication in the presence and absence of cellular miRNAs, were also generated using either the T1L or the T3D strain of reovirus, a segmented dsRNA virus (Fig. 6).

The next virus we examined was VSV, a rhabdovirus bearing a nonsegmented, negative-sense, single-stranded RNA genome. We note that it has previously been reported that VSV is subject to significant repression by cellular miR-93 (28), which is expressed at high levels in wild-type 293T cells (~1.3% of the total miRNA pool) but is obviously lacking in the NoDice cells. In the case of VSV, we analyzed both infectious virus production (Fig. 7A) and the level of intracellular VSV genome expression (Fig. 7B) in the infected 293T and NoDice cell lines. However, we again detected
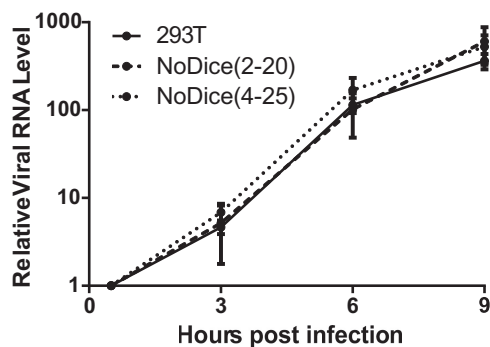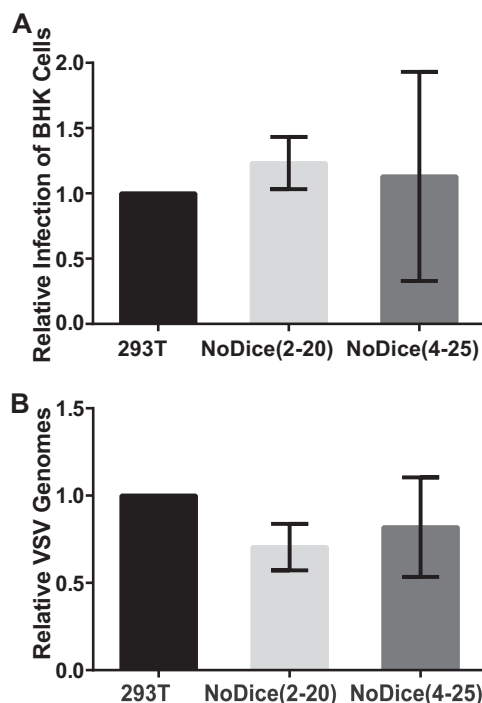
FIG 5 Analysis of the levels of IAV RNA expression in infected cells in the presence or absence of Dicer function. Wild-type 293T cells or their Dicer-deficient derivatives, NoDice(2-20) and NoDice(4-25) cells, were infected with IAV strain WSN at an MOI of 0.1, and viral replication was then assayed at the indicated times postinfection by quantitation of the level of the viral nucleoprotein genome segment by qRT-PCR. Cellular GAPDH mRNA was used as an internal control. Averages of three independent experiments with SDs are indicated.

no significant difference in the replication competence of VSV in the presence or absence of Dicer.

We next turned our attention to HIV-1, which has been reported to be subject to repression by a range of cellular miRNAs, including miR-29a and miR-28 (22–24), both of which are expressed at substantial levels in wild-type 293T cells, comprising ~0.16% and ~1.0% of the total miRNA pool, respectively. In this case, we divided our analysis of the HIV-1 replication cycle into two parts, i.e., from infection to proviral integration and gene expression (Fig. 8A and B) or from viral gene expression through virion release to target cell infection (Fig. 8C). The ability of NoDice cells to support productive HIV-1 infection was analyzed using stocks of HIV-1 indicator viruses bearing either the FLuc gene (Fig. 8A) or the green fluorescent protein (*gfp*) gene (Fig. 8B). The levels of infection of the 293T and NoDice cells were analyzed at 24 h postinfection by luciferase assay (Fig. 8A) or at 48 h postinfection by quantitation of the number of GFP-positive cells by
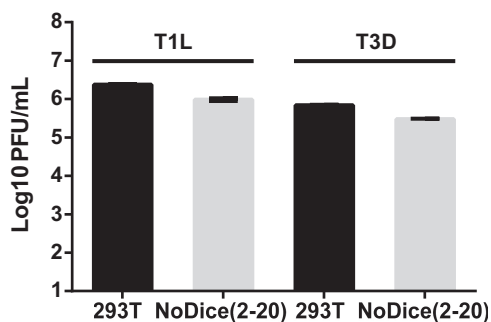


FIG 6 Quantification of the level of reovirus replication in the presence or absence of Dicer function. Wild-type 293T cells or their derivative, NoDice(2-20) cells, were infected with reovirus strain T1L or T3D at an MOI of 3, and viral replication was then assayed at 22 h postinfection by plaque assay on mouse L929 cells. Averages of two wells with SDs are indicated. While results suggest a slightly lower level of replication in NoDice cells, NoDice cells replicate more slowly than the parental 293T cells and were less abundant at the time of harvest (data not shown), likely accounting for the minimal difference in titers.



FIG 7 Quantification of the levels of VSV replication and RNA expression in infected cells in the presence or absence of Dicer. Either wild-type 293T cells or their derivatives, NoDice(2-20) and NoDice(4-25) cells, were infected with a VSV derivative engineered to express GFP. (A) At 9 h postinfection, the supernatant media were harvested and viral progeny production was analyzed on BHK-21 cells by FACS analysis of the level of GFP-positive cells. (B) In parallel, total RNA was harvested from the transfected cells, and levels of VSV genomic RNA were quantified by qRT-PCR. Cellular GAPDH mRNA was used as an internal control. Averages of three independent experiments with SDs are indicated.

fluorescence-activated cell sorter (FACS) (Fig. 8B). No difference in the level of infection was observed in either case.

Next we tested whether NoDice cells would be able to produce infectious HIV-1 virions by transfecting wild-type 293T cells or NoDice(2-20) or NoDice(4-25) cells with a vector encoding a replication-competent HIV-1 provirus. Supernatant media were then harvested and used to infect the indicator cell line TZM-bl, which expresses FLuc only after HIV-1 infection (55). As may be observed (Fig. 8C), we detected at most a modest reduction in the ability of NoDice cells to produce infectious HIV-1 virions and certainly no evidence of enhanced virus production.

In a final experiment, we analyzed the ability of a DNA virus, HSV-1, to replicate in the NoDice cells. HSV-1 encodes at least eight viral miRNAs, several of which are expressed at significant levels in productively HSV-1-infected cells (61), and these viral miRNAs are all dependent on Dicer for their production. Analysis of the level of HSV-1 replication in wild-type 293T cells versus that in the NoDice cells revealed a significant, ~2-fold decrease in viral replication in the NoDice cells (Fig. 9), possibly consistent with the hypothesis that HSV-1 replication is, in aggregate, facilitated by viral and/or cellular miRNAs.
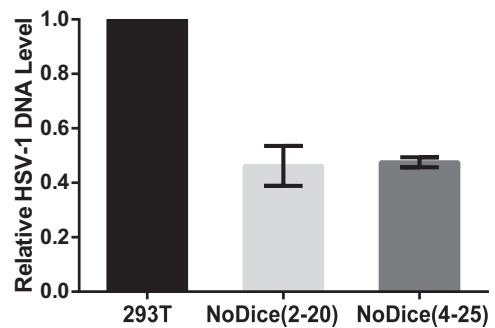
## DISCUSSION

Although several studies have failed to detect siRNAs in virus-infected mammalian cells (13, 14) and the full-length form of
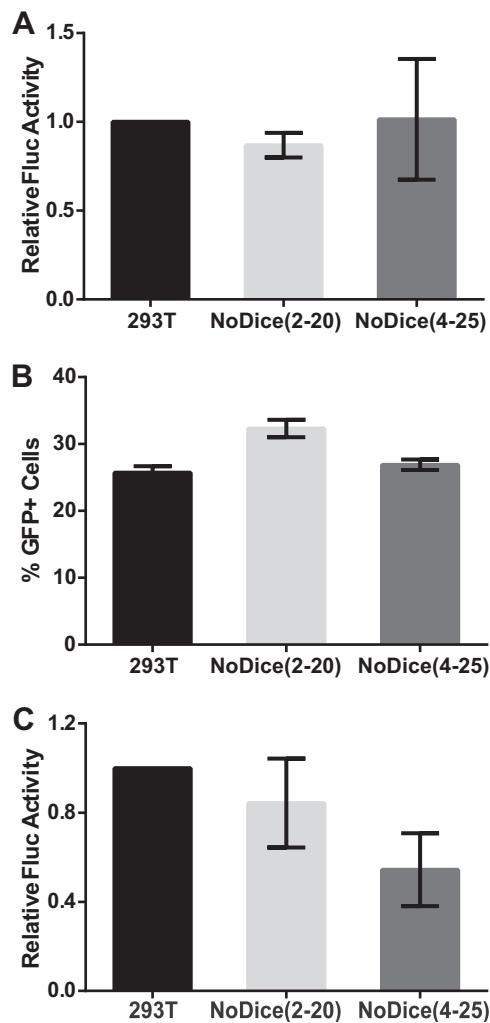
FIG 8 Analysis of HIV-1 infection and progeny production in the presence and absence of Dicer function. (A) Wild-type 293T cells or the NoDice(2-20) and NoDice(4-25) cell lines were infected with an equal amount of a previously described HIV-1 derivative engineered to express FLuc (56). At 24 h postinfection, the cells were lysed and the level of FLuc expression was quantitated. (B) Shown are the results of an experiment similar to that for panel A, except that the cells were infected with an HIV-1 derivative engineered to express GFP. The level of transduced cells was quantitated by FACS at 48 h postinfection. (C) 293T or NoDice cells were transfected with a plasmid encoding a full-length replication-competent HIV-1 provirus together with an internal control plasmid expressing GST. At 48 h posttransfection, the supernatant media and transfected cells were harvested, and the former was filtered and then used to infect the indicator cell line TZM-bl, which expresses FLuc only after HIV-1 infection. Induced FLuc expression levels were determined at 24 h postinfection. In parallel, the level of expression of the GST internal control in the transfected cells was determined by Western blotting and used to correct the level of FLuc expression (54). Averages of three independent experiments are shown in each panel with SDs indicated.



FIG 9 Analysis of HSV-1 infection in the presence and absence of Dicer function. Wild-type 293T cells or the NoDice(2-20) and NoDice(4-25) cell lines were infected with HSV-1 at an MOI of 0.1. Total DNA was harvested at 18 h postinfection, and HSV-1 genomic DNA was quantitated by qPCR. The human 18S rRNA gene served as an internal control. Averages of three independent experiments with SDs are indicated.

mammalian Dicer expressed in somatic cells appears unable to process perfect, long dsRNAs into siRNAs (15, 16), there have also been reports suggesting that siRNAs play a role in the intrinsic antiviral response in mammalian somatic cells (18, 65). Moreover, it has also been suggested that endogenous cellular miRNAs can exert a significant antiviral effect on wild-type viruses in human cells (20–30), and it is certainly true that mutant viruses engineered to contain target sites for a specific cellular miRNA are severely attenuated in their ability to grow in cells that express that miRNA (74–78).

In this study, we first used deep sequencing to examine the small RNAs expressed by two flaviviruses, DENV and WNV, in infected human Huh7 cells. This effort was prompted by recent reports suggesting that DENV replication is significantly repressed by siRNAs and/or miRNAs produced in infected cells and arguing that both DENV and WNV express viral miRNAs in infected insect cells (64–66). In fact, we failed to detect any siRNAs in human Huh7 cells infected by DENV or WNV, and we also failed to detect any viral miRNAs (Fig. 1 and 2). For DENV, this result reproduces previous work which failed to detect either siRNAs or viral miRNAs in infected Huh7 cells (13) as well as previous research that failed to detect any viral miRNAs in DENV-infected mosquitos or cultured Aag2 insect cells, though siRNAs, as expected, were readily observed (62, 63). Similarly, deep sequencing of small RNAs produced in WNV-infected mosquitos failed to identify any virally encoded miRNAs, though viral siRNAs were abundant (67). We therefore conclude that DENV and WNV do not express any viral miRNAs in infected mosquitos or human cells and, further, that neither DENV nor WNV induces the production of viral siRNAs in infected human somatic cells, though both viruses are certainly able to do so in infected mosquito cells (62, 63, 67).

If viral infection of somatic human cells induces siRNA production and/or if endogenous miRNAs are able to bind and inhibit viral mRNAs, then loss of Dicer function, which would block both siRNA and miRNA production, should result in enhanced virus replication. Conversely, if a particular virus is dependent on a cellular mRNA for a step in its replication cycle, as seen with HCV (35), then Dicer-deficient cells should be partly or entirely nonpermissive for the replication of that virus. To globally examine whether the replication of any human virus is affected by miRNA expression, we analyzed the abilities of 10 diverse RNA viruses, as well as one DNA virus, to replicate in human cells that lack Dicer function and, therefore, are defective for pre-miRNA processing and mature miRNA expression (41). Our results demonstrate that most viruses analyzed, including WNV, YFV, SINV, VEEV, MeV, IAV, VSV, and HIV-1, replicate with more or less the same efficiency in human cells expressing or lacking Dicer (Fig. 3B and C, 4, 5, 7, and 8). For some viruses, specifically DENV, reovi-

rus, and HSV-1, we consistently saw slightly lower replication levels in the absence of Dicer (Fig. 3A, 6, and 9). However, as previously reported (41), 293T cells lacking Dicer grow ~2-fold more slowly than wild-type 293T cells, and at least in the case of reovirus, the ~2-fold-lower level of virus seen in the NoDice(2-20) cells can be fully explained by the ~2-fold-lower level of cells in the NoDice(2-20)-infected wells at the time of virus harvest (Fig. 6). This effect likely also explains at least in part, but perhaps not fully, the lower levels of DENV replication seen in the NoDice(2-20) and NoDice(4-25) cells at later time points (Fig. 3A). Alternatively, we note that Seo et al. (79) recently reported that a number of interferon-stimulated genes (ISGs) are subject to repression by cellular miRNAs and presented evidence indicating that this repression can significantly enhance the replication of HSV-1, thus implying that the global loss of miRNA expression, as seen in the NoDice cell lines, might result in enhanced levels of basal ISG expression and a reduction in virus replication. Indeed, in the case of HSV-1, where virus replication was assayed by quantitation of viral DNA at 18 h postinfection (Fig. 9), slower growth of the NoDice cells likely does not explain the observed lower viral growth rate, as the observed signal was normalized to that of the host cell 18S rRNA gene. However, it is also possible that the inability of HSV-1 to express any of the eight miRNAs encoded by this virus (61) explains the slightly lower level of replication of HSV-1 seen in the NoDice cells.

It is important to note that none of the 11 diverse virus species examined here replicated more efficiently in the absence of Dicer and of cellular miRNA species. Therefore, these data argue that none of these viruses is subject to significant inhibition by any of the miRNAs expressed in 293T cells. This is equally true for VSV, which has been reported to be inhibited by miR-93 (28), and HIV-1, which has been reported to be inhibited by miR-29a and miR-28 (22–24), all of which are expressed at readily detectable levels in wild-type 293T cells. We therefore believe that the viruses analyzed here, and possibly all viruses, have evolved to be resistant to inhibition of their lytic replication cycle by endogenous cellular miRNAs. We hypothesize that, as previously discussed (31), viruses likely use one of three nonexclusive mechanisms to avoid inhibition by cellular miRNAs. These are (i) the selective loss of miRNA target sites on viral RNAs due to selection of point mutations in regions complementary to miRNA seed sequences, (ii) the evolution of viral RNA secondary structures that globally block miRNA access, and (iii) lack of significant 3′UTR regions in many viral miRNAs, which represent the only effective binding sites for miRNA-programmed RISCs. As computational analysis does not suggest that miRNA seed target sites on viral genomes are significantly underrepresented relative to random chance (D. L. Corcoran and B. R. Cullen, unpublished observations), we favor the latter two mechanisms, most probably in combination. Indeed, at least in the case of HIV-1, there is evidence demonstrating that the viral RNA genome adopts an extensive secondary structure (80) and that only the rare regions of the HIV-1 genome that are predicted to be single stranded can be effectively targeted by siRNA-programmed RISCs (81).

While our results clearly argue that viral gene expression during the lytic replication cycle is not subject to significant repression by endogenous miRNAs, they do not imply that cellular miRNAs are constitutively unable to repress viral mRNA function. As noted above, introduced artificial target sites for endogenous miRNAs can very effectively alter the tissue tropism of specific viruses (74–78). More importantly, it is also clear that viruses that can establish latent infections, especially herpesviruses, use endogenous cellular miRNAs to attenuate the expression of specific viral mRNAs and thereby facilitate entry into latency and/or maintenance of the latent state (30, 45, 82, 83). Therefore, viruses are able to use endogenous cellular miRNAs to help maintain latency by repressing specific viral mRNAs, yet they clearly avoid repression of viral mRNAs by cellular miRNAs during the lytic replication cycle.

It could be argued that human 293T cells and their NoDice derivatives are not a good model system to examine whether miRNAs play a role in virus replication, as they are not representative of the tissues that these viruses normally replicate in *in vivo*. That is, this question should be analyzed using T cells for HIV-1, neuronal cells for HSV-1, etc. However, we believe that this is not, in fact, the case. In particular, if viruses indeed evolved to avoid miRNA binding by the selection of point mutants that destroy potential miRNA binding sites, then they would be more likely to be resistant to the pattern of miRNA expression seen in their normal target tissues and less likely to be resistant to the novel miRNAs seen in a cell type, such as 293T cells, that they normally would never encounter *in vivo*. Nevertheless, we observed no evidence that cellular miRNAs are able to significantly repress the replication of wild-type viruses. We do not believe that this reflects viral inhibition of miRNA function, as several different viruses engineered to contain miRNA target sites have been shown to be repressed by endogenous miRNAs specific for that target site (74–78). One implication of these data is that virus mutants engineered to lack RNA secondary structure but that encode the same protein components might become sensitive to repression by endogenous miRNAs and exhibit an attenuated phenotype. Whether this approach has any potential for the development of attenuated virus vaccines, as has been suggested for virus mutants bearing inserted miRNA target sites (74, 76, 78), is currently unclear, but such "unstructured" viral mutants likely would be more phenotypically stable, as the miRNA target sites would not be as readily deleted during virus passage. It will therefore clearly be of interest to determine whether viral sensitivity to inhibition by cellular miRNAs can indeed be rescued by the introduction of mutations designed to reduce viral RNA secondary structure.

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