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Research Paper

# Zika Virus Replicons for Drug Discovery

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ABSTRACT

The current epidemic of Zika virus (ZIKV) has underscored the urgency to establish experimental systems for studying viral replication and pathogenesis, and countermeasure development. Here we report two ZIKV replicon systems: a luciferase replicon that can differentiate between viral translation and RNA synthesis; and a stable luciferase replicon carrying cell line that can be used to screen and characterize inhibitors of viral replication. The transient replicon was used to evaluate the effect of an NS5 polymerase mutation on viral RNA synthesis and to analyze a known ZIKV inhibitor. The replicon cell line was developed into a 96-well format for antiviral testing. Compare with virus infection-based assay, the replicon cell line allows antiviral screening without using infectious virus. Collectively, the replicon systems have provided critical tools for both basic and translational research.


## 1. Introduction

Zika virus (ZIKV) is a mosquito-borne member of the genus *flavivirus* within the family *Flaviviridae*. Many flaviviruses, such as the four serotypes of dengue virus (DENV-1 to -4), yellow fever (YFV), West Nile virus (WNV), Japanese encephalitis virus (JEV), and tick-borne encephalitis virus (TBEV), cause significant human diseases. The current epidemic of ZIKV in the Americas poses an international public health emergency. Symptomatic ZIKV infection presents with "dengue-like" manifestations, such as fever, headaches, lethargy, conjunctivitis, rash, arthralgia, and myalgia. Severe diseases associated with ZIKV infection include neurotropic Guillain-Barre syndrome and congenital microcephaly (Shan et al., 2016a). Despite urgent medical needs, neither vaccine nor therapeutics are currently available for ZIKV.

Flaviviruses have a positive single-strand RNA genome of approximately 11,000 nucleotides in length. The genome contains a 5′ untranslated region (UTR), long open-reading frame (ORF), and 3′UTR. The ORF encodes three structural (capsid [C], precursor membrane [prM], and envelope [E]), and seven non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins. The structural proteins form viral particles. The nonstructural proteins participate in viral replication, virion assembly, and evasion of the host immune response (Lindenbach et al., 2013).

In response to the current ZIKV emergence, we urgently need to develop experimental systems that could be used to study why ZIKV has become epidemic with increased disease severity (Guillain-Barre syndrome and microcephaly). It is equally urgent to establish tools for vaccine and antiviral development. Flavivirus replicon is a self-replicative viral RNA. Compare with the complete viral genome, the replicon contains a deletion of viral structural gene(s) (Khromykh and Westaway, 1997). The replicon can be launched through transfection of susceptible cells with in vitro transcribed RNA, or introduced from plasmids that encode the replicon cDNA under a CMV or SV40 promoter. Flavivirus replicon can also be stably expressed in cells when a selection marker (such as Neomycin resistance gene [Neo]) is engineered into the system. Such replicon systems have been established for WNV (Khromykh and Westaway, 1997; Lo et al., 2003a; Shi et al., 2002), YFV (Corver et al., 2003; Jones et al., 2005), DENV (Ng et al., 2007; Puig-Basagoiti et al., 2006), TBEV (Gehrke et al., 2003), and JEV (Li et al., 2013). They have been successfully used to study viral translation/replication (Manzano et al., 2011; Wang et al., 2011), to produce single-round infectious particles as vaccine candidate (Harvey et al., 2004; Qing et al., 2010), and to screen compound libraries for antiviral inhibitors (Lo et al., 2003b; Xie et al., 2011).

We recently developed an infectious cDNA clone for ZIKV (Shan et al., 2016b). Compared with the infectious cDNA clone, replicon systems allow analyzing viral replication events (i.e., viral translation and RNA synthesis) in the absence of virus entry or virion assembly. This feature of replicon is particularly useful when studying antiviral mechanism of inhibitors. Due to the lack of viral structural genes, the replicon system is non-infectious, thus reducing the biosafety concern when used to screen large compound libraries. The goal of this study was to develop the replicon system of ZIKV.

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## 2. Materials and Methods

### 2.1. Cell Culture and Antibodies

Huh7 cells (RRID:CVCL_0336) were maintained in a high-glucose Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT), 1% penicillin-streptomycin (Invitrogen) at 37 °C with 5% $CO_2$. The following antibodies were used: a mouse monoclonal antibody (mAb) J2 anti-double strand RNA (dsRNA) (English and Scientific Consulting, Hungary, Cat# 10010200), a mouse mAb 44-4-7 cross-reactive with DENV and ZIKV NS4B (Xie et al., 2014), and goat anti-mouse IgG conjugated with Alexa Fluor®488 (Thermo Fisher Scientific Cat# A-11029, RRID:AB_2534088).

### 2.2. Plasmid Construction

The *Renilla* luciferase (Rluc) replicon plasmid was constructed from an infectious clone pFLZIKV that contains a T7 promoter and hepatitis delta virus ribozyme sequence (HDVr) at the 5′ and 3′ end of the cDNA sequence of Cambodian strain (FSS13025), respectively (Shan et al., 2016b). Standard overlap PCR was performed to amplify the DNA fragment between unique restriction enzyme sites NotI and SphI. This DNA fragment contains the T7 promoter, 5′UTR, and a DNA cassette ($C_{38}$-Rluc2A-$E_{30}$) in-frame fused with the ORF (Fig. 1). The $C_{38}$-Rluc2A-$E_{30}$ cassette encodes the N-terminal 38 amino acids of C protein ($C_{38}$), Rluc reporter, foot-and-mouth disease virus (FMDV) 2A protease, and the C-terminal 30 amino acids of the E protein ($E_{30}$). The codons of $C_{38}$ contain the flavivirus-conserved cyclization sequence required for viral RNA replication (Hahn et al., 1987; Khromykh et al., 2001). The $E_{30}$ serves as a signal peptide for proper translocation of NS1 into the endoplasmic reticulum (ER) lumen. The purified PCR fragment was cloned into pFLZIKV through the NotI and SphI sites to replace the structural genes, resulting in plasmid pZIKV Rep WT (wild-type). As a control, the flavivirus-conserved polymerase motif GDD (corresponding to residues Gly664, Asp665, and Asp666 in ZIKV polyprotein) was mutated to Ala (G664A-D665A-D66A) using QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies), resulting in plasmid Rep NS5ΔGDD.

The cDNA clone of ZIKV Rep-Neo was constructed through engineering an IRES-Neo cassette into plasmid pZIKV Rep WT (Fig. 2). The IRES-Neo cassette (containing a neomycin phosphotransferase [Neo] gene driven by an internal ribosomal entry site [IRES] from encephalomyocarditis virus) was inserted downstream of the first 28 nucleotides of 3′UTR. The IRES-Neo cassette was amplified by PCR using WNV Rluc/NeoRep as a template (Lo et al., 2003b). Overlap PCR was performed to fuse the IRES-Neo cassette with the 3′UTR, resulting in a DNA fragment spanning restriction enzyme sites EcoRI and ClaI. This fragment was cloned into pZIKV Rep WT through the EcoRI and ClaI sites, resulting in plasmid Rep-Neo. All plasmids were validated by restriction enzyme digestion and DNA sequencing. The complete DNA sequences of plasmids pZIKV Rep WT and Rep-Neo are shown in Supplemental Materials. Primer sequences are available upon request. All restriction enzymes were purchased from New England BioLabs.

### 2.3. RNA Transcription and Transfection

Replicon RNAs were in vitro transcribed as described previously (Shan et al., 2016b). WT replicon or Rep-NS5ΔGDD RNAs (10 μg) were electroporated into Huh7 cells ($8 \times 10^6$ cells) by pulsing once at 0.27 kV/950 μF in 4-mm cuvettes using a GenePulser apparatus (Bio-Rad). After electroporation, $1 \times 10^5$ cells per well were seeded into a 24-well plate. At various time points post-transfection (p.t.), cells were washed twice with PBS and lysed in 100 μl 1 × *Renilla* luciferase lysis buffer (Promega). Lysates (15 μl) were mixed with *Renilla* luciferase substrates (50 μl). Luciferase signals were immediately measured by Cytation 5 (Biotek) according to the manufacturer's instructions.

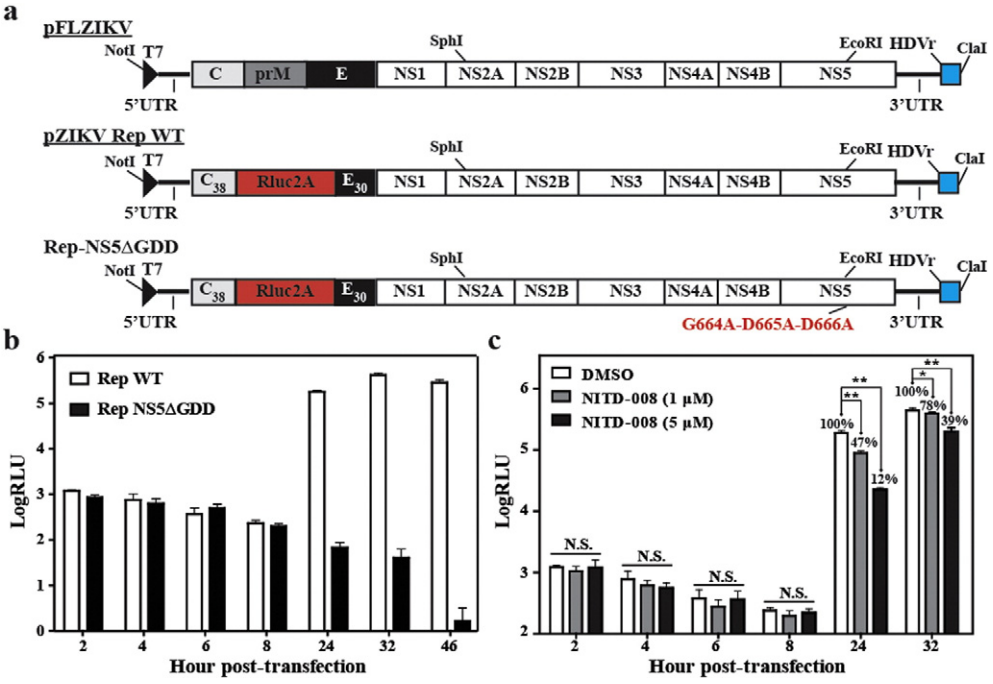


**Fig. 1.** Characterization of ZIKV luciferase replicon. (a) Diagram for ZIKV replicon construction. $C_{38}$ and $E_{30}$ represent DNA sequences encoding the first 38 amino acids of C protein and the last 30 amino acids of E protein, respectively. Rluc2A represents the gene cassette expressing *Renilla* luciferase (Rluc) and foot-and-mouth disease virus 2A protease (Rluc2A). HDVr, hepatitis delta virus ribozyme sequence. (b) Transient replicon assay. Equal amount of wild-type (WT) and NS5ΔGDD mutant RNAs (10 μg) were electroporated into Huh7 cells. Cellular Rluc signals were measured at indicated time points. The means and standard deviations from three independent experiments are shown. (c) Antiviral activity of NITD-008. Huh7 cells were electroporated with 10 μg of WT Rluc replicon. The transfected cells were treated immediately with NITD-008 (1 or 5 μM) or 0.9% DMSO as a control. The relative Rluc activities harvested at 24 and 32 h p.t. are indicated in percentages of the Rluc activities derived from the DMSO control cells (set as 100%). N.S., not significant; *, significant ($p < 0.05$); **, highly significant ($p < 0.01$).

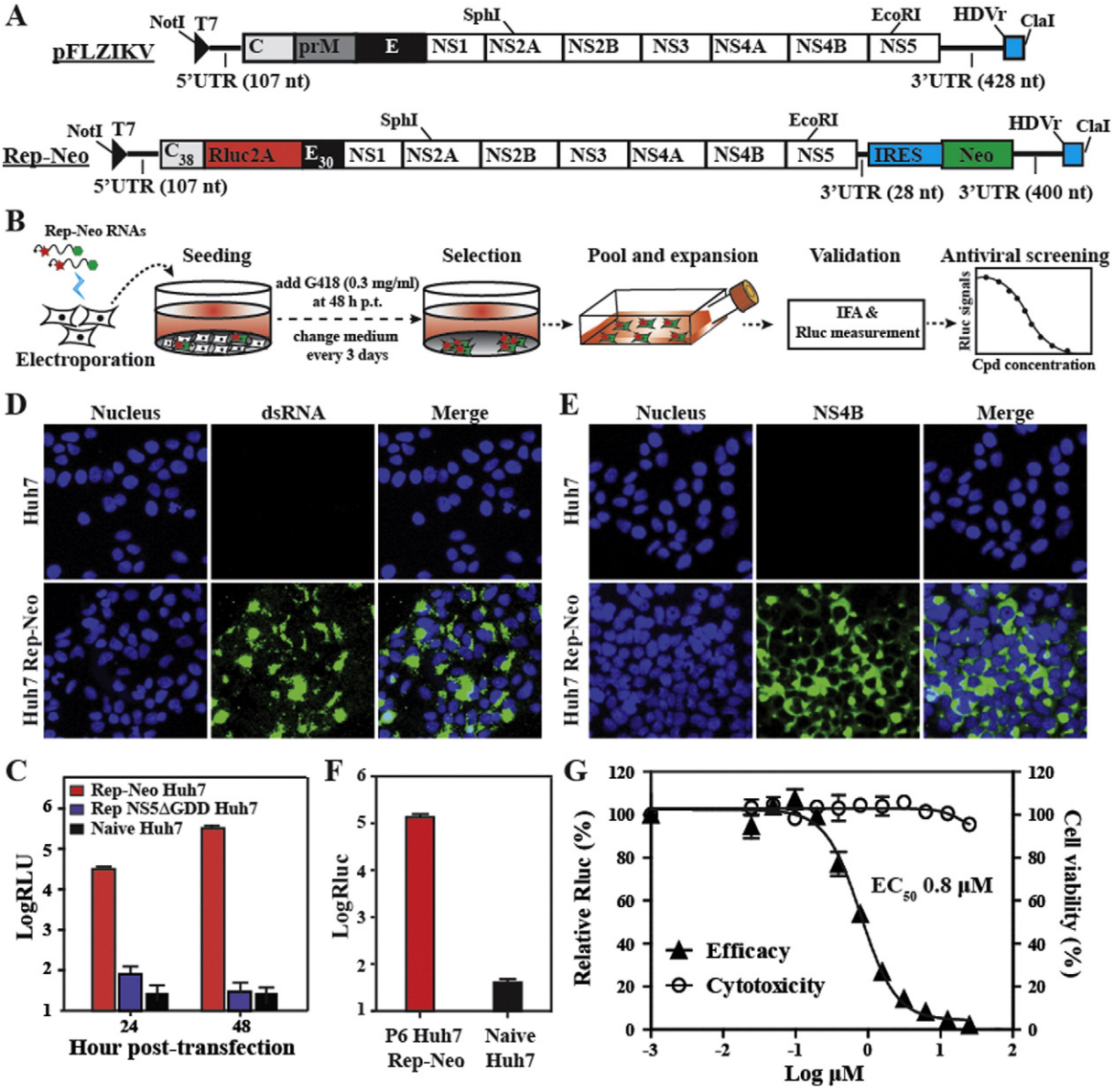


**Fig. 2.** A Huh7 cell line stably expressing luciferase and Neo ZIKV replicon (Huh7 Rep-Neo cell). (a) Schematic diagrams of the full-length cDNA clone of ZIKV (top) and the cDNA clone of ZIKV Rep-Neo (bottom). In the ZIKV Rep-Neo construct, a fragment containing internal ribosome entry site (IRES) and neomycin resistance gene (Neo) was inserted downstream of the first 28 nucleotides of 3′UTR. The nucleotide lengths of complete 5′UTR and 3′UTR are indicated. (b) Development of Huh7 Rep-Neo cell line. The flowchart outlines the major steps to generate the stable replicon carrying cell line. (c) Transient luciferase assay. Huh7 cells were electroporated with equal amounts (10 μg) of WT Rep-Neo RNA or an NS5 polymerase GDD active site mutant replicon (Rep NS5ΔGDD) RNA. At 24 and 48 h p.t., luciferase signals were measured from lysates of about 20,000 transfected cells or naïve Huh7 cells. The average and standard deviations from three independent measurements are presented. (d) Detection of viral dsRNA. Rep-Neo cells (P6) were analyzed for dsRNA by IFA using mAb J2 and goat-anti-mouse IgG conjugated with Alexa Fluor®488 as primary and secondary antibodies, respectively. Nucleus was stained by DAPI. (e) Detection of viral NS4B protein. The expression of NS4B protein was detected in Rep-Neo cells (P6) using mAb 44-4-7 and goat-anti-mouse IgG conjugated with Alexa Fluor®488 as primary and secondary antibodies, respectively. (f) Luciferase assay of P6 Rep-Neo cells. Luciferase activities were measured from the lysates of 20,000 P6 Rep-Neo cells or naive Huh7 cells. (g) Antiviral testing using Rep-Neo cells. Rep-Neo cells (P6) were seeded into a 96-well plate. The cells were incubated with NITD-008 at indicated concentrations and measured for luciferase activities at 48 h post-treatment. See experimental details in Materials and Methods.

### 2.4. Cell Line Selection

Approximately $8 \times 10^6$ Huh7 cells were electroporated with 10 μg Rep-Neo RNA as described above. The transfected cells were seeded in a 10-cm dish. At 48 h p.t., G418 (ThermoFisher Scientific) was added to a final concentration of 0.3 mg/ml in culture medium. Medium was changed every 3–4 days. Cell foci formed after 12 days of G418 selection. All cells were trypsinized and pooled together in a T-175 flask for expansion. The cells were continually cultured under G418 selection for 6 passages (P6 Rep-Neo cells; 3–4 days per passage). The P6 cells were aliquoted in a cryo-medium containing 90% FBS plus 10% dimethyl sulfoxide (DMSO) and stored in a liquid nitrogen tank.

### 2.5. Immunofluorescence Assay (IFA)

IFA was performed according to a previously described protocol (Shan et al., 2016b) with some modifications. In brief, after fixation and blocking, the cells were incubated with primary antibody (anti-dsRNA antibody J2 or anti-NS4B mAb 44-4-7) followed by secondary antibody (goat anti-mouse IgG conjugated with Alexa Fluor®488). The

cells were mounted in a mounting medium with DAPI (4′, 6-diamidino-2-phenylindole; Vector Laboratories, Inc.). Fluorescence images were acquired by a fluorescence microscope equipped with a video documentation system (Olympus).

### 2.6. Antiviral Screening Assay

Approximately $1.5 \times 10^4$ Rep-Neo cells/well were seeded in a phenol red-free DMEM medium (Invitrogen) with 10% FBS in an opaque (for Rluc assay) or a clear (for cytotoxicity assay) 96-well plate (Corning). At incubating the cells at 37 °C for 16 h, medium was replaced with fresh phenol red-free medium containing 2% FBS. The reason for using 2% FBS phenol red-free medium (rather than 10% FBS medium) is to minimize protein binding of compounds. Compounds NITD-008 (a gift from Novartis Institute for Tropical Diseases) with 10-fold serial dilutions were added to cells. Cells were also treated with 0.9% DMSO as a negative control. After 48 h of treatment at 37 °C, cells were assayed for Rluc activity using ViviRen substrates (Promega) or cell viability using Cell counting kit-8 (CCK-8, Sigma) as previously described (Xie et al., 2011). The 50% effective concentration ($EC_{50}$) and 50% cytotoxicity concentration ($CC_{50}$) were estimated by using a four-parameter logistic regression model from the GraphPad Prism 5 software (GraphPad Software Inc., San Diego CA). For the antiviral screening assay, we chose ViviRen substrate, rather than Renilla lysis buffer/substrate (as described for transient replicon assay), to improve the assay throughput. This is because ViviRen substrate is able to penetrate into cells, thus eliminating the step of cell lysis during antiviral screening.

## 3. Results

### 3.1. A Luciferase Reporter ZIKV Replicon

Fig. 1a depicts the ZIKV replicon containing a *Renilla* luciferase (Rluc) gene (pZIKV Rep WT). The pZIKV Rep WT was constructed by replacing the viral structural genes with Rluc using a full-length cDNA infectious clone (pFLZIKV) (Shan et al., 2016b). As a control, Rep-NS5ΔGDD was constructed by mutating the viral polymerase GDD active site to Ala (G664A-D665A-D66A in ZIKV; Fig. 1a). A T7 promoter and a hepatitis delta virus ribozyme (HDVr) sequence were engineered at the 5′ and 3′ ends of the replicon cDNA for in vitro transcription and for generation of the authentic 3′ end of the RNA transcript, respectively. Upon transfection into Huh7 cells, wild-type (WT) replicon RNA generated two distinct Rluc peaks (Fig. 1b). The first peak appeared at 2 h post-transfection (p.t.) and decreased from 4 to 8 h. At 24 h p.t., the Rluc signal increased by 200 folds above that of 2 h post-transfection. At 32 h p.t., the Rluc signal reached the second peak. Mutation of the viral polymerase GDD motif (Rep-NS5ΔGDD) abolished the second Rluc peak, but did not affect the first Rluc peak. Collectively, the results indicate that the first and second Rluc peaks represent the translation of the input RNA and the translation of the newly synthesized RNA, respectively. These results are in agreement with the previously reported flavivirus replicons (Lo et al., 2003a).

We demonstrated the use of replicon in drug discovery by testing an inhibitor NITD-008, an adenosine nucleotide analogue with a broad antiviral spectrum (Lo et al., 2016; Yin et al., 2009). This compound was recently shown to inhibit ZIKV in cell culture and A129 mice (Deng et al., 2016; Shan et al., 2016b). We treated the replicon-transfected cells with NITD-008 (1 or 5 μM in medium containing 0.9% dimethyl sulfoxide [DMSO] because the compound was initially dissolved in 100% DMSO). Fig. 1c shows the Rluc signals collected up to 32 h p.t.; data points after 32 h were not presented because the Rluc signals dramatically decreased. No significant differences in Rluc signal were observed from 2 to 8 h p.t. between the NITD-008-treated cells and DMSO control, indicating that NITD-008 does not affect viral RNA translation. In contrast, at 24 and 32 h p.t., the Rluc signals were markedly suppressed by NITD-008 in a dose-dependent manner (Fig. 1c), indicating that the compound inhibits viral RNA synthesis. Notably, NITD-008 suppressed Rluc signals less efficiently at 32 h than that at 24 h in the transfected cells, possibly due to the difference in conversions of NITD-008 to its triphosphate (the active antiviral form) at different time points. Nevertheless, the results demonstrate the utility of the replicon in analyzing the mode-of-action of antiviral compounds.

### 3.2. A Luciferase and Neo ZIKV Replicon Cell Line

Fig. 2a shows the second ZIKV replicon containing Rluc and Neo genes (Rep-Neo). The dual reporter replicon was constructed by inserting an EMCV IRES (encephalomyocarditis virus internal ribosomal entry site)-Neo into the 3′UTR of the above Rluc replicon. The Rep-Neo RNA were transfected into Huh7 cells; the transfected cells were selected under Geneticin (G418). Fig. 2b depicts the selection flowchart for the Rep-Neo cells. For evaluating the replication competency of Rep-Neo RNA, $1 \times 10^5$ transfected cells/well were seeded into a 24-well plate. At 24 and 48 h p.t., the cells were assayed for Rluc activity; the Rep-Neo RNA-transfected cells produced $>1 \times 10^3$-fold higher luciferase signals than naïve Huh7 cells; almost background levels of Rluc activities were detected in the cells transfected with an NS5 polymerase GDD active site mutant RNA (NS5ΔGDD Rep) (Fig. 2c). The results demonstrate that Rep-Neo RNA is replication-competent.

After 12 days of G418 selection, cells transfected with the Rep-Neo RNA produced foci. All surviving cells were pooled, expanded, and passaged for six generations (P6). In contrast, cells transfected with the non-replicative polymerase GDD mutant RNA did not produce any foci under the identical G418 selection; this is expected because replication of viral RNA is required to express Neomycin resistance gene. Next, we examined the P6 Rep-Neo cells for viral RNA, protein expression, and luciferase signals. Immunofluorescence assay (IFA) using anti-double strand RNA (dsRNA) or anti-NS4B mAb showed viral dsRNA and NS4B protein in all Rep-Neo cells; in contrast, neither dsRNA nor NS4B signals were detected in naïve Huh7 cells (Fig. 2d & e). In addition, Rep-Neo cells generated 2000-fold higher luciferase signals than naïve Huh7 cells (Fig. 2f). Similar IFA and luciferase results were obtained for P1 Rep-Neo cells (data not shown). Altogether, the results demonstrate that the Rep-Neo cell line stably maintains the Rluc-Neo replicon.

The Rep-Neo cells were applied to testing NITD-008 in a 96-well format. As shown in Fig. 2g, NITD-008 reduced Rluc signal in a dose-dependent manner with a calculated 50% effective concentration ($EC_{50}$) of 0.8 μM; no obvious cytotoxicity was observed at the highest tested concentration of 25 μM. The $EC_{50}$ value in Huh7 was more potent than the $EC_{50}$ values previously reported in Vero cells (3.6 and 2.5 μM derived from plaque assay and a luciferase virus infection assay, respectively) (Shan et al., 2016b); this is most likely because the kinases that convert NITD-008 to its triphosphate form are more robust in Huh7 cells than those in Vero cells (Deng et al., 2016). Overall, the data are consistent with the known antiviral activity of NITD-008 against DENV and ZIKV (Shan et al., 2016b; Yin et al., 2009), demonstrating that the ZIKV Rep-Neo cell line could be used for compound library screening.

## 4. Discussion

Replicon-based cell lines have been widely used for flaviviruses, including DENV (Ng et al., 2007), WNV (Khromykh and Westaway, 1997; Lo et al., 2003b), and hepatitis C virus (Blight et al., 2000). The two genes (Rluc and Neo) engineered into the current replicon constructs could be replaced by other reporter (such as NanoLuc, GFP, or mCherry) and selection marker (such as puromycin *N*-acetyl-transferase [PAC]). The ZIKV Rep-Neo cells could be readily adapted for high-throughput screening in a 384- and 1536-well format (Xie et al., 2011). Because replicon assay does not generate infectious particles, it eliminates the risk of laboratory-acquired viral infection. Since the replicon assay examines cell permeability of compounds, inhibitors identified through such assay (in comparison with viral enzyme-based

screening assays) might have a high success rate during preclinical development (Shi, 2002). Although the replicon-based assay covers all targets involved in viral translation and RNA synthesis, it does not include targets required for viral entry and virion assembly/release. Those targets could be covered by using a replicon-derived virus-like particle (VLP)-based assay (Qing et al., 2010). VLPs can be produced through packaging the replicon RNA by structural proteins supplied *in trans* (Harvey et al., 2004; Qing et al., 2010). Besides drug discovery, the VLPs could also be used for potential vaccine development.

In summary, we generated both a transient ZIKV replicon and a Huh-7 Rep-Neo cell line that stably carries a ZIKV replicon. These replicons are useful tools to study ZIKV replication and to develop antiviral therapy. These replicons, together with a previously reported infectious clone (Shan et al., 2016b), are critical experimental systems that could be used to unravel the molecular details of ZIKV replication and pathogenesis, and to aid in the development of vaccines and therapeutics.

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## Conflict of Interest Statement

The authors have no conflict of interest in this study.

## Author Contributions

X.X., J.Z., S.C., Y.Y., and D.B.K. performed experiments and data analysis. X.X, K.D., J.N., and P.Y.S. interpreted the results. X.X., K.D., J.N., and P.Y.S. wrote the manuscript.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ebiom.2016.09.013.

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