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Short communication

Development of a stable *Gaussia* luciferase enterovirus 71 reporter virus

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A B S T R A C T

We report a stable *Gaussia* luciferase enterovirus 71 (Gluc-EV71) reporter virus to facilitate drug discovery. The Gluc-EV71 reporter virus was generated by engineering the *Gaussia* luciferase (Gluc) gene between the 5′ untranslated region and VP4 gene of the EV71 genome. We could recover Gluc-EV71 after transfection of Vero cells with the cDNA clone-derived RNA. The reporter virus efficiently infects and replicates in various cell types (Vero, human rhabdomyosarcoma, and HeLa cells), producing robust luciferase activity. The Gluc-EV71 virus replicates slower than the wild-type virus in cell culture. The reporter virus is stable in maintaining the *Gluc* gene after five rounds of continuous passaging in Vero cells. Using known EV71 inhibitors, we demonstrate that the reporter virus can be used for antiviral testing. However, the Gluc-EV71 infection assay cannot be adapted to a homogenous format for high throughput screen, mainly due to the secreted nature of the Gluc protein and the short half-life of the Gluc luminescence signal. The Gluc-EV71 and its infection assay could be useful for antiviral drug discovery as well as for studying EV71 replication and pathogenesis.

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Enterovirus 71 (EV71) is one of the major causative agents for hand, foot, and mouth disease (HFMD) associated with neurological disease in children (Nishimura et al., 2009). Over the past decades, outbreaks and epidemics caused by EV71 have occurred in Asia-Pacific region frequently. EV71 has become a major threat to public health. Currently, there is no approved antiviral therapy for treatment of its infection. EV71 belongs to the *Enterovirus* genus within the *Picornaviridae* family. It contains a single plus-strand genome RNA of approximately 7400 nucleotides in length. The EV71 genome encodes a polyprotein with a single open reading frame (ORF) flanked by a 5′ untranslated region (UTR) and 3′ UTR with a poly (A) tail (Paul et al., 2000). Viral replication takes place in the cytoplasm of infected cells. The complete viral life cycle includes viral entry, proteins synthesis and processing, RNA replication, and viral particles formation and releasing (De Palma et al., 2009; Kok et al., 2012; Lu et al., 2011; Thibaut et al., 2012; Wu et al., 2010; Yamayoshi et al., 2009). Compounds capable of blocking any steps of viral life cycle could be potentially developed for antiviral therapy. The drug discovery of EV71 requires the development of reliable antiviral assays. Xu et al. and our group previously established different anti-EV71 drug screening systems based on pseudoviruses carrying a luciferase gene or eGFP reporter viruses (Shang et al., 2013; Xu et al., 2014). However, either system inevitably has its respective disadvantages. For instance, the luciferase pseudovirus could not fulfill complete viral life cycles due to lacking the stages of virion assembly and release, and the eGFP reporter virus is not sensitive and quantitative enough to be adapted to compound testing although it could surrogate complete viral life cycle. In this study, we report the development and characterization of a stable Gluc reporter EV71 that is suitable for antiviral drug discovery by overcoming the drawbacks of previous systems.

Gluc-EV71 reporter virus cDNA clone was constructed by engineering *Gaussia* luciferase (Gluc) gene into an infectious cDNA clone of pACYC-EV71-FL (Fig. 1A) following the same strategy as

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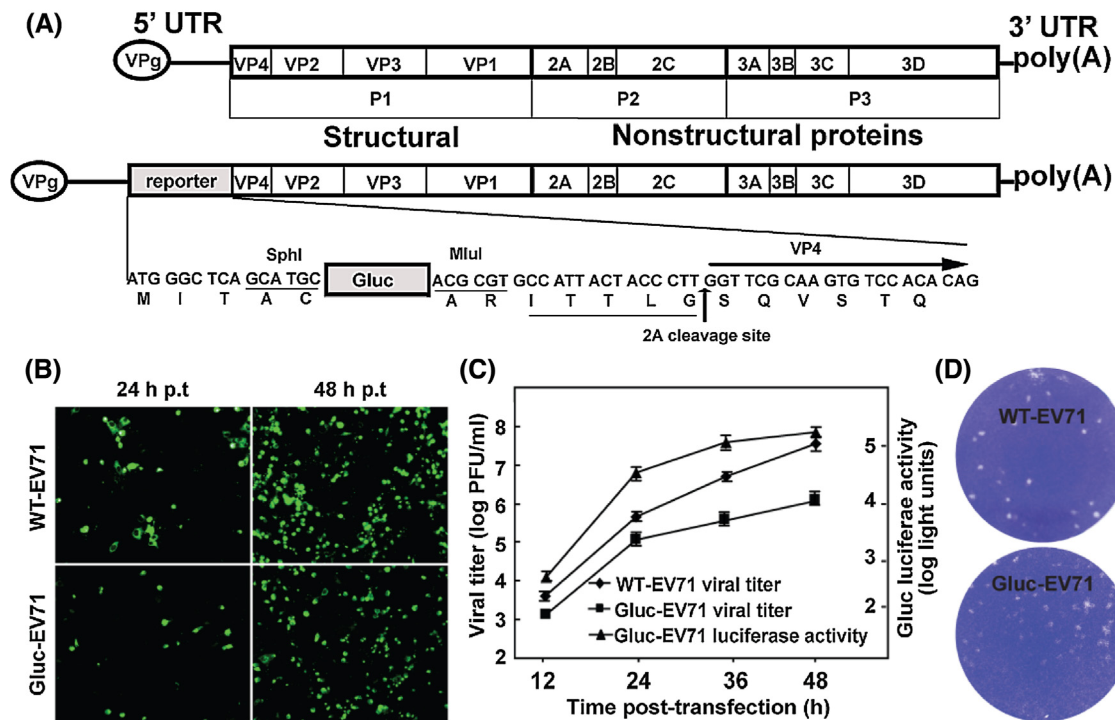

Fig. 1. Construction of the Gluc-EV71 reporter virus. (A) An infectious cDNA clone of pACYC-EV71-FL was used as backbone for construction of EV71 reporter virus. Gluc reporter gene was inserted at the engineered SphI and MluI sites. 2A protease cleavage site (ITTLG) was indicated by an arrow. (B) IFA detection for viral protein expression in cells transfected with full-length WT-EV71 and Gluc-EV71 RNA transcript. The transfected Vero cells were analyzed by IFA using EV71 VP1 rabbit polyclonal antibody as the primary antibody and goat anti-rabbit IgG conjugated with fluorescein isothiocyanate (FITC) as the second antibody. The transfected cells were visualized with a FITC filter set at the indicated time points after transfection. The upper and lower panels represent the view for WT-EV71 and Gluc-EV71 at indicated time points post transfection, respectively. (C) Viral production and luciferase activity measurement of WT-EV71 and Gluc-EV71 RNA-transfected Vero cells at different time points after transfection. Viral titers were calculated as the number of plaque-forming units (PFU) per microliter by plaque assays. Plaque assays were performed as described previously (Shang et al., 2013). The luciferase activities were measured by incubating the culture fluids containing the secreted Gluc with substrate following the manufacturer's protocol (Promega). Left and right *y*-axes indicate viral titer and luciferase activity, respectively. Error bars indicate the standard deviation of triplicate measurements. A representative of three experiments is shown. (D) Plaque morphology of WT-EV71 and Gluc-EV71 on Vero cells.

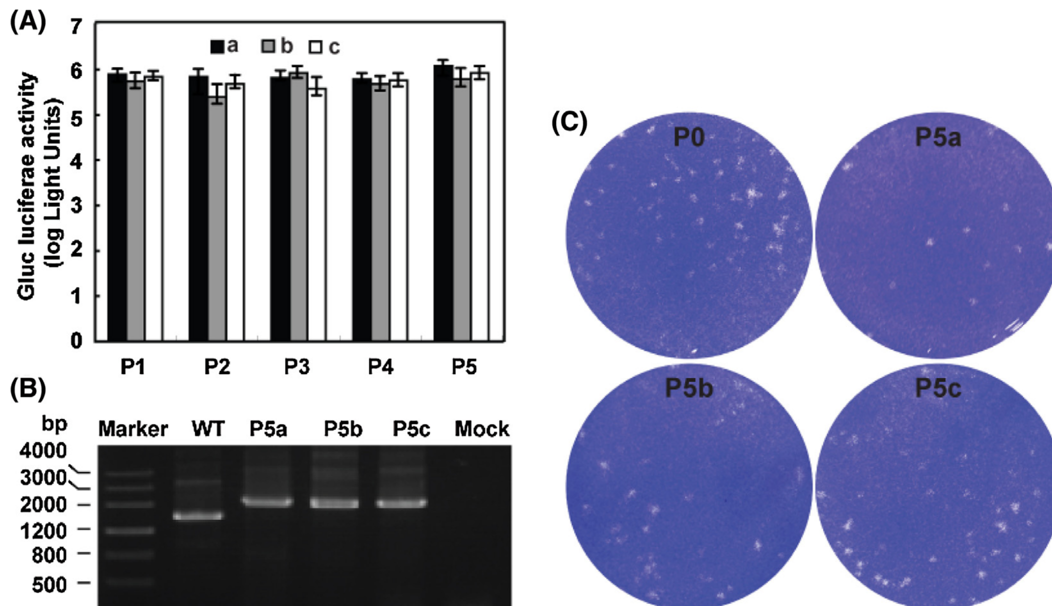

Fig. 2. Stability of Gluc-EV71 reporter virus in Vero cells. (A) Three independent lineages (a, b and c) Gluc-EV71 reporter viruses derived from transfected cells were passaged on Vero cells for five rounds. Viruses from each lineage and each passage were used to infect Vero cells. At 48 h post-infection, luciferase activities were measured from culture supernatants. Error bars represent the standard deviation of triplicate measurements and representative of three experiments is demonstrated. (B) Detection of the Gluc reporter gene of passaged reporter virus. Viral RNA was extracted from culture supernatants of P5a, P5b and P5c passage, respectively. RT-PCR was performed with primer pair locating 5′UTR and VP4 region. The RT-PCR products were resolved by 1% agarose gel electrophoresis. 1.5 Kb and 2.0 Kb bands were detected from wild type and reporter viruses RT-PCR product, respectively. As a negative control, no RT-PCR product was detected from the extracted supernatant of mock infected Vero cells. (C) Plaque morphologies of Gluc-EV71 reporter viruses from P0, P5a, P5b and P5c. Viruses from culture supernatants were measured by plaque assay on Vero cells.

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