Fibroblast growth factor 11 inhibits foot-and-mouth disease virus gene expression and replication in vitro

Hyo Rin KANG1), Mi So SEONG1), Hyung-Soon YIM2), Jung-Hyun LEE2), Sang Ho CHA3) and Jaehun CHEONG1)*

1) Department of Molecular Biology, Pusan National University, Busan, Republic of Korea  
2) Marine Biotechnology Research Center, Korea Institute of Ocean Science and Technology, Busan, Korea  
3) Foot-and-Mouth Disease Research Division, Animal and Plant Quarantine Agency, Gyeongbuk-do, Korea

ABSTRACT. Foot-and-mouth disease virus (FMDV) causes highly contagious disease of cloven-hoofed animals such as cattle, swine, and sheep. Although FMD vaccine is the traditional way to protect against the disease, the use of FMD vaccines to protect early infection is limited. The alternative strategy of applying antiviral agents is required to control the spread of FMDV in outbreak situations. Fibroblast growth factor 11 (FGF11) is a member of the intracellular FGF. Here, we identified the inhibitory effect of FGF11 on FMDV gene expression through the transcriptional and translational regulation. For the quantitative analysis of FMDV transcription/translation level, we firstly constructed a plasmid reporter system (FMDV five prime untranslated region (5′ UTR)-luc) conjugating luciferase encoding gene with FMDV 5′ UTR region, which is a non-coding region to control FMDV transcription/translation and includes cis-acting replication element (CRE) and internal ribosome entry site (IRES). FGF11 decreased the gene expression of FMDV 5′ UTR-luc reporter in a dose-dependent manner. We further confirmed the inhibitory function of FGF11 on FMDV gene expression a replication in the FMDV-infected pig cells. FGF11 expression inhibited RNA production of FMDV RNA polymerase 3D gene in the FMDV-infected cells. In addition, while FMDV cell infection induced cytopathic effect (CPE) within 24 hr, FGF11 expression dramatically repressed CPE at the basal level. These results indicate that FGF11 inhibits FMDV gene expression and replication in vitro, implicating to provide intervention strategy for FMDV pathogenesis and transmission.

KEYWORDS: antiviral drug, fibroblast growth factor 11, five prime untranslated region, foot-and-mouth disease virus

The foot-and-mouth disease virus (FMDV), which causes severe vesicular disease in livestock, is a member of the Aphthovirus genus in the Picornaviridae family [1]. The FMDV has high potential for antigenic and genetic variation; based on their induction of cross-protection in host animals, seven serotypes (A, O, C, Asia1, SAT 1, SAT 2, and SAT3) of FMDV have been identified [5, 11, 16]. Additionally, advances in DNA sequencing have dramatically increased the rate at which genotypic and phenotypic variants of FMDV are identified [4, 8].

Replication and translation of FMDV RNA occur in association with the cell membrane in the cytoplasm of infected cells. The most critical step of FMDV replication is RNA-dependent RNA synthesis by 3D polymerase, which requires a regulatory network involving viral-encoded proteins (3B and 3D), various host factors, and a non-coding structural RNA element [10, 13]. The FMDV five prime untranslated region (5′ UTR) contains two highly structured RNA sequences; the cloverleaf, required for genome replication, and the internal ribosome entry site (IRES), which directs translation initiation [2, 10]. RNA replication is carried out on membranous structures by viral RNA-dependent RNA polymerase in conjunction with other viral and cellular proteins and cis-acting replication element (CRE) and IRES [27, 28]. The viral RNA structure is critical for several essential functions, including replication, translation, and encapsidation [6, 9, 12]. Determining the viral RNA activation has broadened our understanding of its involvement in the viral infection cycle. This reflects a common theme among many eukaryote-infecting viruses, which have evolved a variety of mechanisms to manipulate cellular transcription and translation machinery, in many cases via elegant RNA-centered strategies [14].
The family of fibroblast growth factors (FGFs) has 22 members and plays important various biological functions in differentiation, angiogenesis, cell growth, wound healing and repair, embryonic development, as well as metabolic regulation [23]. FGFs are classified as canonical hormone-like FGFs (hFGFs) and intracellular FGFs (iFGFs). FGF11 is one of iFGFs and functions intracellularly and is independent of FGF receptor [15, 31]. FGF11 was reported to play a critical role in the transcriptional regulation of lipid metabolism gene expression that FGF11 regulated the expression of peroxisome proliferator-activated receptor gamma (PPARγ) through modifying the expression of multiple PPARγ regulators in adipogenesis [24]. In previous our reports, since metabolism-related transcription factors regulated virus gene expression [19–21], it was relevant to determine FGF11 as an antiviral factor for FMDV.

FMDV infections can develop into severe threatening pathologies and economical damage for which no specific prophylactic or therapeutic treatment has been approved to date. It is necessary to develop an analysis tool for evaluation of in vitro gene expression of FMDV. However, the construction of a FMDV reporter as a bio-safe tool to study fundamental viral processes and to test antiviral drugs has not yet been reported. In this study, we developed in vitro FMDV gene expression reporter system and applied to evaluate FMDV gene repression by FGF11. In addition, we confirmed the FGF11 antiviral function in the FMDV-infected cells.

MATERIALS AND METHODS

Cell culture

IBRS-2 cells (obtained from Korea Animal and Plant Quarantine Agency) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 5% heat-in-activated fetal bovine serum (FBS; GIBCO BRL, Grand Island, NY, USA) and 1% (v/v) penicillin-streptomycin (PS; GIBCO BRL) at 37°C in a humidified atmosphere containing 5% CO₂.

Preparation of plasmid constructs

The gene fragment of FMDV 5' UTR region was obtained using Serotype Asia1 isolate (As1/shamir/89, JF739177.1) as a template via PCR with forward primer (5'-GAA TTC GCC ACC ATG GAA GAC GC-3') and reverse primer (5'-GCG GAA AGA TCG CCG TGT AAT CTA GA-3'). We got a luciferase gene using pGL3b as a template with forward primer (5'-GGA TCC TTG AAA GGG GGC GCT-3') and reverse primer (5'-GCC TTT CTC CTT AAT TAC CAC TTC TTA GAA TTC-3'). FMDV 5' UTR gene and luciferase gene were ligated with pcDNA3 cutting with BamHI/EcoRI and EcoRI/XbaI cleavage site each. After ligation, we finally prepared pcDNA3/5' UTR-Luci reporter system.

Transient transfection

A total of 1 × 10⁵ IBRS-2 cells were seeded into each well of 24-well plates for 24 hr before transfection. Transfections were performed using jetPEI transfection reagent (Polyplus transfection-SA, Illkirch, France) and Lipofectamin 3000 (Thermo Fisher Scientific, Waltham, MA, USA) at a ratio of 1 μl transfection reagent to 500 ng DNA, and other procedure were also performed following the manufacture’s instructions. Under this condition, at least 50% of cells could be transfected and could express the plasmid DNA transiently. 100, 200, or 400 ng of expression vector of pFlag-CMV2/FGF11 and pcDNA3-HA/FXRα along with 100 ng of the reporter plasmid (pcDNA3/5' UTR-Luc) were transfected to cells.

Luciferase assay

Cells were seeded in a 24-well culture plate and transfected with the reporter vector and expression plasmid using jetPEI (PolyPlus transfection-SA). The pFlag-CMV2/FGF11 and pcDNA3-HA/FXRα plasmid was added to achieve the same total amount of plasmid DNA transfection with pGL3b-FMDV 5' UTR-luc reporter plasmid. After transfection, the cells were rinsed with ice-cold PBS and lysed with 1 × cell culture lysis buffer (Promega, Madison, WI, USA). Luciferase activity was determined using an analytical luminescence luminometer (Promega) according to the manufacturer’s instructions. Luciferase activity was normalized for transfection efficiency using the corresponding β-galactosidase activity. All assays were performed at least in triplicate.

Analysis of FMDV 5' UTR-directed translation efficiency

IBRS-2 cells were incubated 24 hr at 24 well plate with 6 × 10⁴ cell density in DMEM (5% FBS and 1% PS). The pcDNA3/5' UTR-Luci plasmid was added to achieve the same total amount of plasmid DNA transfection. After transfection, the cells were rinsed with ice-cold PBS and lysed with 1 × cell culture lysis buffer (Promega). Luciferase activity was determined using an analytical luminescence luminometer (GloMax 96, Promega) according to the manufacturer’s instructions. All reporter assays were repeated at least three times. Data shown are mean ± standard deviation (SD) from one representative experiment.

RNA isolation, RT-PCR and quantitative real-time-PCR

Total RNA from cells was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s recommendations. The cDNA was synthesized from 1 μg of total RNA using Moloney-murine-leukemia virus (M-MLV) Reverse Transcriptase (Enzymomics, Daejeon, Republic of KOREA) and oligo dT primer at 37°C for 1 hr. A one-twentieth aliquot of the cDNA was subjected to PCR amplification using gene-specific primers for FGF11 and farnesoid X receptor alpha (FXRα). PCR was carried out using the following forward and reverse primers: FGF11 F, 5'-CCA AGT CCC TTT GCC AGA AGC-3'; FGF11 R, 5'-CCA TGT AGT GAC CCA GCT TGG -3'; FXRα F, 5'-AGG GGA TGA GCT GTG TG T-3'; FXRα R, 5'-CCT GTA TAC...
ATA CAT TCA GCC AAC-3′. The cDNA was amplified by PCR and the PCR products were examined by electrophoresis on a 1% agarose gel. The RT-PCR bands were quantified relative to the β-actin control band. qRT-PCR was performed with TOPreal qPCR 2x PreMIX with SYBR Green (Enzymics). The comparative CT method was used to calculate the relative gene expression levels with β-actin as an endogenous control gene.

**Cell viability**

IBRS-2 cells were incubated 24 hr at 96 well plate with 1 × 10^4 cell density in DMEM (5% FBS and 1% PS). The pFlag-CMV2/FGF11 plasmid was added to dose-dependent amount of plasmid DNA transfection. After transfection, the cells were rinsed with ice-cold PBS and treated with CCK-8 (Abbkine, Wuhan, China) according to manufacturer’s recommendations.

**Western blot**

The cells were prepared by washing with ice-cold PBS and lysed. The protein concentration was determined using Bradford method as BSA standard. Same amount of proteins was loaded and separated by SDS-PAGE and the gels were transferred to PVDF membrane (Millipore, Burlington, MA, USA). For western blotting, after blocking of using skim milk, the membranes were incubated with anti-hemagglutinin (HA) (Roche, Basel, Switzerland), anti-Flag (Cell Signaling Technology, Danvers, MA, USA) anti-β-tubulin (Abcam, Cambridge, UK) and anti-β-actin (Bioworld technology, St. Louis Park, MN, USA) in skim milk for overnight at 4°C. After washing three times with 0.1% TBST, the blotted membranes incubated with 2nd antibodies (goat anti-rabbit antibody HRP conjugation (Enzo, Farmingdale, NY, USA) for anti-flag, goat anti-rat antibody HRP conjugation for anti-HA (Santa cruz biotechnology, Dallas, TX, USA)) for 1 hr at room temperature. After washing three times with 0.1% TBST, the protein bands were detected by the ECL-Western blotting detection system (GE Healthcare, Chicago, IL, USA).

**Copy number of viral RNA genome**

Total RNA from cells was isolated using the RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s recommendations. qRT-PCR was performed with AccuPower FMDV Real-Time RT-PCR Master Mix kit (Bioneer, Daejeon, Republic of Korea). The copy number of viral RNA was calculated by standard nucleic acid.

**Evaluation of cytopathic effect and TCID_{50}**

IBRS-2 cells were incubated 24 hr at 6 well plate with 1.5 × 10^6 cell density in α-MEM (5% FBS and 1% PS). Cells were transfected with pFlag-CMV2/FGF11 plasmid with different amounts using jetPEI (PolyPlus transfection-SA). After 24 hr, FMDV Asia1 Shamir (R) strain was infected IBRS-2 cells according to 10^3 TCID_{50} for 1 hr and changed cell culture medium with α-MEM (2% FBS, 1% PS). After 24 hr, cytopathic effect was evaluated by microscope and numbered by a represented graph. The cytopathic effect (CPE) scores were determined with the following criterion. Score 1; CPE was confirmed in 20% cells. Score 2; CPE was confirmed in 40% cells. Score 3; CPE was confirmed in 60% cells. Score 4; CPE was confirmed in 80% cells. Score 5; CPE was confirmed in 100% cells. TCID_{50} values were determined by serially supernatants of virus stock and performing duplicate infections of cells for 24 hr and confirming CPE after 3 days. Finally, TCID_{50} values were calculated by using Karber method [25].

**Statistical analysis**

Statistical software GraphPad Prism 5.03 (GraphPad Software, Inc., La Jolla, CA, USA) was used for analysis. All experiments are presented as mean ± standard deviation (SD) and analyzed by a one-way analysis of variance (ANOVA) followed by Tukey’s post hoc test. P-value of <0.05 was considered statistically significant.

**RESULTS**

**Construction and functional analysis of FMDV translation reporter plasmid**

The 5′ UTR region of FMDV genome plays a critical role in entire viral RNA genome replication and protein translation of FMDV. It covers CRE and IRES sequences. In principal, FMDV and cellular protein should respond to the 5′ UTR region to lead gene activation for protein translation. We prepared pcDNA3/5′ UTR-Luci reporter system (Fig. 1A). In this reporter system, FMDV 5′ UTR-directed activation increases luciferase expression, resulting in its luminescent activity. To confirm the intensity of pcDNA3/5′ UTR-Luci expression in cells, we identified RNA expression of 5′ UTR in IBRS-2 cells (Fig. 1B). In addition, an increasing expression of 5′ UTR-luci showed a gradual induction of luciferase activity (Fig. 1C). Based on these results, we applied the evaluation experiment of a regulatory molecule to inhibit FMDV gene expression.

**FGF11 inhibits 5′ UTR-directed translation of FMDV**

In order to identify whether FGF11 inhibits FMDV gene expression, we applied 5′ UTR-luci reporter with ectopic FGF11 expression in IBRS-2 cells. At first, we added ribavirin, which is known to inhibit other picornaviruses replication and shows as a functional inhibitor of FMDV 5′ UTR-directed gene expression, to the FMDV 5′ UTR-luci expressing IBRS-2 cells. We treated 200 μM concentration of ribavirin and CoCl_{2} which did not induce the cytotoxicity. As shown in Fig. 2A, ribavirin suppressed the FMDV 5′ UTR-luci expression, indicating the functional integrity of 5′ UTR-luci reporter system. Since hypoxia was known to increase picornavirus gene expression, CoCl_{2}, which is a hypoxia mimic, was added the same as Fig. 2A. CoCl_{2} treatment slightly increased the luciferase expression directed by 5′ UTR (Fig. 2B). In Fig. 2C, increasing amounts of flag-tag FGF11 expression
plasmids were transiently transfected to IBRS-2 cells together with pcDNA3/5′ UTR-Luci reporter. FGF11 expression by the transient transfection gradually inhibited 5′ UTR-directed translation in a dose-dependent manner. For control experiment 5′ UTR-luci activity, we further transfected FXRα gene which is a nuclear receptor. FGF11 is known to be distributed in both cytoplasm and nucleus. FXRα is one of nuclear receptor and is also localized to cytoplasm or nucleus dependent on ligand binding. In several viruses, including hepatitis B virus, FXRα increases viral gene expression [29, 30, 33]. We regarded that FXRα does not inhibit FMDV gene expression. Based on these clue, we used FXRα as the control. As shown in Fig. 2C, FXRα overexpression did not inhibit FMDV 5′ UTR-luci expression (Fig. 2D). In order to confirm whether the inhibitory translation by FGF11 is not derived from cellular toxicity, we determined the cell viability after increasing amounts of FGF11 transfection (Fig. 2E). Transfection of higher amount of FGF11 gene (200 ng/24-well) showed similar value of cell viability at transfection of 50 ng FGF11. These results suggest that FGF11 inhibits FMDV gene expression without cell cytotoxicity.

**FGF11 inhibits RNA expression of FMDV 3D gene in FMDV-infected cells**

We sought to assess the role of FGF11 in FMDV replication by evaluating viral replication in FMDV-infected cells. FMDV genomic copies were quantified by qPCR experiments, and the results showed that there was a significantly lower viral replication in FGF11-overexpressed cells than that in control cells. FMDV infection induced RNA expression of FMDV 3D gene within 24 hr by detecting qPCR. Quantitation of FMDV 3D expression dictated determination of copy number of FMDV. Before FMDV infection, we transfected FGF11 gene in cells with two different gene doses and confirmed FGF11 expression. We confirmed that up to 2 μg of FGF11 transfection did not show the cytotoxicity. The effect of FGF11 for cytotoxicity was evaluated by microscope and MTT assay. After FGF11 induction, we infected FMDV in IBRS-2 cells. As shown in Fig. 3A, FGF11 expression even low dose 1 μg dramatically inhibited 3D mRNA expression in FMDV-infected cells at almost basal level. Together with decreased expression of FMDV 3D gene, we confirmed reverse proportional expression of FGF11 as a control (Fig. 3B). This result indicates that FGF11 highly inhibited copy number production of FMDV in the infected cells.

**FGF11 suppresses CPE induced by FMDV infection**

In IBRS-2 cells, cellular infection of FMDV Asia1 Shamir (R) strain constantly induced a significant CPE within 24 hr. To evaluate whether FGF11 expression inhibits CPE induced by FMDV infection, cells were transfected with pFlag-CMV2/FGF11 plasmid with different amounts before FMDV infection. After 24 hr post-transfection, FMDV Asia1 Shamir (R) strain was infected
Fig. 2. Fibroblast growth factor 11 (FGF11) inhibits foot-and-mouth disease virus (FMDV) five prime untranslated region (5′ UTR)-directed translation. (A) Ribavirin increases FMDV 5′ UTR-directed translation. After transfection of 5′ UTR-luci reporter, ribavirin was treated to cells. (B) CoCl₂ increases FMDV 5′ UTR-directed translation. Data shown are means ± SD of three independent experiments performed in duplicate. (C) FGF11 decreases FMDV 5′ UTR-directed translation. FGF11 gene was transfected together with 5′ UTR-luci reporter. After 24 hr post-transfection, cells were applied to measure luciferase activity. FGF11 expression was confirmed in a dose-dependent manner. **P<0.01, *P<0.05 compared with mock-transfected cells. (D) Farnesoid X receptor alpha (FXRα) expression do not affect FMDV 5′ UTR-directed translation. FXRα gene was transfected together with 5′ UTR-luci reporter. The experiments were similarly performed as in (C). (E) FGF11 overexpression does not affect cell viability. After increasing amounts of FGF11 transfection, we measured the cell viability by CCK-8.

Fig. 3. Fibroblast growth factor 11 (FGF11) inhibits foot-and-mouth disease virus (FMDV) replication in FMDV-infected cells. (A) FGF11 expression decreased FMDV 3D gene expression in FMDV-infected cells. After 24 hr post-transfection of FGF11 gene, FMDV Asia1 Shamir (R) strain was infected to IBRS-2 cells. Within 24 hr post-infection of FMDV, we measured FMDV 3D RNA level by qPCR. (B) Expression level of FGF11 was determined by qPCR and normalized to β-actin.
IBRS-2 cells according to $10^3$ TCID$_{50}$ for 1 hr. After 24 hr post-infection, cytopathic effect was evaluated by microscope and numbered by a represented graph. FMDV virus-containing sample at a concentration that is to be determined was serially diluted until a dilution is reached where no virus is present. Adequate replication of the serial dilution steps and individual monitoring of the CPE together with simple mathematics allowed for extrapolation of the amount of infectious virus particles present in the original sample. In the initial development of CPE, the IBRS-2 cells became smaller and rounded. At this stage, only 20% of the cells developed cytopathic effects. Fifty % of the cells became rounded and some of them began to be detached from the culture flask due to cell death induced by severe cytopathic effects. At this stage, the FMDV-induced CPE were obvious to recognize under the microscope. In correlation result of Fig. 3, FGF11 expression completely suppressed signs of morphology-based CPE by FMDV infection (Fig. 4A). The result of Fig. 4B showed ectopic expression of FGF11 in a dose-dependent manner after transfection. Most studies on FMDV replication kinetics or fitness rely on a particular assay to initially standardize inocula from virus stocks. The most accurate measure of infectious FMDV titers involves a calculation of the dose required for 50% infectivity of susceptible cells in tissue culture (TCID$_{50}$). There were significant correlations between TCID$_{50}$ values and the CPE appearance (Fig. 4C). This result indicates that FGF11 inhibits copy number promotion and pathogenesis in FMDV infection.

**DISCUSSION**

In the current study, we evaluate the antiviral potential of FGF11 against FMDV, a picornavirus that causes widespread disease in animals. For elucidating the inhibitory activity of FGF11 on FMDV gene expression, we prepared the FMDV 5′ UTR-luciferase construct containing all 5′ UTR region including CRE, which is essential element for FMDV RNA replication, and IRES, which is critical to regulate FMDV protein translation shown in Fig. 1. Herod *et al.* described that replication can be assessed by the

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**Fig. 4.** Fibroblast growth factor 11 (FGF11) inhibits cytopathic effect (CPE) induction in foot-and-mouth disease virus (FMDV)-infected cells. (A) IBRS-2 cells were transfected with pFlag-CMV2/FGF11 plasmid with different amounts. After 24 hr post-transfection, FMDV Asia1 Shamir (R) strain was infected IBRS-2 cells according to $10^3$ TCID$_{50}$ for 1 hr. After 24 hr post-infection, cytopathic effect was evaluated by microscope and numbered by a represented graph. (B) Expression level of FGF11 was determined by qPCR and normalized to β-actin. (C) Samples were collected every 24 hr postinfection ($x$ axis) and were quantified by TCID$_{50}$ ($y$ axis) in the presence or absence of FGF11 expression. The experiment was performed in triplicates. TCID$_{50}$ values were calculated by using Karber method.
expression of a GFP reporter gene, by comparison to a replication-defective construct, which indicates the level of input translation [13]. Using this FMDV sub-genomic replicon, they identified arbidol as a specific inhibitor for FMDV replication in vitro. Kanda et al. described that they constructed a bicistronic reporter plasmid containing the FMDV-IRES element located between Renilla luciferase and firefly luciferase to evaluate FMDV IRES-mediated translational activity and calculated the ratio of IRES-mediated translation to cap-dependent translation [17].

FMDV spreading can occur in an extremely rapid manner for a variety of reasons, including the small amount of virus required to initiate infection, the large amount of virus excreted by affected animals, and the multiple routes of infection [26, 35]. Additionally, the rate by which a population of viruses, including the FMDV, evolves can be influenced by genomic mutation rates, genomic architecture, and the speed of replication and recombination. The use of FMDV vaccines to protect early infection is limited, and the FMDV mutants frequently escape the immune system [7]. FMD vaccine is the traditional way to protect against the disease, which greatly reduces its occurrence. However, the use of FMD vaccines to protect early infection is limited, because the vaccines take protection effect until after 7 days [3]. Additionally, North America and Western Europe are considered as virus-free regions where the animals are not vaccinated. Therefore, the alternative strategy of applying antiviral agents is required to control the spread of FMDV in outbreak situations [7].

FMDV is capable of manipulating host cell machinery for viral replication and evading the host immune response, but the mechanisms are not fully understood [22, 32]. The functional inhibition of FMDV gene expression can be applied to develop antiviral intervention for FMDV transmission using the reporter assay system. In Fig. 2, we have tried to treat ribavirin to IBRS-2 cells and confirmed the inhibitory effect on FMDV 5′ UTR activity. Ribavirin is well known to inhibit viral replication of other picornaviruses including poliovirus and enterovirus [18, 34]. However, ribavirin has serious side effects, such as red blood cell breakdown, liver problems, and allergic reactions. In this study, the FMDV gene expression was inhibited by FGF11 with dose-dependency. Besides, the FGF11 target of the antiviral effect was the early phase of FMDV translation. The results reveals that FGF11 has potential as an effective anti-FMDV drug. The results of Figs. 3 and 4 indicated that cellular FGF11 expression inhibited the productive copy number and CPE in FMDV infection. FGF11 is different with other FGF family proteins which are mostly extracellular soluble proteins. Intracrine FGF11 plays a role in intracellular cytoplasm or nucleus, possibly interacting with various host cellular proteins. Some host factors interacting with FGF11 may regulate FMDV replication and translation, which lead to inhibition of FMDV copy number and CPE induction.

We have recently identified the amino acid variations of FGF11 from different animal species. The different amino acid composition of FGF11 protein may make differential protein stability and antiviral activity. We can use the FMDV 5′ UTR-luc reporter construct for the quantitative evaluation of amino acid variants of FGF11. In addition to FGF11, the reporter system can be used to interrogate large libraries of compounds to identify those with therapeutic potential against FMDV. Based on that, the mechanism of the antiviral effect of FGF11 on FMDV infection is need to in-depth research.

POTENTIAL CONFLICTS OF INTEREST. All authors declare that they have no conflict of interest.

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