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A safe and sensitive enterovirus A71 infection model based on human SCARB2 knock-in mice

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ABSTRACT

Enterovirus A71 infection has become a severe threat for global public health. Vaccines for controlling and preventing Enterovirus A71 epidemics are highly demanded, however, vaccine evaluation has been hindered by the lack of suitable Enterovirus A71 infection animal models. Here we established an *hSCARB2* knockin mouse model for real-time monitoring of enterovirus A71 infection *in vivo*. This model was sensitive to the infection of both replication-competent virus rEV71(FY)-EGFP and single round pseudotype virus pEV71(FY)-Luc. The intensity of bioluminescence correlated well with viral loads in infected tissues ($R = 0.86$, $P < 0.01$). Pathological changes recapitulated human infectious and clinical features of enterovirus A71, including both general characteristics of "hand foot and mouth" and the severe symptoms in the CNS. A formalin-inactivated enterovirus A71 vaccine can elicit antibodies in *R26-hSCARB2* mice, which play effective roles in protecting knockin mice against enterovirus A71 infection as indicated by bioluminescence. Therefore, this work provides a safe, sensitive and visualizing model for exploring mechanisms of enterovirus A71 infection and examining human enterovirus A71 vaccines and antiviral therapies.

1. Introduction

Enterovirus A71 (EV-A71) is an etiological agent that causes hand-foot-and-mouth disease (HFMD) and severe neurological complications in children, including aseptic meningitis, and fatal encephalitis with cardiopulmonary complication [1,2]. EV-A71 outbreaks have occurred frequently in the Asia-Pacific region, posing a severe global public health threat [3,4]. Since vaccine is the most efficient approach for controlling and preventing EV-A71 epidemics, various vaccine candidates are under research and development [5–10]. To facilitate vaccine developments, suitable and relevant EV-A71 infection animal models are urgently needed.

Both non-human primate and mouse models have been developed for EV-A71 infection. Cynomolgus and rhesus monkeys, which are susceptible to EV-A71 infection, have been used as EV-A71 non-human primate models [11–13]. However, the application of EV-A71 non-human primate model is limited by ethical and economic considerations. As murine models, neonatal and immunodeficient mice are susceptible to EV-A71 caused muscular lesions, paralysis and death. However, they are not ideal models for evaluating protective effect of vaccines. Although neonatal mice can be passively protected by maternal antibodies generated by immunized pregnant mice against EV-A71 challenge, the direct correlation between antibodies elicited by vaccination and the protection effect is hard to be quantitatively evaluated [14–16]. Therefore an adult EV-A71 infection murine model with mature immune system is demanded for vaccine effect evaluation *in vivo*.

To establish proper animal models for EV-A71 infection, two transgenic (Tg) mice carrying the known human EV-A71 receptor gene, scavenger receptor class B member 2 (*hSCARB2*) [17], were established [18,19]. Transgenic mice developed by Lin et al., were only susceptible to EV-A71 infection up to two-week-old, with pathological features similar to suckling mouse model [18]. While in the mouse model developed by Fujii et al., young *hSCARB2*-Tg

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mice (six weeks old) were susceptible to infection by EV-A71 and displayed neuropathology. However, the major symptoms of HFMD is absent in the adult *hSCARB2*-Tg mice [19]. Here we generated *hSCARB2* knockin mice, which are not only susceptible to EV-A71 infection but also exhibit common features of HFMD in humans.

Traditional mouse models for viral infection relies on either survival rate or viral load as a readout, and needs laborious monitoring and dissecting. Besides that, pathogens residing in unexpected anatomical sites might be omitted. Here we developed a sensitive, safe and convenient EV-A71 murine model, in which, the infection of firefly luciferase (Luc) recombinated EV-A71 pseudovirus and antibody-mediated protection can be real-time monitored and quantitatively evaluated *in vivo* in *hSCARB2* knockin mice by bioluminescent imaging (BLI).

2. Material and methods

2.1. Ethics statements

Mice used in the study were housed and handled strictly in accordance with the institutional (National Institute for Food and Drug Control) guidelines for animal care and use. The study protocol was approved by the NIFDC Institutional Animal Care and Use Committee.

2.2. Construction of hSCARB2 knockin mice

To construct the targeting vector, cDNA encoding human *SCARB2* fused with a F2A sequence and tdTomato reporter were inserted into a modified vector Ai3, which contains DNA sequences of *ROSA26* recombination site, as well as neomycin selection marker and a loxP-flanked STOP sequence (triple polyadenylation sequence) under a CAG promoter. The targeting vector was electroporated into C57BL/6 embryonic stem (ES) cells. G418-resistant colonies were picked and expanded. PCR was further used to screen ES cells for homologous recombination, with primer set 1 (Supplemental Table 1). Correctly targeted ES cells confirmed by southern blot were injected into BALB/c blastocysts, and then subsequently implanted into pseudopregnant mice. Male chimeras were bred with C57BL/6 female mice to produce offspring carrying the *hSCARB2* gene.

2.3. Genotyping

Tail genomic DNA was isolated using Tissue Genomic DNA Extraction Kit (Generay, Shanghai, China) and then subjected by PCR to verify the insertion of *hSCARB2* gene. Genomic DNA of *hSCARB2* knockin mice and wildtype mice could be amplified with primer set 1 or 2 (Supplemental Table 1), with expected PCR products of 561 bp or 469 bp respectively. Primer set 3 (Supplemental Table 1) was used to identify Cre in Tg(EIIa-cre) mice, with an expected PCR product size of 100 bp.

2.4. Southern blot

Genomic DNA prepared from ES cells or mouse tail clips was digested with restriction enzymes *Hind* III (NEB) and *Mfe* I (NEB), concentrated by precipitation and resolved on a 0.8% agarose gel. Upon transfer to nylon membrane and UV cross-linking, prehybridization and hybridization were carried out according to instructions for DIG Easy Hyb Granules (Roche) at 42 °C. The membrane was then developed using DIG Detection Kit (Roche), following manufacturer's instructions. Primers used for probe template synthesis were as follows: Rosa-5′-probe-F AACAAGTGCTCCATGCTGGAAG, Rosa-5′-probe-R CTGTGTACTGATGCTGCAGCCC; Rosa-3′-probe-F ACTGACATGTAGAAGTGTTTGTCC, Rosa-3′-probe-R GTCAAGCCACATTTGTAAGCT. The developed membrane was exposed to KODAK films.

2.5. Real-time PCR

Total RNA was extracted from tissues using TRIZOL reagent (Life Technologies, Carlsbad, CA) following the manufacturer's instructions, and then converted into cDNA with the reverse transcription (RT) PCR kit (Takara, Shiga, Japan). The synthesized cDNA was subjected to real-time PCR analysis (the Light Cycler 480 Real-Time PCR system) using primer set 4 for *hSCARB2* (Supplemental Table 1). Mouse *Gapdh* mRNA was used as an internal control. Each reaction was performed in triplicate. Viral loads were quantified by real-time RT-PCR using primer set RT-FY-3D-F/R (Supplemental Table 1) and Taqman probe: 5′Fam-TGCCAGCCTTAGCCCTGTCTGG-3′Tamra, and then represented as Lg virus RNA copies/GAPDH.

2.6. Western blot

Mouse tissues were homogenized in RIPA buffer (50 mM Tris–Cl pH 7.4, 150 mM NaCl, 100 mM EDTA, 1% NP-40) supplemented with 1x Proteinase Inhibitor (PI) (Roche, Basel, Switzerland). The hSCARB2 was detected by immunoblotting with anti-hSCARB2 (Sigma, St. Louis, MO) at 1:5000 dilution and GAPDH was detected by immunoblotting with anti-GAPDH (Abcam, Cambridge, UK) at 1:80,000 dilution, followed by peroxidase-conjugated anti-rabbit IgG (Santa Cruz, CA) at 1:10,000 dilution.

2.7. EV-A71 infection

Production of EV-A71 pseudotype virus pEV71(FY)-Luc and replication-competent rEV71(FY)-EGFP virus were performed as described previously, which was derived from FY strain (accession number: EU703812, genotypic subgroup C4) [20]. Four-week-old *R26-hSCARB2* mice were challenged with viruses at a given dose by intravenous (I.V.), intraperitoneal (I.P.), intragastrical (I.G.), or subcutaneous (S.C.) routes, and observed for fluorescence signals and clinical signs at indicated time points. Four additional C57BL/6 control mice were injected with MEM as controls.

2.8. Bioluminescence imaging (BLI)

Bioluminescence imaging was performed with IVIS-Lumina II imaging system (Xenogen, Baltimore, MD) as described previously [21,22]. Mice were anesthetized by an I.P. injection of pelltobarbitalum natricum (240 mg/kg body weight). Luminescence was acquired with an exposure time of 60 s, and regions of interest (ROIs) were analyzed using Living Image software (Caliper Life Sciences, Baltimore, MD). For rEV71(FY)-EGFP infection, considering the emission spectra overlap between EGFP and tdTomato, the color scale of radiant efficiency was adjusted to clear the tdTomato fluorescence. For pEV71(FY)-Luc infection, mice were anesthetized and followed by an I.P. injection of the substrate, D-luciferin (50 mg/kg body weight, Xenogen-Caliper Corp., Alameda, CA). Mice were imaged 10 min later. The relative intensities of emitted light were presented as pseudocolored images, with colors ranging from red (the most intensive) to blue (the least intensive), and presented as photon flux in photon/(s cm^2 sr).

2.9. Immunohistochemistry

Mouse tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned (2 μm). For histopathological examination, tissue sections were stained with hematoxylin and eosin. For immunohistochemistry, tissue sections were rehydrated

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