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Exosomes-mediated transmission of foot-and-mouth disease virus in vivo and in vitro

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ABSTRACT

Exosomes are small membrane-enclosed vesicles that participate in intercellular communication between cells. Numerous evidences suggested that exosomes derived from virus-infected cells can mediate virus transmission or/and regulate immune response. Foot-and-mouth disease virus (FMDV) is the prototype member of the *Aphthovirus* genus of the *Picornaviridae* family. It can cause highly infectious disease of cloven-hoofed livestock and significantly increase public awareness. However, the role of exosomes in the transmission of FMDV has still remained unknown. In this study, full length of FMDV genomic RNA and partial viral proteins were identified in purified exosomes isolated from FMDV-infected PK-15 cells with qRT-PCR and /MS. Exosomes from FMDV-infected cells were capable of transmitting infection to naive PK-15 cells and suckling mice. Furthermore, exosome-mediated infection cannot be fully blocked by FMDV-specific neutralizing antibodies. This finding highlights that FMDV transmission by exosomes as a potential immune evasion mechanism.

1. Introduction

Foot-and-mouth disease (FMD) is an extremely contagious disease caused by foot-and-mouth disease virus (FMDV), affecting cloven-hoofed livestock and wildlife species, such as cattle, pigs, and sheep (Grubman and Baxt, 2004). FMDV is a positive-sense, single-stranded RNA virus with a genome size about 7.8 kb, that encodes several structural and nonstructural proteins (Alexandersen and Mowat, 2005; Wernery and Kaaden, 2004). The titer of FMDV-specific neutralizing antibodies (NAbs) is an important index to evaluate the immune effect of FMD vaccine, and NAbs are believed to be as a key factor for anti-FMDV immunity (Barrionuevo et al., 2018; Jin et al., 2007; Xiao et al., 2007). However, FMDV can still be isolated in the presence of high level of FMDV-specific NAbs and nonstructural protein (NSP) antibody (Farooq et al., 2018), which led to achieve one of the evidences that FMDV can antagonize the innate and adaptive immune responses and establish a persistent infection facing NAbs (Bao et al., 2011; Eschbaumer et al., 2016; Farooq et al., 2018; Huang et al., 2011; Vosloo et al., 1996). Characteristics of quasispecies for FMDV can generate viral escape mutants (also called “cloud mutants”) which constitute another important mechanism related to FMDV persistence in the present of NAbs. Are there any other mechanisms for FMDV transmission or/and its escape of NAbs?

Exosomes are small membrane-encapsulated lipid bilayer vesicles that secrete into the extracellular environment. The size of exosomes 30 to 150 nm (Thery et al., 2002). Lipids, proteins, and RNAs are carried and delivered by exosomes (Mathivanan and Simpson, 2009; Thery, 2011). One of the main functionof exosomes is to mediate intercellular communication and signal transduction through the transmission of signal proteins or functional RNAs during various biological processes, such as immune responses, viral pathogenesis, etc. (Alenquer and Amorim, 2015; Chahar et al., 2015; Simons and Raposo, 2009; Valadi et al., 2007). Virus can mediate exosomes to modulate the host immune response, and facilitate its replication and survival (Liu et al., 2014; Madison and Okeoma, 2015). Important roles of exosomes during virus infection have been studied and reported, involving enterovirus 71 (EV71)(Fu et al., 2017), herpes simplex virus 1 (HSV-1) (Heikkila et al., 2016), porcine reproductive and respiratory syndrome virus (PRRSV) (Wang et al., 2018), hepatitis B virus (HBV) (Aly et al., 2016; Zhao et al., 2014), hepatitis C virus (HCV) (Devhare et al., 2017; Elgner et al., 2016; Liu et al., 2014; Ramakrishnaiah et al., 2013), Human immunodeficiency virus-1 (HIV-1) (Shelton et al., 2012), Dengue virus

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(DENV) (Zhu et al., 2015b), respiratory syncytial virus (RSV) (Chahar et al., 2018), The Epstein–Barr virus (EBV) (Gallo et al., 2017), and Marek's disease virus (MDV) (Nath Neerukonda et al., 2018). These studies highlighted the potential and crucial roles of exosomes in viral transmission and infection; however, it's a pity that the functions of exosomes during FMDV infection have not been fully elucidated yet.

In the present study, exosomes derived from FMDV-infected PK-15 cells were isolated and purified. Evidences showed that most viral proteins (except for L, 2 A and 3B) and complete genomic RNA (except 5' and UTRs) were contained in the exosomes. The exosomes viral RNA could be transferred and replicated in vivo and in vitro. Exosome-mediated FMDV transmission is not inhibitted by NAbs. These findings revealed a new insight in pathogen-host interactions, highlighting that FMDV can exploit a cellular exosomal-based delivery system to transmit viral components that can establish a productive infection in the presence of NAbs.

2. Materials and methods

2.1. Virus and cell culture

The following porcine kidney cell were used: PK-15 and IBRS-2 cells were cultured in Dulbecco's modified Eagle's medium (Gibco, Waltham, MA, USA), which supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, 100 mg/mL streptomycin, and 2 mM L-glutamine (Invitrogen, Carlsbad, CA, USA) to reach 80% confluency, in a humidified incubator at 37 °C with 5% CO2. FMDV serotype A strain (GenBank accession number, KF450794) was provided by China Reference Laboratory Network for Foot-and-Mouth Disease. Lentiviral expression vector pLVX-AcGFP1-N1 (Lv-pLVX) (TaKaRa, Cat. No.) allowed target genes to be expressed in the form of fused GFP protein.

2.2. Ethics statement

All animal experimental protocols were approved by the requirements and management guidelines of the Gansu animal experiments inspectorate and the Gansu ethical review committee (License No. SYXK 2014-003).

2.3. Exosome isolation and purification

Exosomes derived from culture supernatants of FMDV-infected PK-15 cells were characterized and isolated as previously described (Thery et al., 2006). Briefly, FMDV-PK-15 cell supernatants and mock PK-15 cell supernatants were collected simultaneously at 24 hpi and centrifuged at 500 × g for 5 min to remove larger debris and the cells. Cell debris was further removed by centrifuging for 10 min at × g. Next, a centrifugation step was undertaken for 45 min at 12,000 × g to pellet microvesicles, and then, 0.2 μm filter. Ultracentrifugation(UC)was carried out at 120,000 × *g* for 2 h. Exosome pellets were washed and resuspended in 1 mL phosphate buffered saline (PBS) and UC was repeated in the same way as described. Finally, exosome pellets were resuspended with 50 to 500 μL PBS and stored at −80 °C. To further purify exosomes, the CD63 antibody-labelled exosome isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) was used according to the manufacturer's instructions.

2.4. Transmission electron microscopy (TEM)

Exosomes derived from FMDV or mock-infected PK-15 cell culture supernatants obtained after UC and purification were spotted onto formvar coated grids (200 mesh). The adsorbed exosomes were directly stained by phosphotungstic acid (PTA) for contrast enhancement, and then observed using a transmission electron microscope (Hitachi H-7000FA, Tokyo, Japan) at 80 KV. For subsequent analyses, at least 10 views were imaged and representative images were saved and shown.

2.5. Nanoparticle tracking analysis (NTA)

Mean size and size distribution profile of exosomes particles derived from FMDV or mock-infected PK-15 cell culture supernatants after isolation and purification were analyzed as described previously (Fu et al., 2017; Nath Neerukonda et al., 2018). In brief, exosomes samples were diluted before analysis to between 2×10^8 and 2×10^9 particles per ml, and the relative concentration was calculated based on the dilution factor. Samples were analyzed using gain adjustments and manual shutter, which resulted in speed of 15 or 30 ms, with camera between 280 and 560. Data analysis was performed with NTA 3.2 software (Malvern Panalytical Ltd., Malvern, Worcestershire, UK), and evaluated using a Nanosight NS300 instrument (Malvern Panalytical Ltd., Malvern, Worcestershire, UK). Each sample was analyzed five times and the counts were averaged.

2.6. Mass-spectrometry(MS) and data analysis

To perform MS analysis, the purified exosomes was eluted with 200 μL isolation buffer provided by the CD63 antibody-labelled exosome isolation kit. 50ul protein pellets were lysed in 250 μL of lysis buffer (7 M urea, 2 M thiourea, 2% (w/v) CHAPS) containing a complete protease inhibitor cocktail described previously (Wisniewski et al., 2009). After digestion of the peptide separation was performed on reversed phase column, then the column eluent was directly sprayed into the electrospray ionization source of mass spectrometer. Mascot Distiller 2.3 software (Matrix Science Inc., Boston, MA, USA) was used to analyze raw data files and create peak lists. Searching database, containing all FMDV proteins sequences and all pig protein sequences was undertaken by Mascot search algorithm (version 2.2). The GO and KEGG analysis were conducted based on identified host proteins.

2.7. Generation of CD63-GFP stable expression of PK-15 cell lines and FMDV infection

Generation of CD63-GFP stable expression of PK-15 cells based on lentivirus vector was carried out as described previously (Case et al., 1999; Demaison et al., 2002; Godecke et al., 2018). Briefly, primers of CD63 gene were designed and synthesized based on the sequences which published in GenBank (Accession No. XM_005663878.2). Besides, CD63 gene was cloned into the multiple cloning site (MCS) of Lv-pLVX, named Lv-CD63. HEK293FT cells (Invitrogen R700-07) were cultured in DMEM containing glucose and glutamine (Gibco), supplemented with 100 μg/mL PenStrep (Gibco) and 10% FBS (Gibco). Plasmids of Lv- pLVX and Lv-CD63 were transfected into 293FT cells to package lentivirus mediated by Lipofectamine2000 (Thermo Fisher Scientific, Waltham, MA, USA). PK-15 cells infected with packaged lentivirus and stable cell lines (Lv-CD63-PK-15) were selected by limited dilution methods with the presence of 3 μg/ml puromycin . The expression level of fusion protein CD63 + GFP was examined by RT-qPCR and observation of green fluorescent protein by using a microscope.

The release of exosomes can be enhanced by increasing CD63 expression (Gauthier et al., 2017; Hurwitz et al., 2017; King et al., 2012). To evaluate the effect of FMDV infection on CD63 gene expression in PK-15 cells, green fluorescence was observed 24 h after Lv-CD63-PK-15 infection with FMDV. Replication of FMDV was identified by indirect immunofluorescence assay (IFA) with VP3 monoclonal antibody. Lv-CD63-PK-15 cells without FMDV infection were used as the negative controls.

2.8. Western blot analysis

Western blot (WB) analysis was performed with the following protocols. Briefly, purified exosomes were lysed with radio-immunoprecipitation assay (RIPA) buffer (Santa Cruz Biotechnology,

Dallas, TX, USA), and cleared lysate was collected by centrifugation for protein separation on 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. The separated proteins were transferred onto 0.45 μm polyvinylidene difluoride (PVDF) membranes (Millipore, USA) after electrophoresis. Next, the membranes were blocked for 1h with tris-buffered saline containing Tween 20 (TBST) with 5% fat free milk. The blots were then incubated by primary antibody at 4 °C overnight. The primary antibodies against FMDV serotype A, CD63 (Abcam, Cambridge, UK), CD9 (Abcam, Cambridge, UK) and Alix (Cell Signaling Technology, Waltham, MA, USA) were used. After three times washing with TBST, horseradish peroxidase (HRP)-labeled secondary antibody (Proteintech, Chicago, IL, USA) was incubated for 2 h at room temperature. Finally, the proteins were visualized with Clarity enhanced chemiluminescence (ECL) WB substrate (Bio-Rad Laboratories, Hercules, CA, USA).

2.9. Exosomes labeling and uptake

Exosomes from infected and non-infected cells were labeled by DiO (Invitrogen, Carlsbad, CA, USA; Em: 501 nm, Ex: 484 nm; green fluorescence), and a fluorescent lipophilic tracer to trace their uptake by PK-15 cells, which performed as previously described (Jung et al., 2018). Briefly, exosome pellet was incubated with 1 mM of DiO for 15 min at 37 °C, and the fluorescence-labeled exosomes (about 100 mg/mL) were purified and incubated in PK-15 cells for 8 and 16 h, respectively. Nucleus was stained by DAPI. The uptake of the fluorescentexosomes by PK-15 cells was visualized with a fluorescence microscope (Leica, Germany).

2.10. RNA extraction, reverse transcription, and RT-qPCR

RNase (sigma) added to purified exosomes and incubation for 1 h at 37°C before RNA extraction. To detect FMDV RNA, total RNA was extracted from exosomes using a total exosome RNA isolation kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Then, cDNA was synthesized by using the respective specific or random primers with superscript III reverse transcriptase. The whole genome of FMDV (except and UTRs) was divided into seven overlapping fragments to be amplified, in which primers used for each fragment are listed in Table 1. To quantify the RNA copies of FMDV in FMDV-infected or exosome-treated cells, RT-qPCR was performed by using TaqMan universal PCR master mix (Thermo Fisher Scientific, Waltham, MA, USA) and 6-carboxyfluorescein (FAM)minor groove binder probes for FMDV. Each sample was examined at least three times.

Table 1
Primer sequences used in this study.

Genome position	Primer sequences 5'-3'
FMDV 402-950	CGRCGTTAAAGGGAGGTAACC(forward) GGCATCCTTAGCCTGTCACC(reverse)
FMDV 830-1420	GCCTGAATAGGYGACCGGAG(forward) CCGTTGAGYGGTTCTTGATCG(reverse)
FMDV 1398-1958	GAACCATTCTTTGATTGGGTC(forward) CCATGGAGTTCTGGTACTGCTGC(reverse)
FMDV 1900-2600	AATCGAGTGTGGGGGTCACC(forward) GCTGTTTTGGGGTCTGTTGTCACC(reverse)
FMDV 2530-3280	CACCCGAGGCAGCACTG(forward) CTTCTGAGGCGATCCATG(reverse)
FMDV 3068-4130	CATCAGGAACGGCCTCGACG(forward) GGGTGGAAGCCAAAGTCTG(reverse)
FMDV 4060-7485	GACCAGCATCGGAATCTAGC(forward) TCGAAGCCTTAGTGAGAT(reverse)
FMDV 3D	ACTGGGTTTTACAAACCTGTGA(forward) GCGAGTCCTGCCACGGA(reverse) 5'FAM TCCTTTGCACGCCGTGGGAC 3'TAMRA
Pig-GAPDH	ACATGGCCTCCAAGGAGTAAGA(forward) GATCGAGTTGGGGCTGTGACT(reverse)

Note: Primers were disigned based on type A isolate A/HY/CHA/2013 strain (GeneBank accession No, KT968663).

2.11. Transmission of infection via exosomes

Exosomes derived from FMDV or mock-infected PK-15 cell culture supernatants were isolated and purified. RNase (sigma) added to purified exosomes and incubation for 1 h at 37 °C. Total exosomes protein was quantified with Micro BCA™ protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Then, 15 within 3-day old BALB/C suckling mice were randomly divided into 3 groups, and 120 μg (150 μL) FMDV-exosomes was subcutaneously inoculated to each mouse in one group. In the second group, equal mock-exosomes protein was injected subcutaneously into each mouse. About 1×10^5 $TCID_{50}$ of free FMDV and equal volume (150 μL), PBS was subcutaneously injected into each mouse respectively in the third group. All the mice were euthanized 72 h after inoculation. Survival rate in each group was calculated with graphPad prism software. The dead and dying mice were collected and cryopreserved. Total RNA from each mouse carcass was extracted using a total exosome RNA isolation kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was synthesized using specific primers with superscript III reverse transcriptase. FMDV load was evaluated by RTqPCR for each mouse carcass in every group.

PK-15 and IBRS-2 cells were seeded on 24-well plates with the density of 2×10^5 cells per well. About 2×10^5 $TCID_{50}$ of free FMDV and exosomes derived from FMDV or mock-infected cell culture supernatants were added to the cells. All the exosomes was treated with RNase and incubation for 1 h at 37 °C before they added to cells. After incubation in 5% CO2 for 2 h at 37 °C, the medium was replaced with a fresh maintenance medium. Cells were collected for performing RT-qPCR at 30 h after infection. Exosome-mediated infection was confirmed by indirect immunofluorescence assay (IFA) with FMDV-specific antibodies.

2.12. Exosomes treatment with FMDV-specific neutralizing antibody

To evaluate the effects of FMDV antibody on transmission of infection via exosomes, FMDV antibody was added to the purified exosomes. FMDV neutralizing antibody (NAbs) against FMDV A/GDMM/CHA/2013 was determined by the method of virus neutralization test (VNT), according to the OIE manual of diagnostic tests and vaccines for terrestrial animals (2009). FMDV NAbs (the titer of neutralizing antibody above 1:1024) were added to FMDV-exosomes, and free FMDV maintained at 37 °C for 2 h before cell experiment. FMDV-exosomes and free FMDV treated with FBS were used as negative control. Replication of FMDV was evaluated by qPCR.

3. Results

3.1. Isolation and purification of exosomes from FMDV-infected PK-15 cells

Exosomes were isolated from culture supernatants of FMDV-infected PK-15 cells, following a commonly used differential centrifugation protocol. Further purification was performed with the use of CD63-immunoaffinity kit after isolation. Cup-shaped lipid bilayer vesicles of representative exosomes images were observed with transmission electron microscopy (TEM) (Fig. 1A). As shown in Fig. 1B, the size of 30 and 150 nm exosomes was evaluated with nanoparticle tracking analysis (NTA) method. The purified exosomes were characterized and identified by exosomal markers, including Alix, CD63, and CD9 with Western blotting (WB) (Fig. 1C). Results showed that exosomes were isolated, purified, and identified successfully from culture supernatants of FMDV-infected PK-15 cells.

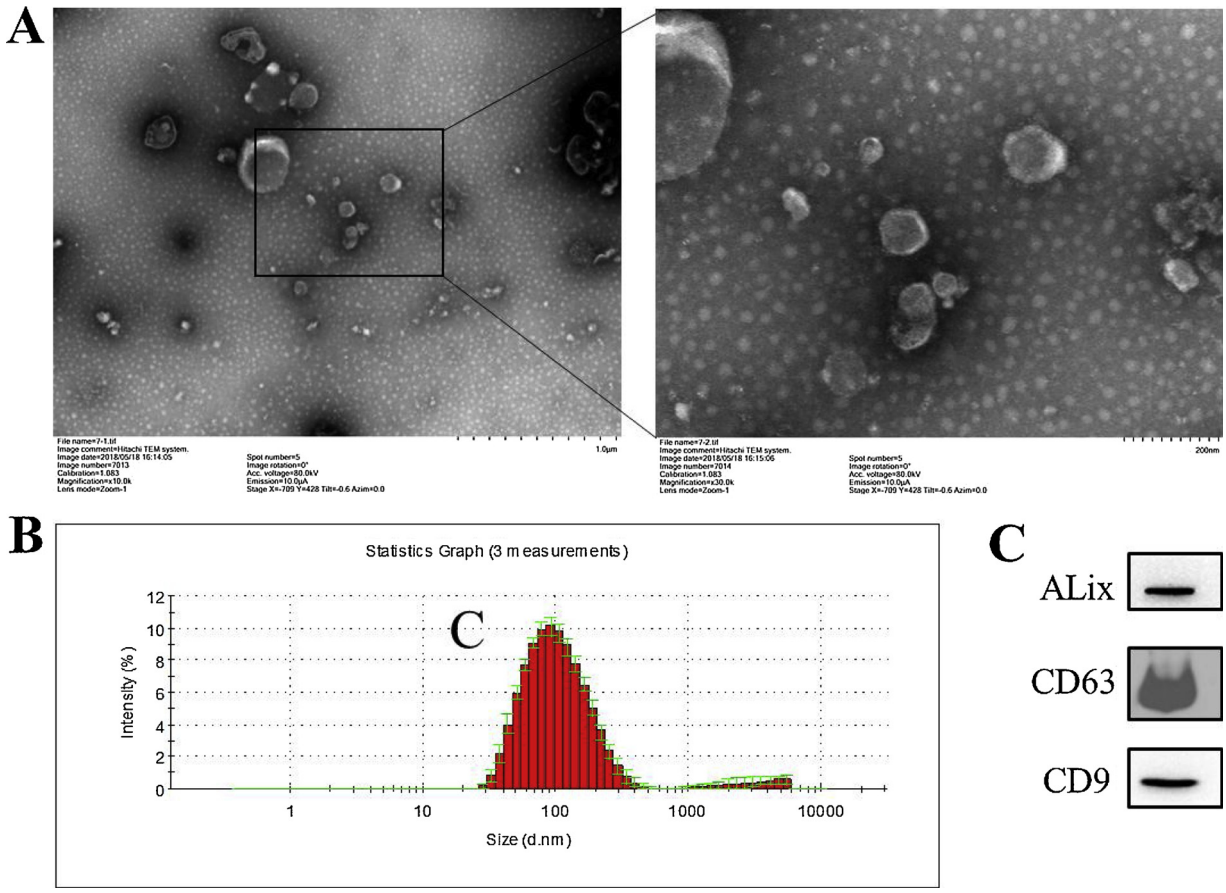

Fig. 1. Isolation and purification of exosomes derived from culture supernatants of FMDV-infected PK-15 cells. (A) TEM observations of negatively stained purified exosomes derived from culture supernatants of FMDV-infected PK-15 cells. (B) Histogram displaying the mean size and size distribution profile of exosomes particles derived from culture supernatants of FMDV-infected PK-15 cells by NTA method. (C) Purified exosomes derived from FMDV-infected cells were analyzed by WB with antibodies against exosomes marker proteins, including Alix, CD63 and CD9.

3.2. FMDV infection increases CD63 expression in PK-15 cells

Compared with negative control group, the green fluorescence significantly increased in Lv-CD63-PK-15 cells at 24 h after FMDV infection (Fig. 2-CD63 + GFP and Fig. 2-Merge). FMDV replication was also confirmed by VP3 antibody IFA (Fig. 2-FMDV-VP3). Because CD63 expression is positively correlated with the release of exosomes, it can be concluded that FMDV infection can increase exosomes secretion

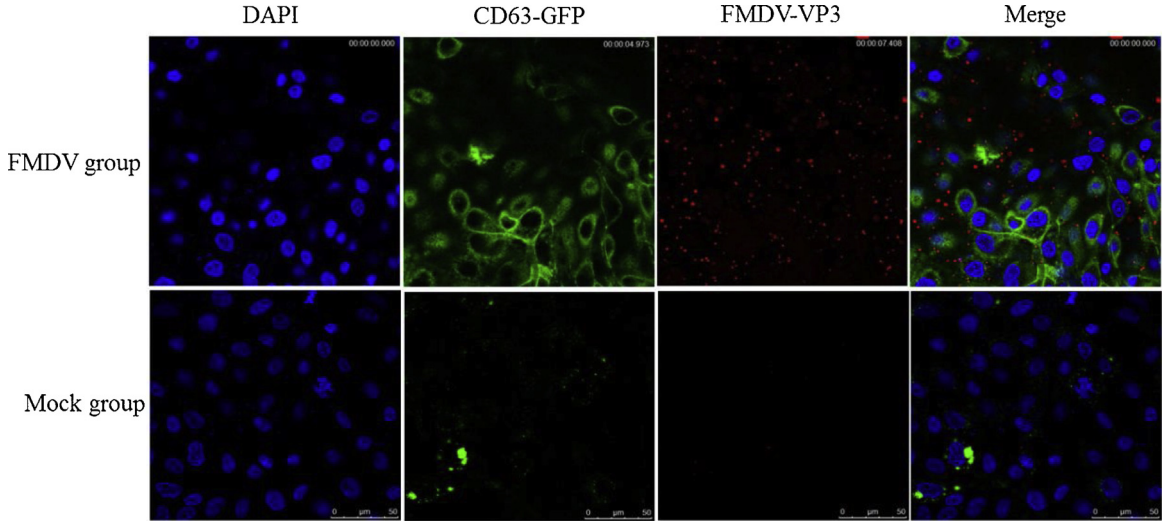

Fig. 2. FMDV infection increase exosomes secretion. FMDV replication was also identified by VP3 monoclonal antibody IFA in FMDV group, as shown by red color (Fig. 2FMDV-VP3); Green fluorescence increased significantly in FMDV group 24 h after FMDV infection, as illustrated by green color (Fig. 2CD63-GFP and Fig. 2Merge); FMDV group: Lv-CD63-PK-15 cells 24 h after FMDV infection; Mock group: normal Lv-CD63-PK-15 cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

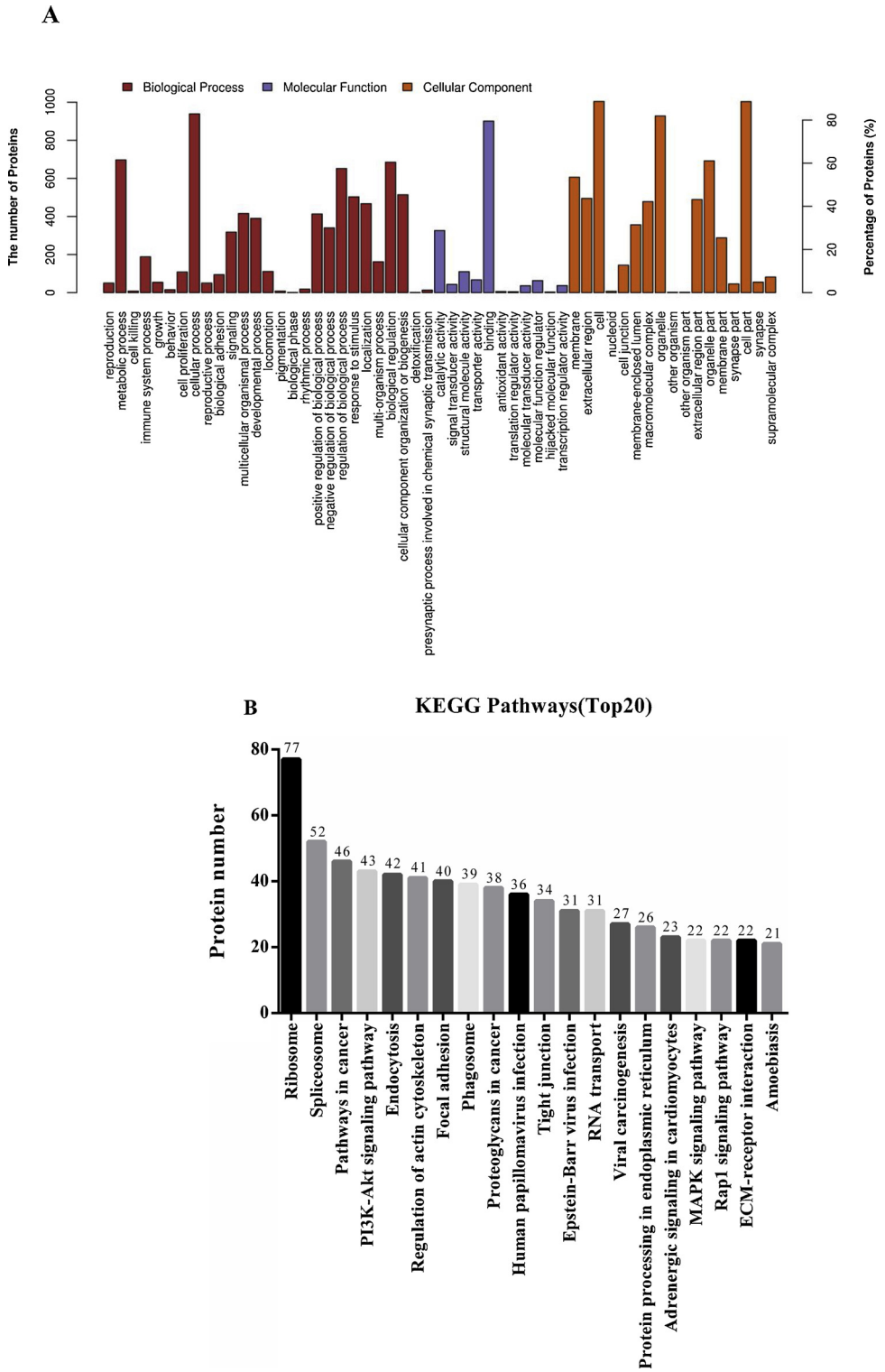

Fig. 3. GO and KEGG analysis of host proteins in FMDV-infected exosomes. (A) Gene ontology (GO) analysis based on identified FMDV-infected exosomes host proteins with /MS method. Biological processes, molecular functions, and cellular components of identified exosomes-protein components derived from culture supernatants of FMDV-infected PK-15 cells are shown. (B) KEGG analysis based on identified FMDV-infected exosomes host proteins with /MS method. Signaling pathways of identified exosomes-protein components derived from FMDV-infected PK-15 cell culture supernatants are shown.

from PK-15 cells.

3.3. Mass spectrometry (MS) identification and components analysis of FMDV-infected cells exosomes

A total of 1133 proteins were identified in purified exosomes from culture supernatants of FMDV-infected PK-15 cells by liquid chromatography-mass spectrometry ()/MS. All identified proteins were analyzed by Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG), which described the properties of genes and gene products in organisms by biological processes (BP), molecular functions (MF), and cellular components (CC). BP analysis showed that identified

proteins are associated with positive/negative regulation of biological process and metabolic process (Fig. 3A). Most of proteins were ranked in binding and catalytic activity based on MF analysis (Fig. 3A), and the results of CC indicated that a portion of the proteins were classified cell membrane or membrane-related proteins (Fig. 3A). Proteins belonged to PI3K-akt signaling pathway, mitogen-activated protein kinase (MAPK) pathway, and cancer-related pathways were explicitly based on KEGG (Fig. 3B). Marker proteins of exosomes, such as CD63 and CD9 were also identified by culture supernatants of FMDV-infected PK-15 cells.

3.4. Viral components are present in FMDV-infected exosomes

/MS analysis revealed that all the FMDV proteins were packaged in FMDV-infected exosomes except for L, 2 A, and 3B proteins. Viral nucleic acids of HBV, PRRSV, and EV71 were confirmed respectively in exosomes which derived from infected host cells (Fu et al., 2017; Ramakrishnaiah et al., 2013; Wang et al., 2018). In this study, reverse transcription polymerase chain reaction (RT-PCR) was used to determine whether the exosomes derived from FMDV-infected cells contain viral RNAs. The whole genome of FMDV (except and UTRs) was divided into seven overlapping fragments to be amplified based on the primers(as shown in Table 1). Results of RT-PCR and DNA electrophoresis indicated that all seven overlapping fragments could be amplified from the exosomes isolated from FMDV-infected cells (Fig. 4). Sequence comparative analysis confirmed that the complete FMDV genomic RNA was contained in exosomes after sequencing of each fragment. Taken together, the above-mentioned results showed that viral proteins(except L, 2 A, and 3B) and the whole FMDV genomic RNAs (except and UTRs) were included in exosomes, which were isolated from culture supernatants of FMDV-infected PK-15 cells.

3.5. Exosomes can transmit FMDV and establish productive infection in vitro and in vivo

To investigate the functional role of exosomes in the transmission of infection, exosomes were labeled by lipophilic green fluorescent dye (DiO), and it was added to PK-15 cells. After 8 and 16 h, nucleus was stained by, 6-diamidino-2-phenylindole (DAPI), and the uptake of the

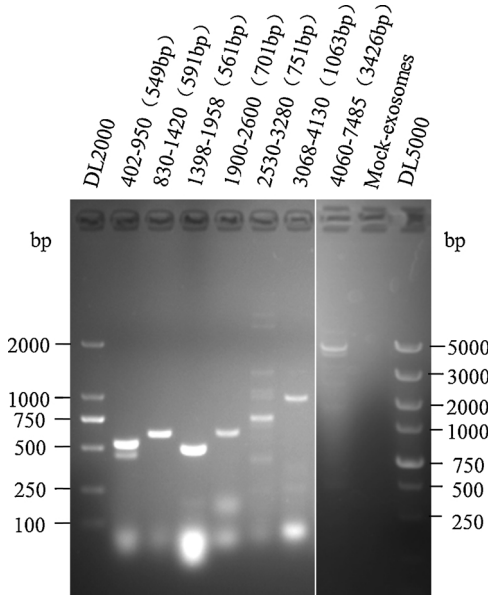

Fig. 4. FMDV genomic RNAs amplification (except and UTRs) in exosomes isolated from FMDV-infected PK-15 cells were detected by RT-PCR. Seven overlapping fragments were designed based on the genome sequence of FMDV strain A/GDMM/CHA/2013.

fluorescent-labelled exosomes by cells PK-15 was visualized with a fluorescence microscope. As shown in Fig. 5A, exosomes can be uptaken by PK-15 cells.

The exosomes purified from FMDV-infected cells were incubated with PK-15 and IBRS-2 cells, and indirect immunofluorescence assay with FMDV-specific antibodies was performed at 24 h.p.i. Cells inoculated with exosomes and isolated from mock-infected cell supernatants were used as the negative controls. The positive controls were set by cells infected through free FMDV with the same method. As shown in Fig. 5B, fluorescence specific to FMDV induced by FMDV-exosome in PK-15 and IBRS-2 cells was observed. To further prove that the FMDV derived from FMDV-exosome can replicate in PK-15 and IBRS-2 cells, quantitative (RT-qPCR) was carried out for FMDV at 24 hpi. The replication of FMDV derived from FMDV-exosome in host cells can be confirmed in Fig. 5C.

Suckling mouse is sensitive to FMDV and can be used for FMDV research (Basagoudanavar et al., 2018; Chen et al., 2004; Usharani et al., 2017). To further characterize FMDV RNA from FMDV-exosome in vivo, an equal copy number of FMDV-exosome or free FMDV was injected into C57BL/C suckling mice. Exosomes isolated from mock-infected cell supernatants were used as the negative controls. Positive controls were set by suckling mice injected with equal copy number free FMDV. Survival percent was calculated and FMDV copy number in mice was evaluated by RT-qPCR. As illustrated in Fig. 6A, mice death appeared at 36 hpi in FMDV-exosome group, and the final survival rate was only 20%. All the suckling mice died at 48 hpi in positive control group. In negative control group, 100% survival rate in mock-exosomes group was achieved (Fig. 6B and D), while only 20% survival rate in FMDV-exosomes group was obtained (Fig. 6A and D). RT-qPCR results confirmed that FMDV derived from FMDV-exosome can replicate in suckling mouse (Fig. 6C). In brief, these results confirmed that host cells can uptake exosomes, and exosomes are able to transmit FMDV and establish productive infection in vitro and in vivo.

3.6. Exosomes-mediated FMDV transmission infection is resistant to NAbs

Next, we investigated whether infection of exosome-mediated transmission was inhibited in the presence of NAbs. Serum FMDV serotype A was provided by the China Reference Laboratory Network for Foot-and-Mouth Disease. The titer of serum neutralization against FMDV was > 1:1024 determined by VNT. Immunoglobulins (IgGs) were purified and added to FMDV-exosome, MOCK-exosome, and free FMDV separately, after incubation for 1 h at 37 ℃, and then transferred into a monolayer of PK-15 cells. At 36 h.p.i, CPEs of PK-15 cells were observed and copy number of FMDV RNAs was determined by RT-qPCR. As shown in Fig. 7, expectedly, CPEs were not observed when free FMDV was treated with NAbs, indicating that FMDV infection was efficiently neutralized by purified NAbs (Fig. 7C). Typical CPEs were observed in FMDV-exosome group when treated with FMDV-specific NAbs (Fig. 7A).

To further elucidate whether FMDV contained in NAbs-treated FMDV-exosomes can replicate in host cells, RT-qPCR results showed that FMDV can replicate very well in FMDV-exosome group when treated with FMDV-specific NAbs (Fig. 7E). However, replication of FMDV could not be detected in free FMDV group (Fig. 7E). A conclusion can be made that exosome-mediated FMDV transmission is not blocked by FMDV NAbs.

4. Discussion

Several evidences showed that exosomes can establish vehicles for the shuttling of cellular proteins, microRNAs (miRNAs), and mRNAs (Fu et al., 2017), affecting many biological processes (Colombo et al., 2014; Kowal et al., 2014). The contents of exosomes secreted from virally infected cells and normal cells are different. Although exosomes and their contribution to replication and pathogenesis of viruses have

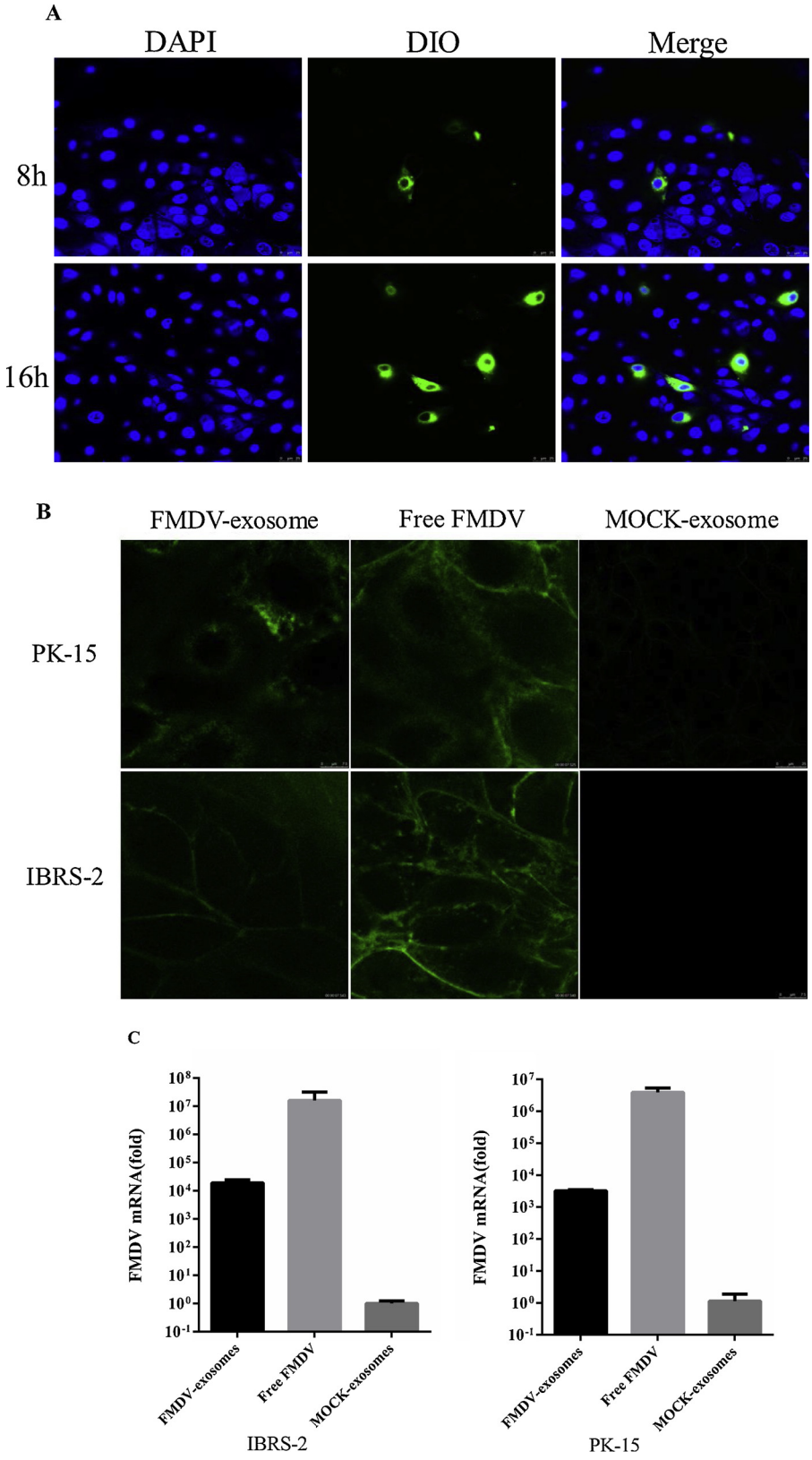

Fig. 5. Exosomes derived from PK-15 cell culture supernatants can entry into PK-15 and IBRS-2 cells and establish productive infection. (A) PK-15 cells were incubated with DiO labeled exosomes. After 8 and 16 h, nuclear acid was stained by DAPI, and the uptake of the DiOexosomes by cells PK-15 was visualized with a fluorescence microscope. DAPI, 6-diamidino-2-phenylindole; DiO, cell-Labeling Solutions, green fluorescence. Bar scale = 25 μm. (B) The exosome-mediated infection confirmed by indirect immunofluorescence assay in PK-15 and IBRS-2 cells induced by FMDV-exosomes were observed (left), there was no fluorescence in MOCK-exosomes group (right). Compared with positive control group(middle), the fluorescence was slightly lower in FMDV-exosomes group. (C) Replication of FMDV in PK-15 and IBRS-2 cells were detected with RT-qPCR after inoculation with FMDV-exosomes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

still remained unclear, numerous viruses have been researched, such as HTLV-1, EV71, HIV-1, HCV, Dengue virus, and PRRSV (Fu et al., 2017; Heikkila et al., 2016; Wang et al., 2018; Zhu et al., 2015a, b). In the current study, we demonstrated for the first time that exosomes derived from FMDV-infected cells can package the viral genomic RNA and proteins, thereby transferring productive infections in vitro and vivo, even in the presence of FMDV-specific NAbs.

There are different methods for isolation and purification of exosomes, such as classical differential UC (Thery et al., 2002), size exclusion chromatography (Lobb et al., 2015), total exosome isolation reagent (Alvarez, 2014), ultrafiltration (Merchant et al., 2010), microfluidic devices (Contreras-Naranjo et al., 2017), etc. However, UC is still

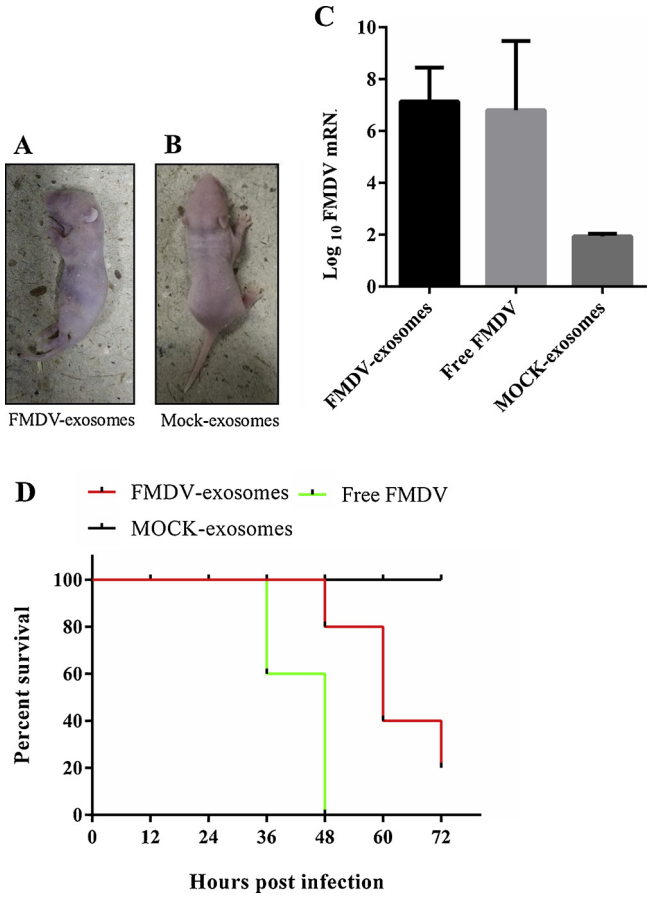

Fig. 6. Characterization of infectivity of FMDV-exosomes in vivo. (A) About 36 h after FMDV-exosomes subcutaneous injection into suckling mice, dying symptoms begin to appear. (B) All the mice in PBS negative control group were alive from beginning to the end. (C) RT-qPCR indicated that FMDV in FMDV-exosomes can replicate and induce high mortality in suckling mice. (D) The results of survival rate calculation showed that all the mice were dead 36 h after inoculated with free FMDV, and 100% survival rate was achieved in mock-exosomes group and PBS group, while only 20% survival rate was found in FMDV-exosomes group. In brief, exosomes can transmit FMDV and establish productive infection in vivo.

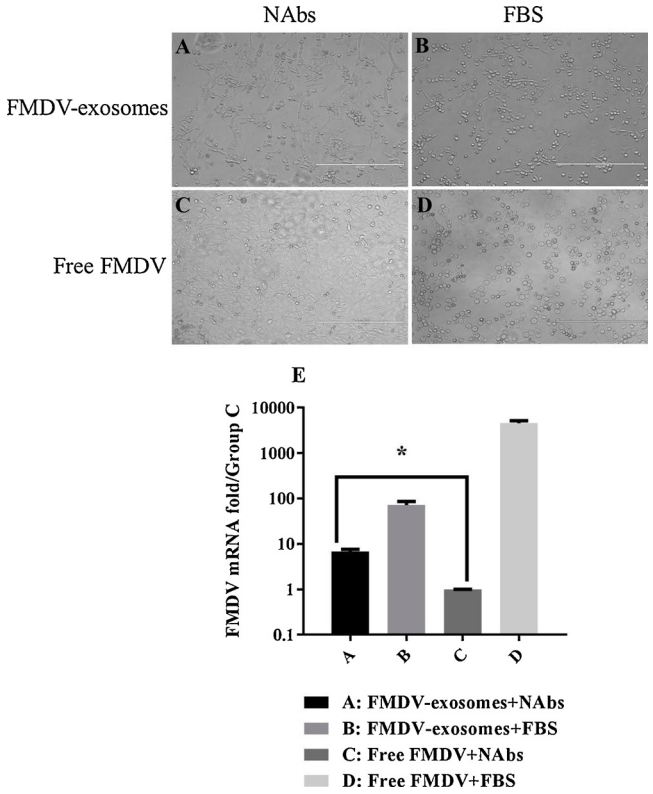

Fig. 7. Exosome-mediated transmission of FMDV was still established after treated with FMDV-specific NAbs. Purified FMDV-positive exosomes or free FMDV were incubated with FMDV-specific NAbs (the titer of serum neutralization against FMDV was > 1:1024 determined by VNT) and FBS for 1 h separately. Then, PK-15 cells were exposed to the NAbs/or FBS-treated exosomes or free FMDV for 2 h. The exosomes or viruses were washed off with 37°C PBS, and the medium was replaced with fresh maintenance medium for 24 h. qPCR was used to evaluate FMDV replication in PK-15 cells. (A) CPE was observed still be observed in FMDV-exosomes + NAbs group. (B) Typical CPE appeared when FMDV-exosomes treated with FBS. (C) No CPE in free FMDV + NAbs group. (D) CPE was observed when free FMDV treated with FBS. (E) FMDV in FMDV-exosomes can still replicate when treated with NAbs or FBS, FMDV can hardly replicate in free FMDV NAbs treated group.

considered as the gold standard for exosomes isolation from many biofluids, that is efficacious, while laborious (Markowska et al., 2017). Due to the similarity of FMDV and exosomes in their size, sedimentation velocities and buoyant densities were observed as well (Valadi et al., 2007). Thus, how to isolate pure exosomes free of contaminating viruses from virus-infected cells is still a main challenge. At present, CD63 or composite magnetic bead purification is the best method to completely separate exosomes and viruses (Raab-Traub and Dittmer, 2017). Thus, in this study, exosomes were isolated and purified with the methods of CD63 immunomagnetic bead affinity after UC, thereby ensuring that the FMDV RNA was intra-exosomal. The results of infectivity of exosomes isolated from FMDV-infected cells were not blocked by FMDV-specific NAbs, which confirmed our expectations.

CD63 is regarded as a major vesicular tetraspanin protein increased in these EVs, and it can regulate EBV LMP1 exosomal packaging, as well as enhancement of vesicle production, and non-canonical nuclear factor-κB (NF-κB) pathway (Hurwitz et al., 2017). Because CD63 expression is positively correlated with exosomes release, CD63-luciferase fusion protein, which stably expressed in Hela and HT-29 cell lines, was used to quantify the exosome secretion (Fu et al., 2017). In the present study, CD63-GFP stable expression PK-15 cell line was generated and used. As shown in Fig. 2, the green fluorescence significantly increased in Lv-CD63-PK-15 cells after FMDV infection. Thus, it can be concluded that FMDV infection can enhance exosomes secretion in PK-15 cells.

The up-take of exosomes by host cells is the key and the first step for its function. In our research, exosomes derived from FMDV-infected PK-15 cells can be up-taken by normal PK-15 cells, which was confirmed after exosomes labeled by DiO (Fig. 5). In addition, /MS analysis of FMDV-infected exosomes showed that 1133 host proteins were identified, including marker proteins of exosomes CD63 and CD9. GO and KEGG analysis showed that proteins associated with positive/negative regulation of biological process and metabolic process (Fig. 3A), belonged to PI3K-akt signal pathway, MAPK pathway, and cancer-related pathways, were explicitly based on KEGG analysis (Fig. 3B). Identified proteins are ranked in binding and catalytic activity, in addition to classified cell membrane or membrane-related proteins (Fig. 3A). The complete FMDV genome RNA (except and UTRs) packaged in FMDV-exosomes was confirmed by RT-PCR and sequence analysis. Most proteins encoded by FMDV were identified by /MS. Unfortunately, viral proteins could not be detected in FMDV-exosomes by WB and immune-electron microscopy (data were not shown). The reason for this result was method of /MS maybe more sensitive than WB and immune-electorn microscopy.

To date, ever-increasing researches suggested that the released exosomes contain viral genomes, viral particles, miRNAs, host proteins, or mRNAs play important roles in viral transmission, pathogenesis, and replication. For example, exosome-mediated infection was not fully

blocked by PRRSV-specific neutralizing antibodies (Wang et al., 2018). NAbs for HCV transmission by exosomes as a potential immune-evasion mechanism (Ramakrishnaiah et al., 2013). Transfer suppresses type I interferon response by exosome-mediated miR-146a facilitates EV71 infection (Fu et al., 2017). Glycoprotein B of HSV-1 diverts HLA-DR into exosome pathway (Temme et al., 2010). Intercellular communication mediated by exosome can activate hepatic stellate cells (HSCs) for liver fibrosis in HCV infection (Cho et al., 2018; Devhare et al., 2017). Exosome-like vesicles provide protection against NAbs (Ramakrishnaiah and van der Laan, 2014), and exosome-associated HCV as an alternative for HCV infection and transmission (Liu et al., 2014). Similar to HCV and PRRSV, we demonstrated that exosome-mediated transmission of FMDV also takes advantage of the exosomal pathway to transfer virus and establish productive infections (Fig. 5). Further, in vivo study indicated that RNA of FMDV in FMDV-exosomes can replicate and lethal in suckling mice (Fig. 6).

Previous researches demonstrated that exosome-mediated transmission of HAV, HCV, EV71, and PRRSV cannot be fully blocked by NAbs (Cosset and Dreux, 2014; Feng et al., 2013; Fu et al., 2017; Mao et al., 2016; Ramakrishnaiah et al., 2013; Wang et al., 2018). Surprisingly, IgGs from patients enhanced the transmission of exosomes isolated from HCV sub-genomic replicon cells (Cosset and Dreux, 2014). The above-mentioned evidences suggested that viral exosomes can escape the humoral immune response. In the present research, it was demonstrated that exosomes-mediated FMDV transmission infection is not blocked by NAbs, in which the results remind us that FMDV-exosomes may establish vehicles for the shuttling infectivity that can escape the humoral immune response. FMDV can be detected and isolated from persistent infected animals in the presence of FMDV high specific antibody (Farooq et al., 2018). Whether exosomes contribute to this phenomenon or not is a main challenge that is worth being researched.

It is well-known that viral RNA, virions, viral proteins, viral or host miRNAs may be contained in exosomes isolated from virus-infected, which can increase the infectivity of viral exosomes or/and regulate host immune responses. Analysis of changes of exosome protein content induced by FMDV infection in host cells, and whether FMDV-exosomes contain viral or/and miRNAs, and also whether these miRNAs contribute to the exosome-mediated transmission of FMDV need to be investigated in the future studies.

In conclusion, our study demonstrated that exosomes derived from FMDV-infected PK-15 cells contained FMDV RNAs and most viral proteins, thus they can establish productive infection in vitro and in vivo. Furthermore, we showed that the exosome-mediated FMDV infection transmission is not blocked by NAbs. Taken together, these data provide a novel and advanced information for better understanding that viral transmission through exosomes contributes to the known immune evasive properties of FMDV.

Conflict of interests

The authors declare they have no competing interests.

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