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Letter

Development of a replicon cell line-based high throughput antiviral assay for screening inhibitors of Mayaro virus

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Dear Editor,

Mayaro virus (MAYV), a member of the *Alphavirus* genus in the *Togaviridae* family, is primarily transmitted by mosquitoes and usually classified as a biosafety level 3 (BSL-3) pathogen. The recent re-emergence of MAYV has drawn increasing attention. The typical symptoms of MAYV infection include acute fever, headache, arthralgia/arthritis, and rash, most of whichare similar to those of chikungunya virus (CHIKV), dengue virus (DENV), and Zika virus (ZIKV) infections. This may be the most significant reason for the potential prevalence of MAYV, particularly in urbanized areas. To date, there are no approved vaccines or specific therapeutics for the prevention and treatment of MAYV. This situation highlights the urgent need to develop antiviral drugs.

Currently, the cell-based antiviral assays for MAYV drug discovery mainly rely on viral infection of susceptible cells (Carvalho et al., 2014; Amorim et al., 2017; Caldas et al., 2018; Camini et al., 2018). Viral RNA quantification and viral titer determination serve as the primary methods for assessing antiviral activity. However, the low-throughput nature of these assays and the high requirements for equipment handling live viruses have significantly limited their application in large compound libraries. Therefore, developing a safe, reliable, high-throughput screening (HTS) method that can be conducted in BSL-2 laboratories is essential for discovering anti-MAYV drugs.

In this study, we first constructed a dual-reporter MAYV replicon (MAYV-Pac-Rluc-Rep) that carries the Renilla luciferase (*Rluc*) reporter gene and the puromycin-N-acetyltransferase (*Pac*) gene (Fig. 1A). The *Pac* and *Rluc* genes were used for the selection of the replicon cell line and the quantification of viral replication, respectively. Foot-and mouth disease virus (FMDV) 2A sequence was engineered to enable cleavage and release of the Pac and Rluc proteins, respectively. To characterize the replication of MAYV-Pac-Rluc-Rep, BHK-21 cells transfected with replicon RNA were subjected to immunofluorescence assay (IFA) and luciferase assays. IFA revealed sporadic positive signals between 24 and 96 hours post transfection (hpt) (Fig. 1B), indicating RNA replication without infectious particle assembly or cell-to-cell transmission. Meanwhile, the luciferase activities at different time points after transfection were also measured (Fig. 1C). The Rluc signal at 2 hpt reflected the translation of the input RNA, and the signal after 8 hpt was used to quantify replication of the replicon RNA. The Rluc signal of transfected cells increased from 8 hpt to 24 hpt and gradually decreased after 24 hpt, demonstrating the replication profile of MAYV-Pac-Rluc-Rep. Collectively, the MAYV-Pac-Rluc-Rep was replication competent, and the luciferase activity could be used to monitor the replication of the replicon RNA.

As replicons can also be stably maintained in cells, they can be used to establish HTS assays. BHK-21 cells were transfected with MAYV-Pac-Rluc-Rep RNA and cultured with medium containing puromycin 0.5 μg/mL) to screen stable cell line steadily expressing MAYV-Pac-Rluc-Rep. With continuous puromycin selection, resistant monoclonal cell clusters were obtained and expanded for luciferase activities quantification. The same number of cells resulted in different levels of luciferase signals, and four colonies (#1, #2, #4, #5) yielded higher luciferase activity (Fig. 1D). At the same time, antibodies against CHIKV nsP1 that cross-react with MAYV nsP1 were used to detect the expression of the viral protein by the IFA assay. The results showed that the above four colonies produced over 90% nsP1-positive cells (Fig. 1E). The #1 colony was randomly chosen for the following experiments and designated as BHK21-MAYV-Rep.

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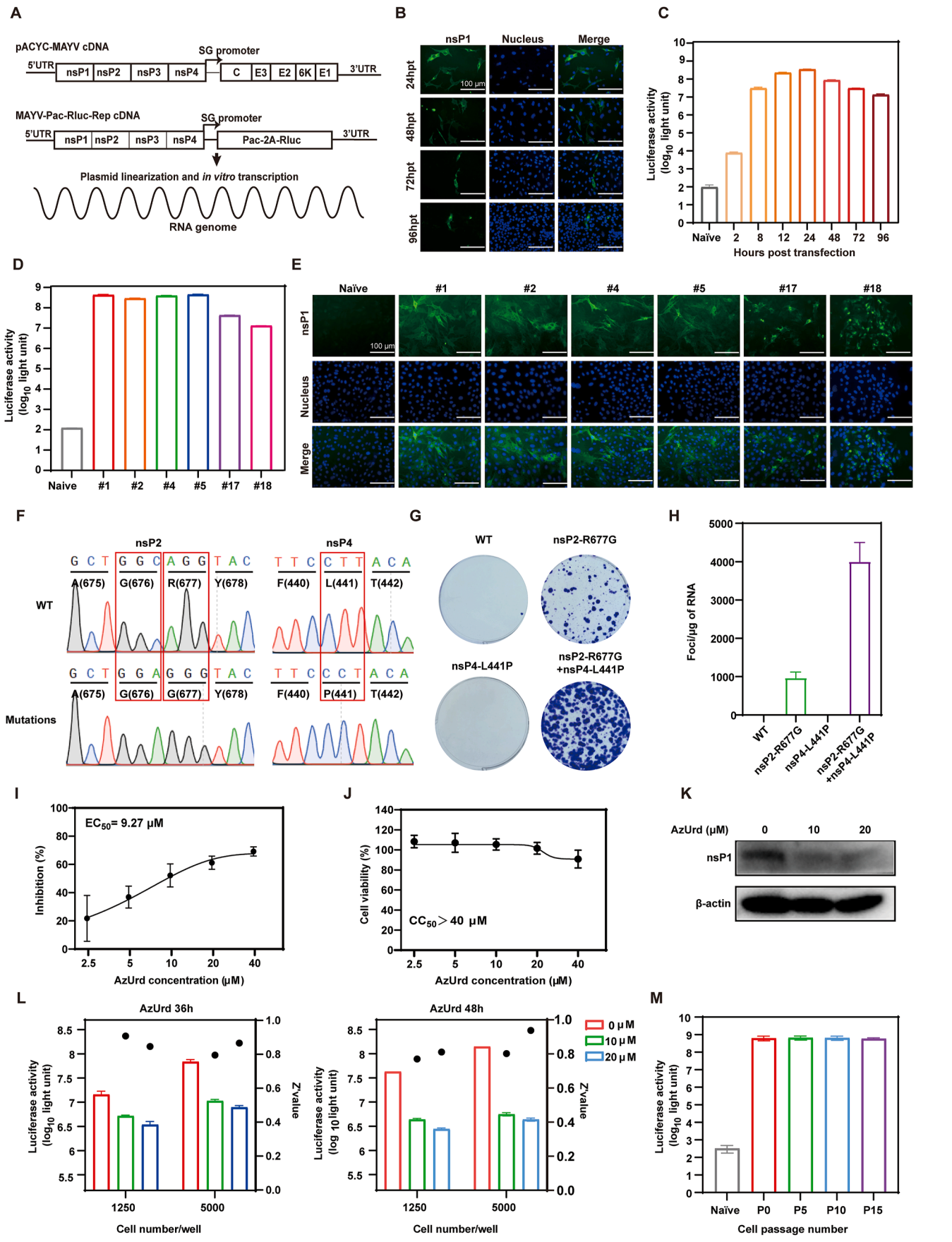

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It has been reported that the replication of alphavirus replicons can cause cytotoxicity and lead to cell death. Given this pattern, we considered the possibility of adaptive mutations emerging in the replicon that allow its replication to adapt to the host cell. Thus, we utilized RT-PCR to amplify the cDNA encompassing the entire replicon RNA genome obtained from BHK21-MAYV-Rep and performed direct sequencing of the PCR products (Supplementary Fig. S1A). The sequencing results showed three mutations in the replicon genome C3711A, A3712G and T6854A, resulting in nsP2G676G, nsP2-R677G, and nsP4-L441P (Fig. 1F). nsP2-G676G is a synonymous mutation, suggesting that nsP2-R677G and nsP4-L441P may play a crucial role in replicon adaptation to cell lines.

To determine whether these mutations provided an adaptive phenotype, we introduced the individual or combined mutations into the original replicon and transfected the recombinant replicon RNAs into BHK-21 cells to analyze their effects on the formation of replicon colonies. Specifically, equal amounts of *in vitro*-transcribed MAYV replicon RNA were electroporated into BHK21 cells and cells were treated with puromycin selection. After about a week of screening, colonies can be observed in the nsP2-R677G and the nsP2-R677G + nsP4-L441P group, but not in the WT and nsP4-L441P groups (Fig. 1G). Quantitative analysis revealed that the colony formation efficiency of the nsP2-R677G + nsP4-L441P group was approximately five-fold higher than that of the nsP2-R677G group (Fig. 1H). The results indicated that the nsP2-R677G mutation is a key factor enabling the replicon to adapt to the host cell, and the nsP4-L441P mutation has a synergistic effect. Meanwhile, we measured the Rluc signals after electroporating the replicon RNA into cells. We found that mutation nsP2-R677G had a relatively small impact on the replication level while mutation nsP4-L441P and combination mutations all had a significant inhibitory effect on replicon replication (Supplementary Fig. S1B). This indicated that the two mutations can affect the adaptability of the replicon to the host cell through different pathways.

Replicon cell lines are powerful tools for the development of antiviral drugs (Zhang et al., 2017; Li et al., 2018). Therefore, we tested whether the selected MAYV replicon cell lines could be used for antiviral drug screening. Here, we examined the antiviral activity of 6-Azauridine in the BHK21-MAYV-Rep cell line. 6-Azauridine is a broad-spectrum antimetabolite that inhibits MAYV virus infection effectively (Li et al., 2019). Luciferase activity by 6-Azauridine exhibited a dose-dependent effect. Its effective concentration of 50% (EC_{50}) was 9.27 μM (Fig. 1I). At the same time, the CC_{50} of 6-Azauridine exceeded 40 μM (Fig. 1J). To verify whether the suppression of luciferase activity stemmed from reduced replicon replication, we performed Western blot assay to measure nsP1 protein levels in BHK21-MAYV-Rep cells. As the concentration of 6-Azauridine increased, the expression of nsP1 protein showed a dose-dependent decrease (Fig. 1K). These results not only validated the reliability of Rluc signals as indicators of MAYV replicon activity but also demonstrated the efficacy of the MAYV replicon cell line for antiviral screening.

For development of a replicon-based HTS assay, we used 6-Azauridine as the positive drug. The Z'value evaluated the performance and signal robustness of the HTS assay and a Z'value between 0.5 and 1 is considered excellent for HTS assays (Zhang et al., 1999; Li et al., 2009; Elshabrawy et al., 2014). The replicon cell line was treated with 10 μM and 20 μM of 6-Azauridine, which caused varying degrees of reduction in luciferase signals. During the development of the HTS assay, cell density and incubation time can be optimized. In this study, we seeded 1250 and 5000 cells per well in a 96-well plate. Luminescence was measured 36 and 48 hours post-6-Azauridine treatment, followed by Z'values calculation. As shown in Fig. 1L, all experimental groups exhibited Z'values between 0.5 and 1, indicating the feasibility of establishing the HTS assay based on the BHK21-MAYV-Rep cell line. Next, we used Ribavirin, another broad-spectrum antiviral drug that had been reported to have inhibitory effect on alphavirus replication to evaluate the system's effectiveness. We performed a full dose–response curve of the replicon-based cell line to ribavirin and determined the actual EC_{50} to be 3.559 μM (Supplementary Fig. S2A), meanwhile, the CC_{50} of ribavirin was above 70 μM (Supplementary Fig. S2B). Therefore, the antiviral drug screening system based on the BHK21-MAYV-Rep cell line that we have established is effective.

Due to the necessity of continuous passages of cell lines during the experimental process, we examined whether the replicon could be stably maintained during passaging with a comparable high luciferase expression level (Tiong-Yip et al., 2014). We continuously passaged BHK21-MAYV-Rep cells for 15 rounds. The original cell stocks of BHK21-MAYV-Rep were defined as P0, with subsequent passages labeled P1 to P15. Every five passages of the BHK21-MAYV-Rep cell line (P0, P5, P10, P15) were subjected to IFA and luciferase assays. All the tested passages detected high levels of Rluc signals (5×10^8~8×10^8 light units per 150,000 cells) (Fig. 1M). Similarly, the consistent results of IFA further demonstrated that replicons could stably exist in cells for at least 15 passages (Supplementary Fig. S3). These results demonstrated that the BHK21-MAYV-Rep cell line was stable and had the potential for application in HTS assay.

In summary, we engineered a dual-reporter MAYV replicon clone (MAYV-Pac-Rluc-Rep) that carries the Renilla luciferase (*Rluc*) reporter gene and the puromycin-N-acetyltransferase (*Pac*) gene. Through puromycin selection, we established a BHK-21 cell line maintaining this replicon. Notably, based on the replicon cell line, we developed and optimized a robust HTS assay using a known MAYV inhibitor. This replicon-based HTS assay will be a safe platform for MAYV antiviral screening without dealing with wild-type virus, however, it can only be used to screen antiviral drugs targeting the replication and translation stages of MAYV, and cannot cover other steps in the viral life cycle.

Fig. 1. Development of a replicon cell line-based high throughput antiviral assay for screening inhibitors of Mayaro virus. **A** Strategy for the construction of MAYV-Pac-Rluc-Rep replicon. The structural genes were replaced with the *Pac* gene and the *Rluc* gene. Foot-and mouth disease virus (FMDV) 2A sequence was engineered to enable cleavage and release of the Pac and Rluc proteins, respectively. The MAYV-Pac-Rluc-Rep replicon clone was transcribed *in vitro* to obtain replicon RNA. **B** Detection of viral protein expression. BHK21 cells transfected with MAYV-Pac-Rluc-Rep replicon were performed for immunofluorescence assay (IFA) using a polyclonal antibody against MAYV nsP1 as the primary antibody. The nucleus was stained with DAPI. **C** Luciferase activity of the cells transiently transfected with MAYV replicon. One representative experiment with two replicates was displayed. Error bars represent standard deviations. **D, E** BHK-21 cell lines stably expressing MAYV-Pac-Rluc-Rep were subjected to luciferase assay (**D**) and IFA (**E**) to compare replicon replication in these cell lines. **F–H** Identification of adaptive mutations in the BHK21-MAYV-Rep cell line (**F**). BHK-21 cells were electroporated with 10 μg wild-type or mutated replicon RNA, followed by puromycin selection (0.5 μg/mL) initiated 24 h post-transfection. After one week of selection, surviving colonies were washed, fixed in 4% paraformaldehyde, stained with crystal violet (**G**), and counted (**H**). Representative data are from three independent experiments. **I–K** Antiviral assay of 6-Azauridine using BHK21-MAYV-Rep cell line. BHK21-MAYV-Rep cells were treated with different concentrations of 6-Azauridine for 36 h. The inhibition curve (**I**), cytotoxicity (**J**), and viral protein levels (**K**) were measured as described in Materials and Methods. In **I** and **J**, three independent experiments were conducted, and average results of all experiments were presented with error bars. **L** Establishment and optimization of BHK21-MAYV-Rep cell line for HTS assay in a 96-well plate format. Cells were seeded at different densities of 1250 and 5000 per well along with 6-Azauridine (10 and 20 μM) or DMSO (0.1%) and incubated for 36 h and 48 h. **M** Stability of BHK21-MAYV-Rep cell line for HTS assay. BHK21-MAYV-Rep cells were continuously passaged for 15 rounds. The cells at passage 0 (P0), 5 (P5), 10 (P10), and 15 (P15) were subjected to luciferase activity assays. Three independent experiments were conducted, and average results of all experiments were presented with error bars.

FOOTNOTES

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