Cell Surface Vimentin is an Attachment Receptor for Enterovirus 71

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Abstract
Enterovirus 71 (EV71) is a highly transmissible pathogenic agent that causes severe central nervous system diseases in infected infants and young children. Here, we reported that EV71 VP1 protein could bind to vimentin intermediate filaments expressed on host cell surface. Soluble vimentin or an antibody against vimentin could inhibit the binding of EV71 to host cells. Accompanied with the reduction of vimentin expression on cell surface, the binding of EV71 to cells was remarkably decreased. Further evidence showed that the N terminal of vimentin is responsible for the interaction between EV71 and vimentin. These results indicated that vimentin on host cell surface may serve as an attachment site that mediated the initial binding and subsequently increased the infectivity of EV71.

Importance
This study delivers important findings on the roles of vimentin filaments in relation to EV71 infection and provides information that not only improved our understanding of EV71 pathogenesis, but also presents us with potentially new strategies for the treatment of diseases caused by EV71 infections.
**Introduction**

Enterovirus 71 (EV71) is a single-stranded RNA virus that belongs to the human enterovirus species A of the genus Enterovirus within the Picornaviridae family. EV71 was thought to be one of the main pathogenic agents that cause foot, hand and mouth disease (HFMD) in young children [1-4]. In recent years, outbreaks of EV71-related HFMD have been reported in Southeast or East Asia, including Taiwan, Malaysia, Singapore, Japan and China [5-7]. Particularly, since 2008 million EV71-related HFMD cases were reported each year in China, including hundreds of fatal cases per year. Because of its danger and high frequency of infection, EV71-related HFMD has raised considerable public health concerns [8]. However, available treatments for EV71 infection are limited as there is currently no effective chemoprophylaxis or vaccination against infection.

Unlike CA16 and other enteroviruses, EV71 infection is usually accompanied by severe neurological complications such as aseptic meningitis, acute flaccid paralysis, encephalitis and other rarer manifestations [9, 10, 2]. The EV71-associated neurological complications can sometimes be fatal and neurogenic pulmonary edema is thought to be the main pathogenic cause in fatal cases [11-13]. It has been postulated that overwhelming virus replication in combination with tissue damage, and the induction of toxic inflammatory cytokines and cellular immunity are the possible process of pathogenesis [14, 15]. Although the initial viral illness often is self-limited, EV71 infection may result in long term neurologic and psychiatric effects.
on central nervous system (CNS) in children [16]. EV71 infection involving the CNS, and cardiopulmonary failure may be associated with neurologic sequelae, delayed neurodevelopment and reduced cognitive functioning [16, 17, 10].

As a non-enveloped virus, EV71 enters host cells via a receptor-mediated clathrin-dependent endocytotic pathway [18]. Several kinds of cell receptors for EV71 have been identified. Human P-selectin glycoprotein ligand-1 (PSGL-1) and scavenger receptor B2 (SCARB2) are two functional receptors believed to determine EV71 host range and tissue tropism [19, 20]. PSGL-1 is a sialomucin membrane protein expressed on leukocytes which have a major role in the early stages of inflammation [21-23]. The tyrosine sulfation at the N terminal region of PSGL-1 has been proven to interact with EV71 and thus may facilitate virus entry [24]. Human SCARB2, the second reported cell receptor for EV71, belongs to the CD36 family [25, 26]. SCARB2 is one of the most abundant proteins in the lysosomal membrane and participates in membrane transport and the reorganization of the endosomal and lysosomal compartment [27]. PSGL-1 is expressed mainly on neutrophils, monocytes and most lymphocytes, while SCARB2 is widely expressed on most types of cells including neurons [19, 20]. Amino acids 144 to 151 of SCARB2 have been proven to be critical for binding to EV71 VP1 [28]. Thus, SCARB2 is believed to be directly involved in EV71 infection of the brain. In addition, SCARB2 can be utilized by most EV71 strains as an entry receptor, while PSGL-1 can only mediate infection by certain strains. More EV71 virus binds to L-PSGL-1 cells (mouse L cells that expressed
human PSGL1) than to L-SCARB2 (mouse L cells that expressed human SCARB2) cells due to a higher affinity of PSGL1 for the virus. However, EV71 could infect L-SCARB2 cells more efficiently than L-PSGL-1 cells [29, 30]. SCARB2 is capable of virus binding, virus internalization and virus uncoating, while PSGL1 is only capable of virus binding [30]. Thus, PSGL-1 may act as a binding receptor but not an uncoating receptor for EV71. Other receptors, such as sialylated glycan and annexin II, have also been shown to facilitate EV71 infection in various kinds of cells, and cell surface heparan sulfate glycosaminoglycan was recently reported to be an attachment receptor for EV71 in RD cells [31, 32]; however, as inhibition of these receptors by antagonists did not completely abolish EV71 infection, it was suggested that multiple receptors are involved during EV71 infection. Vimentin is the major intermediate filament protein of astrocyte cells and cells adapted to tissue culture [33]. An increasing number of reports recognized an important role for cell surface vimentin as a component of the pathogen attachment and endocytotic pathways [34-39]. In this study, we further demonstrate that EV71 VP1 specifically binds to vimentin intermediate filament located on the cell surface and that the vimentin expressed on the cell surface is involved in the attachment of EV71 to host cells.
Materials and Methods

Reagents and antibodies

N-decyl-D-maltopyranoside (DDM) was obtained from Affymetrix, CA, USA. Protease inhibitor cocktail and anti-SCARB2 antibody were obtained from Sigma Aldrich, MO, USA. Anti-EV71 monoclonal antibody was obtained from Millipore, MA, USA. Anti-PSGL-1 antibody was obtained from R&D system, MN, USA. FuGENE transfection reagent was obtained from Roche, IN, USA. Rabbit anti-vimentin polyclonal antibody, mouse anti-vimentin, mouse anti-β-actin, mouse anti-GST, mouse anti-Flag monoclonal antibody, rhodamine (TRITC)-conjugated anti-rabbit IgG antibody, FITC conjugated anti-rabbit IgG antibody, FITC conjugated anti-mouse IgG, HRP conjugated anti-mouse IgG and HRP conjugated anti-rabbit IgG used in immunofluorescence studies, infection inhibition assays and western blots were all obtained from Santa Cruz Biotechnology, CA, USA. PSGL-1 and SCARB2 protein used in infection inhibition assays were obtained from Sino Biological, Beijing, China.

Cell culture, virus isolates and virus infection

The prototype Enterovirus 71 (EV71) BrCr strain was a gift from Prof. Qi Jin (Institute of Pathogen Biology, Chinese Academy of Medical Sciences, Beijing, P. R. China). The prototype EV71 clinical strains Hunan 09 and HeN 09 were gifts from Prof. Zhang Bo (Wuhan Institute of virology, Chinese Academy Of Sciences, Wuhan, P. R. China). The human astrocytoma (U251) cell lines, human rhabdomyosarcoma (RD) cells, African green monkey kidney epithelial (Vero) cells, human cervical (HeLa) cells and human T lymphocyte (Jurkat) cells and mouse embryonic fibroblast (3T3) cells were propagated and maintained in either Double Modified Eagle’s Medium
(DMEM), Modified Eagle’s Medium (MEM) or 1640 medium, all supplemented with antibiotics
(penicillin and streptomycin) and 10% fetal bovine serum (Invitrogen, CA, USA), at 37°C in the
presence of 5% CO₂. In all experiments, cells (other than 3T3 cells) were infected with the
respective virus at a multiplicity of infection (MOI) of 4 PFU cell⁻¹. The 3T3 cells were infected at
a MOI of 20.

Plasmids and protein expression

To create the expression vectors expressing either EV71 VP1, VP2, VP3 or 3C, EV71 genomic
RNA was extracted from the culture fluid of virus infected RD cells using a virus genome
extraction kit. Single-stranded cDNA was then synthesized from the purified virus RNA by
reverse transcription (RT) (Promega). Each of the VP1, VP2, VP3 and 3C genes was amplified
from the cDNA by PCR over 34 cycles of denaturation at 98 °C for 10 sec, primer annealing at 55
°C for 30 sec and extension at 72 °C for 1.5 min, using VP1 sense primer 5’-
CGCGATCCGACAGAGTGGCAGATGTGATTG-3’ and anti-sense primer 5’-
CCGGAATTCTAGAGCGTAGTGATTGCCGTTC-3’; VP2 sense primer
5’-CCCAAGCTTTTCTCCCTCTGCTGAAGCATGTGGC-3’ and anti-sense primer 5’-
5’-CCCAAGCTTTTACTGCCTACTGCGGGCTTATGAC-3’; VP3 sense primer
5’-CCCAAGCTTGGTTTCCCCACTGAATTGAA-3’ and anti-sense primer
5’-ACGGCGCTCGACTATTAGATGTCGGCTTGC-3’; 3C sense primer 5’-CGCGGATCC
CCCAGCTTAGACTTCGCTTC-3’ and anti-sense primer 5’-CCGGAATTCTTTAT
CTGCTTATGTC-3’. Each of the PCR products was separated by
electrophoresis in 1.0% agarose gels, gel purified and then cloned into the corresponding
restriction sites of the pcDNA-Flag vector to produce plasmids expressing VP1-Flag, VP2-Flag, VP3-Flag and VP3C-Flag, respectively.

To create expression vectors expressing either vimentin or 3C, the full length VP1 and 3C cDNAs were generated by PCR using the VP1 sense primer 5'-CGGAATTCTCCACCAGGTCCGTGTCCTCG -3' and anti-sense primer 5'-CGGCGGCCGCTCTTCAAGGTCATCGTGATGTG-3'; 3C sense primer 5'-CGGAATTCCCAGCTTAGACTTCGCCTTGTCT-3' and anti-sense primer 5'-GCGGCCGCTTTGCTCGCTGGCAAAATAACTCCT-3'. The PCR products were separately cloned into the pGEX-4T-1 expression vector. Full length human and mouse vimentin cDNA, and the truncated human vimentin cDNA sequences were generated by PCR using these primers:

vimentin (1-230) sense primer: CGGGATCCGTCCACCAGGTCCGTGTCCTCG-3' and anti-sense primer 5'-CCCAAGCTTCTCTTCTTGCAAAGATTCCAC-3'; vimentin (231-467) sense primer: 5'-CCCAAGCTTTTCAAGGTCATCGTGATGCTG-3' and anti-sense primer 5'-CCCAAGCTTGGCGAAGCGGTCATTCAGCTC-3'; vimentin (116-230) sense primer: CGGGATCCGAACTACATCGACAAGGTGCGC-3' and anti-sense primer 5'-CCCAAGCTTCGGAGGCGTAGAGGCTGCGGCT-3'; vimentin (1-56) sense primer: CGGGATCCGTCCACCAGGTCCGTGTCCTCG-3' and anti-sense primer 5'-GGGGATCCGTCCCCGGCGGCGTGTATGCC-3'. The respective PCR products were cloned into the pET20b vector and expressed in E. coli BL21. Solubilizing and refolding of the recombinant proteins in the inclusion body was performed as described before [40-42]. The recombinant proteins were purified using affinity chromatography (Ni-NTA or Glutathione Sepharose; GE) followed by
In order to elucidate potential roles for vimentin in EV71 infection, a vimentin knockdown cell line (VK-U251) was constructed using a retrovirus vector that stably expressed the siRNA specific to vimentin. To build VK-U251, a shRNA targeting the human vimentin gene (shVim) as reported previously and a control shRNA (shControl) were designed [43]. In order to facilitate the formation and processing of the shRNA, a loop sequence (TTCAAGAGAGA) was designed in the middle area of all shRNAs. The sequences of the two shRNAs were: shVim, 5’-GATCCGCTATTGACCACATCCACTTCAAGAGAGTGGATGTGGTCACATAGCTTTTTTG-3’; and shControl, 5’-GATCCCCACCATGCATGTCATTCAAGAGATGACATACGTGCATGGTGTTTTTG-3’. The corresponding complementary oligonucleotides were also synthesized to produce DNA duplexes of each of the shRNAs. The shRNA oligonucleotides were annealed and ligated to the BamHI and EcoRI sites behind the human U6 promoter of pSIREN-RetroQ vector, and the constructs were confirmed by DNA sequencing. Phoenix cells were plated and transfected with the resultant pSIREN-RetroQ-siVim or pSIREN-RetroQ-siControl plasmid in the presence of a helper plasmid by using the FuGENE transfection reagent. At 48h post transfection, cell culture fluid containing the recombinant retroviruses were harvested and used to infect U251 cells. The infected cells were screen using puromycin at a concentration of 5µg ml⁻¹. Vimentin expression in VK-U251 and control cells (C-U251) was analyzed by western blot analysis. To decrease vimentin expression in mouse 3T3 cells, the cells were transfected with siRNA control or siRNA specific to mouse vimentin [44]. The expression levels of vimentin were determined by size-exclusion chromatography (Superdex 200, GE healthcare).
western blot analysis.

Virus propagation, purification and titer determination

Virus was propagated and purified as described before [45]. To determine virus titers, RD cells were plated into 96 well dishes, incubated overnight, and then infected with the serially diluted virus. After adsorption for 30min, the virus suspension was replaced with Modified Eagle's Medium containing 2% fetal bovine serum. The cultures were incubated at 37°C for 5 days and plates that displayed cytopathic effects were counted. Virus titers were determined by Reed-Muench method. All virus titers data presented are mean ±SD based on three independent experiments.

Western blot, pull down assay and co-immunoprecipitation

Western blots and pull down assays were performed as described before [46]. For co-immunoprecipitation, cell lysates were prepared and pre-immunoprecipitated with protein G-agarose beads. After a short centrifugation, the precipitates were either incubated with mouse IgG-conjugated agarose beads, EV71-conjugate d agarose beads or vimentin monoclonal antibody-conjugated agarose beads. After 2 h of incubation at 4°C, the beads were washed with lysis buffer and heat-denatured in sample-loading buffer (50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue and 10% glycerol). After a brief centrifugation, the proteins in supernatants were separated by SDS-PAGE followed by western blot analysis. The blots were developed using the chemiluminescence reaction (GE Healthcare).
Quantitative RT-PCR, indirect immunofluorescence and flow cytometry

Quantitative RT-PCR and indirect immunofluorescence analysis were performed as described before [47, 48]. For flow cytometry, cells were harvested at the indicated times and fixed in 0.01% formaldehyde for 10-15 minutes at room temperature. After three washes in PBS, the cells were permeabilized with 0.1% NP40 in PBS for 20 min. Cells were blocked with 5% BSA in PBS and incubated for 1h at room temperature. After washing, the cells were resuspended in PBS with 1% BSA and incubated for 2h at 4°C with the primary antibody at a final concentration of about 1μg ml⁻¹. Cells were then incubated for 1h with the fluorescent secondary antibody. The cells were then subjected to flow cytometry analysis after they had been washed with PBS. For cell surface staining, the cells were not permeabilized but the other steps remained the same.

Virus binding inhibition assay

To examine the effect of soluble vimentin on virus binding to host cells during infection, we mixed EV71 with either vimentin or BSA and incubated them for 60 min at 4°C prior to infection. The cells were then incubated with the above treated EV71 at 4°C for 1 h, washed three times with culture medium and subjected to flow cytometry or western blot analysis.

To examine the inhibition effects of pretreating host cells with specific antibodies on virus binding, the cells were preincubated with polyclonal antibody against either vimentin, SCARB2, PSGL-1 or rabbit IgG for 60min at 4°C before being incubated with EV71 for 1 h at 4°C. The cells were
then subjected to flow cytometry or western blot analysis as mentioned above.

To study the effects of vimentin and vimentin antibody on virus replication, cells were incubated with EV71 at 4°C for 1 h in the presence of either vimentin or vimentin antibody. Then, fresh medium was added to the infected cells and infection was allowed to proceed for the indicated times at 37°C. Virus replication of the infected cells was determined by measuring the virus titers in the culture fluids and cell lysates.

**Statistical analysis**

Data were subjected to one-way analysis of variance with factors of treatment and expressed as means ±SD. Comparisons between any two groups were performed by unpaired student’s t-tests. **Significant difference at p < 0.02 compared with control. *Significant difference at p < 0.05 compared with control.**
Results

To investigate whether EV71 can interact directly with vimentin, we incubated U251 cell lysates with EV71 strain BrCr particles immobilized on agarose beads conjugated with anti-EV71 VP1 monoclonal antibody and analyzed the precipitated proteins by western blot. The results (Fig. 1A) showed that a 55KD band corresponding to vimentin was detected by a vimentin monoclonal antibody, whereas no vimentin was detected in the IgG and control groups. This interaction between EV71 and vimentin was further verified by a co-immunoprecipitation experiment which showed that EV71 co-immunoprecipitated with vimentin (Fig. 1B). Neither EV71 nor vimentin was detected in the IgG control group. These results thus indicated that EV71 can interact directly with vimentin. To determine whether the interaction with vimentin was specific to the test virus strain, or is a feature common to other circulating EV71 strains in general, we tested two other EV71 clinical isolates for their interaction with vimentin by co-immunoprecipitation as described above. The results showed that both of these strains which belong to the C4 genotype of EV71, were captured by vimentin and migrated at the appropriate size position in a western blot probed with the anti-EV71 antibody (Fig. 1C). This interaction with vimentin was shown to be specific for EV71 as a parallel co-immunoprecipitation experiment with Coxsackievirus A16 (CA16), another common pathogen of HFMD belonging to the same species of human enterovirus A, failed to capture the CA16 virus (Fig. 1C).

To determine if vimentin could act as an attachment receptor for EV71 entry, we
first investigated the cell surface expression of vimentin by flow cytometry and immunofluorescence studies. The cytometry results (Fig. 2A) showed that there were strong immunofluorescence signals in the EV71 permissive U251, Vero, RD and Hela cells when stained with the vimentin antibody, but not in the corresponding cells stained with mouse IgG, indicating that vimentin was expressed on the surface of these cells. However, the amounts of cell surface vimentin in RD and Vero cells were greater than those in U251 and HeLa cells. Additionally, immunofluorescence studies of non-permeabilized U251 cells stained with vimentin monoclonal antibody demonstrated that some vimentin was located on the surface of these cells (Fig. 2B). The results showed that when EV71 was incubated with non-permeabilized cells, the rhodamine-labeled EV71 was found localized on the cell surface and was colocalized with the cell surface vimentin stained with FITC (Fig. 2B). In contrast, when U251 cells were stained with vimentin antibody in the permeabilized states, the vimentin filaments were seen to be distributed throughout the cytoplasm (Fig. 2C).

We next determined whether EV71 binds to vimentin through its VP1 protein, the most external, surface accessible and immuno-dominant protein that is believed to be responsible for host-virus binding. A pull-down assay was initially performed to analyse the interaction between VP1 and vimentin. As shown in figure 3A, a band representing vimentin could be detected in the eluate of cell lysates from the VP1 binding column. In contrast, no such protein band was observed in eluate from the control resins. To determine whether EV71 VP1 could directly bind with vimentin, pull
down assays were performed by incubating VP1 with vimentin protein immobilized on anti-vimentin monoclonal antibody-conjugated agarose beads. The results showed the presence of VP1-GST protein band in the vimentin protein pulled down eluate but not in control GST eluate (Fig. 3B). The capsid of EV71 consists of 60 subunits comprising the four capsid proteins, VP1, VP2, VP3 and VP4. VP1, VP2 and VP3 are exposed on the virion surface and believed to mediate cell receptor binding or endocytosis [49]. To investigate which capsid protein mediates the interaction between the EV71 particles and vimentin, we transfected U251 cells with plasmids expressing either VP1-Flag, VP2-Flag or VP3-Flag. Co-immunoprecipitation experiments performed on these cells followed by western blot analysis using anti-vimentin and anti-Flag antibodies showed that vimentin could be precipitated by VP1-Flag, but not VP2-Flag nor VP3-Flag (Fig. 3C, 3D). Together, these studies provided strong evidence that EV71 VP1 could bind directly and specifically to vimentin and that the EV71 VP1 mediated the interactions between the EV71 particle and cell surface vimentin.

An assay was done to investigate if the addition of exogenous vimentin would competitively inhibit the binding of the virus to host cells and thus virus infection. EV71 was preincubated with either vimentin (0, 5, 10, 20, 30 and 40 μg ml⁻¹) or BSA (40 μg ml⁻¹) for 60 min at 4°C before being used to infect U251 cells (MOI of 4). At 1 h postinfection, samples of the cells were washed three times with culture medium and analysed for bound virus particles by western blot and flow cytometry. The blotting results (Fig. 4A) showed that preincubation with exogenous vimentin reduced
the binding of EV71 particles to the cells when compared to preincubation with BSA. This inhibition effect was specific to EV71 as exogenous vimentin failed to block the binding of CA16 to U251 permissive cells (Fig. 4A). Analysis by flow cytometry showed that preincubation with vimentin at a concentration of 10 µg ml⁻¹ dramatically reduced the amount of EV71 binding on U251 cell surface while preincubation with BSA had no effect (Fig. 4B). The binding of EV71 to U251 cells was also analyzed by using quantitative PCR, and the results showed that the EV71 RNA levels observed were inversely proportional to the concentrations of soluble vimentin added. However, the reduction of EV71 RNA levels was not observed when the concentration of vimentin was increased from 10 µg ml⁻¹ to 20 µg ml⁻¹ (Fig. 4C). Similarly, the virus yields in both the culture medium and infected cells were significantly reduced and the levels of reduction were proportional to the concentrations of vimentin added (Fig. 4D). Moreover, the lower virus yields in the vimentin pretreated cells were associated with an attenuation of the typically severe cytopathic effect (CPE) of EV71 seen in the infected untreated control or the BSA pretreated cells. These results indicated that preincubation with exogenous vimentin competitively inhibited EV71 infection (Fig. 4E). To further understand the effect of exogenous vimentin on EV71 infection, the binding of EV71 was measured by quantitative PCR in U251 cells treated with vimentin at a concentration of 20µg ml⁻¹ at various times after the initiation of infection. The results showed that the binding of EV71 to the cells decreased proportionately with the increased time delay in treatment with vimentin postinfection such that treatment at 30min postinfection had no
significant effect on EV71 binding (Fig. 4F). These results indicated that the inhibitory effect of exogenous vimentin was at the binding stage of EV71 to U251 cells.

The question of whether vimentin also participates in the binding of EV71 in the infection of HeLa, Vero and RD cells, the most well-known permissive cell lines for EV71 infection and replication, was investigated. Virus column were preincubated with either vimentin (20 μg ml⁻¹) or BSA (20 μg ml⁻¹) for 60 min at 4°C before being used to infect U251 cells and the amounts of virus particles bound to the cells were determined at 1 h postinfection by western blot and quantitative PCR. The results showed that preincubation with vimentin also inhibited the binding of EV71 to HeLa, Vero and RD cells as reflected in the reduction of both the EV71 protein fluorescence signals as well as EV71 RNA levels in infected cells pretreated with vimentin compared to those pretreated with BSA (Fig. 5A, 5B). Moreover, the inhibition effect of vimentin observed in these cell types was collaborated by a reduction in virus yield when they were infected with vimentin pretreated EV71 (Fig. 5C). However, the levels of reduction in EV71 binding and virus yields differed between the cell types. The decrease in EV71 binding and virus yield was greater in RD and Vero cells than in HeLa cells (Fig. 5B, 5C).

To provide further evidence for the participation of cell surface vimentin in EV71 infection, U251 cells were preincubated with either a polyclonal antibody to vimentin...
(0, 20, 40, 60 and 80 μg ml⁻¹) or the isotype IgG (80 μg ml⁻¹) for 45 min before being infected with EV71. After infecting for 1 h at 4°C, samples of the cells were subjected to western blot analysis. The blotting results (Fig 6A) indicated a partial inhibition of EV71 binding as reflected by a decrease in VP1 proteins observed in cells preincubated with vimentin antibody, while no change was detected in the corresponding rabbit IgG preincubated cells. In addition, the lower virus yield obtained in the vimentin antibody pretreated cells was associated with an attenuation of the typical CPE observed in EV71 infected untreated control or the isotype antibody pretreated cells (Fig 6B). The binding of EV71 to U251 cells was also measured by quantitative PCR, and the results indicated a partial inhibition of EV71 binding as reflected by a decrease in EV71 RNA levels (Fig 6C). However, preincubating cells with vimentin antibody at a concentration of 60 μg ml⁻¹ did not decrease EV71 binding any further (Fig 6C). At 24 h postinfection at 37°C, the vimentin antibody pretreated U251 cells also suggested a reduction in virus replication compared to the IgG control, as demonstrated by a decrease in virus titers in the infected cell culture medium and lysates (Fig 6D). To determine the effect of vimentin antibody pretreatment of cells on infection by other EV71 strains, U251 cells were pretreated with vimentin antibody as described above and then infected with EV71 Hunan 09 and HeN 09 for 1 h at 0°C. Analysis of cell surface bound EV71 in these cells by quantitative PCR indicated a decrease in the amounts of EV71 Hunan 09 and HeN 09 virus binding to the U251 cells, implicating that vimentin was also involved in binding of other strains of EV71 to host cells (Fig 6E). However, the
inhibition effect of vimentin on the binding of EV71 Hunan 09 and BrCr strains to U251 cells appeared stronger than that in HeN 09 strain.

A vimentin knockdown cell line (VK-U251) with little vimentin expression was built using retrovirus-based RNAi vectors and was employed to verify the requirement of cell surface vimentin for the binding of EV71 to U251 cells. Flow cytometry analysis of vimentin expression on the cell surface of the knockdown line showed that the vimentin on the VK-U251 cell surface was present at a very lower level compared to C-U251 and U251 cells (Fig 7A). To analyze the effect of this decrease in vimentin expression on the binding of EV71 to the cell surface, VK-U251 and C-U251 cells were incubated with EV71 and subjected to flow cytometry 30 min later. The results (Fig 7B) showed that a reduction in vimentin expression was accompanied by a dramatic decrease in virus binding in the VK-U251 cells when compared to C-U251 and U251 cells. The same result was obtained when the amounts of virus binding to the cells was assessed by western blot and quantitative PCR analysis (Fig 7C, 7D). As expected, inhibition of EV71 binding by vimentin antibody could not be detected in VK-U251 cells as indicated by no reduction of EV71 RNA after vimentin antibody treatment (Fig 7C). In addition, the replication of EV71 in VK-U251 cells was also reduced as shown by the presence of a relatively lower virus yield and an attenuation of the typical CPE found in cells with normal vimentin expression (Fig 7E, 7F).

To investigate the inter-relationships between vimentin and two previously identified
EV71 cellular receptors, SCARB2 and PSGL-1, we first examined the cell surface
SCARB2 and PSGL-1 in U251, HeLa, RD, Vero and Jurkat cells. Flow cytometry
analysis showed that U251, HeLa, RD and Vero cells all expressed large amounts of
SCARB2 on their cell surface (Fig 8A), whereas only a small amount of SCARB2
was detected on the cell surface of Jurkat cells (Fig 8A). Interestingly, no PSGL-1
could be detected on the U251, Vero and HeLa cell surface (Fig 8A). Conversely,
Jurkat cells expressed large amounts of PSGL-1 on its cell surface (Fig 8A). Further
evidence suggested that SCARB2 might have relatively less influence on the binding
of EV71 to U251 and VK-U251 cells, as only a small reduction of virus RNA levels
was obtained from the cell surface of cells preincubated with SCARB2 antibody
compared to that observed in cells preincubated with vimentin antibody (Fig 8B).
Moreover, SCARB2 antibody also had no influence on the blocking effect of
vimentin antibody on EV71 binding in both U251 and VK-U251 cells (Fig 8C).
Strangely, an obvious decrease in virus yield was detected in U251 and VK-U251
cells pretreated with SCARB2 antibody (Fig 8D, E), suggesting that SCARB2 may
not be responsible for the initial binding between virus and host cells. In addition,
pretreating cells with vimentin antibody did not affect the inhibition effect of
SCARB2 antibody on EV71 binding. Pretreating cells with SCARB2 antibody also
did not affect the inhibition effect of vimentin antibody on EV71 binding. These
results indicated that EV71 binds to vimentin and SCARB2 at different sites.
Competitive inhibition assays with exogenous SCARB2 and vimentin were also done
to investigate the effect of SCARB2 on EV71 and vimentin binding. EV71 particles were preincubated with vimentin, SCARB2 or BSA before being used to infect U251 cells. At 1 h postinfection at 0°C, samples of the cells were washed three times with culture medium and analysed for bound virus particles by quantitative PCR. The results showed that there was a remarkable change (about 32% reduction) in the binding of EV71 in the vimentin pretreated group compared to those of BSA or untreated groups (Fig 9A). However, pretreating the virus with SCARB2 had only a limited effect on EV71 binding as reflected by no significant reduction of EV71 protein signals in this group (Fig 9A). Analysis of EV71 replication in these samples at 24h postinfection revealed that pretreatment of virus with SCARB2 resulted in a dramatic decrease of virus replication (Fig 9B); however, the reduction in virus replication was less in the vimentin preincubated group than in the SCARB2 treated group (Fig 9B).

To determine whether the phenomenon observed above was a unique feature of U251 cells or a feature common to other EV71 permissive cells, we investigated the inhibition of EV71 binding by SCARB2 and vimentin antibody in HeLa, Vero and RD cells. These cell types were incubated with SCARB2 and vimentin antibody and the binding of EV71 were analyzed by using quantitative PCR as described above. The results showed a dramatic reduction of EV71 RNA in the vimentin antibody treated HeLa, Vero and RD cells (Fig 10A) while, as in the U251 cells, only a small amount of inhibition of EV71 binding was detected in the corresponding SCARB2 antibody
treated cells (Fig 10A). In addition, an obvious decrease in virus yield was detected in
the SCARB2 antibody treated U251 cells (Fig 10A). Pretreating cells with vimentin
antibody also resulted in a reduction of virus yield in HeLa, Vero and RD cells as well
as U251 (Fig 10B). Unexpectedly, the decrease in virus yield in the vimentin antibody
treated cells was less than that in the SCARB2 antibody treated cells, implicating that
the decrease in virus yield was not directly related to the reduction of virus binding. The
effect of SCARB2 and vimentin antibody on the binding of EV71 Hunan 09 and HeN
09 strains to RD cells were also compared. Quantitative PCR analysis showed that
pretreating cells with vimentin antibody also inhibited Hunan 09 and HeN 09 virus
binding and replication in U251 cells (Fig 10C), whereas pretreating cells with
SCARB2 antibody did not influence the inhibition effect of vimentin antibody on the
binding of these strains (Fig 10C).

To investigate the role of host cell PSGL-1 on the binding of EV71 to cells, different
permissive cell types were preincubated with PSGL-1 antibody (60 μg ml⁻¹) or isotype
antibody (60 μg ml⁻¹) for 60 min at 4°C before being infected with EV71. At 1h
postinfection, virus particles bound to the cells were analyzed by quantitative PCR.
Result showed that PSGL-1 antibody did not affect EV71 binding to U251, HeLa,
Vero and RD cells (Fig 11A). However, preincubation of Jurkat cells with PSGL-1
antibody led to a dramatic reduction of EV71 binding in these cells (Fig 11B).
Moreover, pretreating Jurkat cells with vimentin antibody did not alter the effects of
PSGL-1 antibody on EV71 binding (Fig 11B), implicating that the sites of EV71
binding with PSGL-1 and vimentin are different. The virus yield in PSGL-1 antibody
treated Jurkat cells was also dramatically decreased compared to the IgG control
group. The inhibition of EV71 infectivity by PSGL-1 antibody was not influenced by
vimentin antibody (Fig 11C).

It has been shown that certain mouse cell lines cannot efficiently support EV71
replication [50, 51]. It is believed that mouse SCARB2 or PSGL-1 lacked the property
required to mediate efficient EV71 infection [52, 11]. The binding and replication of
EV71 in mouse 3T3 cells was therefore studied. The results showed that the virus was
able to bind to the 3T3 cell surface (Fig 12A). Further analysis showed that 3T3 cells
also have vimentin expressed on their cell surface (Fig 12B), and vimentin antibody
treatment of 3T3 cells also inhibited the binding of EV71 to the cells. In contrast,
pretreating these cells with SCARB2 antibody or PSGL-1 antibody had no influence
on EV71 binding (Fig 12C). In addition, unlike human cell lines, 3T3 cells allowed
only highly inefficient EV71 replication. Pretreating 3T3 cells with vimentin antibody
also resulted in a reduction of virus yield (Fig 12D). Co-immunoprecipitation was
further performed to investigate the interaction between mouse vimentin and EV71
VP1. The results showed that a band corresponding to mouse vimentin could be
detected in VP1-Flag protein eluate but not in control eluate (Fig 12E), indicating that
mouse vimentin could also interact with EV71 VP1. To further analyze the roles of
mouse vimentin on EV71 binding, we performed EV71 binding experiments on 3T3
cells with reduced vimentin expression on their cell surface (produced by transfecting
the cells with siRNA to vimentin) (Fig 12F). We observed that a reduction of vimentin expression on the 3T3 cell surface was accompanied by a significant reduction in the binding of EV71 to the cells (Fig 12G). These results indicated that the vimentin on the surface of mouse cells also contributed to virus-cell binding.

To identify the domains responsible for VP1 and vimentin interaction, pull-down assays involving VP1 and vimentin truncates were performed. U251 cells were transfected with either full-length vimentin (aa 1-466), vimentin (1-230), vimentin (231-466), vimentin (1-115), vimentin (116-230), vimentin (1-56) or vimentin (57-115) and then lysed and incubated with either VP1-GST or GST, both precombined with glutathione agarose. The protein samples were then analyzed by western blot to detect for VP1 binding. The results showed that shortening the vimentin polypeptide from the N-terminus that included amino acids 1-56 abolished the VP1 binding capacity of vimentin (Fig 13A), suggesting that the N-terminal aa 1-56 of vimentin contained the domain directly responsible for the specific binding of EV71 VP1 to host cells. The role of the N-terminal vimentin (aa 1-56) as a EV71-binding domain was further validated using functional assays in which all the vimentin truncates containing the N-terminal vimentin (aa 1-56) tested were able to inhibit EV71 binding. The vimentin (aa 1-56) was also shown to be more effective in reducing virus yields than other vimentin truncates (Fig 13B). Thus, these results implied that specific regions within vimentin (aa 1-56) played a role in host-virus interactions that facilitates cell entry.
Discussion

Specific interaction of virion constituents with cellular surface components is essential for infection of target cells by extracellular virus particles. For example, to initiate the infection process, interactions between virus particles and cell surface receptors mediate the initial attachment of the virus to the cell surface [53, 54]. Vimentin is the major intermediate filament protein of astrocyte cells and is believed to be responsible for maintaining cell shape, integrity of the cytoplasm and stabilizing cytoskeletal interactions [55]. It also plays a significant role in supporting and anchoring the nucleus, endoplasmic reticulum and mitochondria. Its expression has been detected in cells of mesenchymal origin and is also present in cells adapted to tissue culture and many transformed cell lines [33]. Several reports have suggested a conserved role for surface vimentin as a general attachment receptor for pathogen entry [34]. However, it is still not clear how does vimentin contributes to pathogen attachment and internalization. It is supposed that the interactions between vimentin and pathogens may increase the attachment of pathogens to the other cell surface molecules and subsequently enhances pathogen endocytosis. In this study, we have demonstrated that EV71 could attach to cell surface vimentin through VP1 and provided evidence that this interaction of EV71 with vimentin contributed to the establishment of EV71 infection since EV71 replication could be reduced by using either exogenous vimentin or an antibody against the cell surface vimentin. In addition, the decreased expression of vimentin on cell surface resulted in a reduction of virus binding. Thus, our results
indicated that vimentin expressed on the cell surface very likely serves as a capture receptor and acts as an attachment site for EV71. However, our results also suggested that the vimentin receptors could merely function as an additional route for virus entry into host cells as virus infection was not completely eliminated by increasing the concentrations of vimentin antibody or exogenous vimentin. This suggests that cell surface vimentin is not essential for EV71 infection but helps the initial binding of virus to host cell, and that multiple receptors are involved during EV71 infection. Alternatively, the interaction of vimentin with EV71 VP1 might facilitate EV71 attachment to the other virus binding cellular receptors and subsequently enhances virus endocytosis into host cell.

SCARB2 and PSGL-1 have been reported to be the cellular receptors for EV71 [56, 19, 20]. A comparison of the reported roles of SCARB2 and PSGL-1 in the virus infection process showed that most EV71 strains were able to utilize SCARB2 as an entry receptor since all mouse cells that expressed human SCARB2 became susceptible to EV71 strains when tested [30]. In contrast, mouse cells that expressed PSGL-1 were only susceptible to PSGL-1–binding strains (EV71-PB) and certain strains of CVA16 [30]. In addition, cell surface expression of SCARB2 could be detected in most cell lines, while PSGL-1 expression could only be detected in myeloid cells and stimulated T lymphocytes [19]. These reports are in accord with our observations that SCARB2, and not PSGL-1, could be detected on the cell surface of the non-leukocyte cell lines HeLa, Vero, U251 and RD. Although cell surface expression of PSGL-1 was observed in Jurkat T cells, in contrast,
cell surface vimentin was expressed in all the cell lines tested. SCARB2 has been reported to play additional essential roles in the internalization and induction of virus uncoating, a step after virus attachment [57]. Although numerous EV71 particles (a PB strain) were found binding to PSGL-1, this interaction did not induce conformational alterations in the cells [29]. In addition, SCARB2 and PSGL1 were reported to bind to different sites of the virion, and the affinity of the SCARB2-EV71 interaction was found to be different from that of the PSGL-1-EV71 interaction [30]. In cells that expressed human SCARB2 and PSGL-1, EV71 was found to infect and spread more efficiently via SCARB2 than PSGL1, despite more virus particles had bound to L-PSGL1 cells than to L-SCARB2 cells [30]. This result is consistent with our observation that pretreating cells with SCARB2 antibody had relatively less influence on virus binding when compared to cells pretreated with vimentin antibody or PSGL-1 antibody. However, this latter observation was not in accord with our other observation that SCARB2 antibody pretreatment resulted in a dramatic decrease in virus titers compared to those of vimentin and PSGL-1 pretreated cells. This implied that the amount of virus attaching to the cell surface might not solely determine the infection efficiency. Various reports suggest that vimentin may have roles in virus attachment and internalization but not in uncoating [34, 37]. It is possible that the virus captured by PSGL-1 and vimentin could be uncoated either by SCARB2 in human cells that expressed these receptors or by other unknown molecules.

It is worth mentioning that the capacity for a virus to replicate is determined not only
by the availability of host cell receptors and molecules that mediate virus entry but
also by a host cell environment that favors virus gene replication, protein expression,
and virus assembly and release. Several studies have indicated that mouse cells could
not support robust EV71 infection [58-61]. Other studies have shown that EV71
virion particles could attach to the surface of mouse L929 cells [28, 30] and low levels
of virus replication can be detected in mouse L929 cells infected with EV71
(SK-EV006 and Nagoya strains) [20]. This implies that, in oppose to human SCARB2
and PSGL-1, other unspecified molecules on the surface of mouse cells might interact
with EV71 and mediate its infection. One of these studies have also shown that
infectious particles could be readily recovered from mouse cells transfected with
EV71 genomic RNA [20], suggesting that the restriction of EV71 infection in mouse
cells probably occurred at one of the early steps of infection and that mouse cells
might lack the cell receptors essential for productive infection. However, in our study,
we have observed that EV71 could bind to mouse 3T3 cells by interacting with cell
surface vimentin, leading to a very limited replication of EV71 in 3T3 cells. In
addition, a reduction of vimentin expression on 3T3 cell surface did result in a
decrease in the binding of virus particles to the cells. Thus, the low efficiency of
EV71 infection and replication in mouse cells might not be due to a lack of
attachment receptors but mainly attributed to an absence or low expression of
essential cell receptors that mediate virus internalization, endocytosis and/or
un-coating. Furthermore, it is unlikely that mouse SCARB2 and PSGL-1 are involved
in virus-cell interaction since pretreating 3T3 cells with SCARB2 and PSGL-1
antibody did not decrease EV71 binding to these cells. This suggests that mouse cells could possess other molecules that lead to very limited infection.

We have found that vimentin mediated the interaction between vimentin and VP1. Dramatic decreases in virus binding and virus yields were observed in cells pretreated with vimentin fragments that contained the N terminal amino acids 1-56 of vimentin.

A vimentin monomer, like all other intermediate filaments, has a central α-helical domain, capped at each end by a non-helical amino (head) and carboxyl (tail) domain, respectively [62]. Two vimentin molecules form a coiled-coil dimer which is the basic subunit of vimentin assembly [63, 64]. Thus, we can envisage that the head domains of the cell surface vimentin interact with EV71 VP1, and increase the attachment of EV71 to host cells. In contrast, it was demonstrated that the binding of SCARB2 to EV71 occurred within the luminal domain of SCARB2 at amino acids 142 to 204, and that amino acids 144 to 151 were particularly important [28]. It has been shown that EV71 VP2 could participate in EV71 -PSGL-1 binding [50]. In our study, we have found that EV71 might bind with vimentin at a different site to those of SCARB2 or PSGL-1 since vimentin binding did not influence the interaction between EV71 and SCARB2 or PSGL-1. In addition, the virus-vimentin interaction was shown to be common to other selected C4 genotype of EV71, suggesting that the interaction with vimentin could be a common feature of most, if not all, of the circulating EV71 viruses. In consideration of the broad distribution of vimentin in animal tissues, we hypothesize that vimentin might contribute to the proliferation of virus infection by
broadening the target cells in vivo. Moreover, it might also be more than an attachment site on the host cell surface.

In conclusion, we believe this study delivers important findings on the roles of vimentin filaments in relation to EV71 infection. This work provides information that not only improved our understanding of EV71 pathogenesis, but also presents us with potentially new strategies for the treatment of diseases caused by EV71 infections. For example, it might be possible to develop some antibodies, peptides or small compounds for the prevention and/or treatment EV71 infections by blocking the binding between EV71 and vimentin. However, the functional significance of vimentin-EV71 interaction during the natural infection process of these important human viruses remains to be elucidated.
Acknowledgements

This work is supported by grants from the National Basic Research Program of China (973 Program) (No. 2011CB504703, 2010CB530102), and the National Natural Science Foundation of China (NSFC, Grant No. 81321063, 31270211, 31370201).

We thank Dr. Paul Chu for his help during the preparation of the manuscript. We also thank Zhao Tong, Institute of Microbiology, CAS, for technical assistance with flow cytometry and Xiaolan Zhang, Institute of Microbiology, CAS, for technical assistance with confocal microscopy. We also thank Fulian Liao and Weihua Zhuang for their technical assistance with the experiments.

All the authors declare that they have no conflicts of interest with regards to this publication.
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Figure legends

Figure 1. Experiments showing specific interaction between EV71 and cellular vimentin. A: Detection of specific interactions between EV71 BrCr virus particles and cellular vimentin in uninfected U251 cell lysates by immunoprecipitation assays and western blot performed as described in Materials and Methods. The figure shows a western blot of the following precipitated protein samples using anti-EV71 VP1 and anti-vimentin antibodies: lane Input = untreated cell lysate, lane Control = cell lysate incubated with agarose beads treated with purified EV71 particles, lane anti-EV71 = cell lysate incubated with anti-EV71 VP1 monoclonal antibody-conjugated agarose beads treated with purified EV71 particles, lane IgG = cell lysate incubated with mouse IgG-conjugated agarose beads treated with purified EV71 particles. B: Detection of specific binding of EV71 with vimentin in EV71 infected U251 cells using co-immunoprecipitation assays. U251 cells were infected with EV71 and lysates prepared as described in Materials and Methods. Infected cell lysates were then incubated with either mouse IgG- (IgG) or vimentin monoclonal antibody (Anti-Vimentin)-conjugated agarose beads for 2 h at 4°C. The precipitated proteins were blotted with anti-EV71 VP1 and anti-vimentin antibodies. Lane Input = EV71 and vimentin markers. Lane Control = proteins from cell lysate incubated with IgG-conjugated agarose beads. Lane Anti-Vimentin = proteins from cell lysate incubated with vimentin monoclonal antibody-conjugated agarose beads. C: Analysis of the specific interactions between various strains of EV71 viruses and U251 cellular vimentin by immunoprecipitation assays. Lanes CA16, EV71 BrCr, Hubei 09 and HeN 09 = cell lysates incubated with anti-vimentin monoclonal antibody-conjugated agarose beads treated with purified CA16, EV71 BrCr, EV71 Hubei 09 and
EV71 HeN 09 particles, respectively. Lane Control = cell lysate incubated with agarose beads treated with purified EV71 BrCr particles.

Figure 2. **A**: Detection of vimentin expression on the cell surface of U251, RD, Vero and HeLa cells by flow cytometry. Cells were fixed and incubated with either mouse IgG (black line) or antibody to vimentin (gray line). The cells were then incubated with the fluorescent secondary antibody and subjected to flow cytometry analysis as described in Materials and Methods. Y axis (Counts) = cell counts. X axis (FL1-H) = fluorescence density. **B**: Analysis of the cell surface distribution of vimentin and cell surface-bound EV71 by indirect immunofluorescence in U251 cells. Cells were infected with EV71 BrCr (+EV71) at 4°C for 1h and then stained with specific antibody to either EV71 (red) or vimentin (green) and analysed by confocal fluorescence microscopy. Bar = 20µm. **C**: Analysis of the distribution of cell vimentin by indirect immunofluorescence in U251 cells (-EV71). Cells were fixed and permeabilized as described in Materials and Methods. Cells were then stained with antibodies to vimentin (Vim, green fluorescence) and EV71 (EV71, red fluorescence) and subjected to confocal microscopy analysis. An overlay of the vimentin and EV71 fluorescence is also shown (Merged). Cell morphology (Phase) was assessed by light microscopy.

Figure 3. Experiments showing the interaction between EV71 VP1 protein and vimentin. **A**: Detection of binding between VP1 and vimentin using GST-pull down assays and western blotting. The eluates obtained as described in Materials and Methods were blotted with anti-vimentin and
anti-GST antibodies and the resultant VP1 protein band observed is indicated. Lanes: Input =
untreated cell lysate, Control = U251 cell lysate incubated with glutathione-Sepharose beads; VP1,
3C and GST = U251 cell lysate incubated with VP1-GST, 3C-GST or GST pre-combined
glutathione-Sepharose beads, respectively. B: Pull down assays and western blot analysis showing
the interaction between VP1 and vimentin. GST or VP1-GST protein was incubated with
anti-vimentin monoclonal antibody-conjugated agarose beads that were pre-incubated with
vimentin protein. The precipitated proteins were blotted with antibodies to either vimentin or GST
and the resultant VP1-GST protein band observed is indicated. C: Analysis of the binding of VP1
to vimentin by co-immunoprecipitation and western blot. U251 cells were transfected with a
plasmid expressing VP1, lysed and co-immunoprecipitated with vimentin antibodies (anti-Vim) or
mouse IgG. The precipitated proteins were blotted with antibodies to vimentin and EV71 VP1.
Lanes: Input = cell lysate; Control = agarose beads incubated either with no antibodies or with
IgG; anti-vimentin and mouse IgG = agarose beads incubated with vimentin antibody and mouse
IgG, respectively. D: Co-immunoprecipitation and western blot analysis of the binding of
vimentin to VP1, VP2 and VP3. U251 cells were transfected with plasmids expressing either VP1,
VP2 or VP3. Co-immunoprecipitation was performed with Flag antibody. The precipitated
proteins were analysed by western blotting using antibodies to vimentin and Flag. Lane Control =
precipitated proteins from cells with no plasmid transfection. Lanes VP1, VP2, VP3 = precipitated
proteins from cells transfected with VP1, VP2, VP3 plasmids, respectively. The figure shows only
EV71 VP1 interacted with vimentin and not VP2 or VP3.

**Figure 4.** Experiments on the role of vimentin in the attachment of EV71 to U251 cells. A:
Analysis of the binding of EV71 or CA16 to U251 cells using competitive inhibition assays and western blot. Cells were infected with EV71 or CA16 at 4°C for 1 h after the virus inoculum was preincubated with vimentin or BSA at the concentrations indicated (μg ml⁻¹). Control = cells infected with untreated virus. Infected cell lysates were subjected to western blot analysis using antibodies to EV71, CA16 and β-actin (internal control). The figure shows the inhibition of EV71 binding but not CA16 binding after pretreatment of the virus inoculum with vimentin. 

**B:** Flow cytometry analysis of the binding of EV71 to U251 cells after pretreatment of the virus inoculum with increasing concentrations of vimentin (5μg ml⁻¹, black heavy line; 20μg ml⁻¹, black dotted line) or BSA (20μg ml⁻¹, gray hairline). Black hairline (Mock)= infected cells with no vimentin or BSA added. Gray filled line (Control)= cells with no EV71 infection. X axis (FL1-H) = fluorescence density.

**C:** Analysis of the binding of EV71 to U251 cells using quantitative RT-PCR. Cells were infected with EV71 at 4°C for 1 h after the virus inoculum was preincubated with vimentin or BSA at the concentrations indicated. Control = cells infected with untreated EV71.

**D:** Infectivity of vimentin-pretreated EV71 in U251 cells. EV71 were pretreated with various doses of vimentin (Vim) at 4°C for 1 h prior to infecting U251 cells. The total virus yield at 48 h post-infection was determined. Virus titer from cells infected with EV71 that had no vimentin pretreatment was used as reference (100%) to calculate the % reduction in TCID50 (50% tissue culture infective dose) in the vimentin-pretreated groups.

**E:** CPE of EV71 infections viewed under the visible light phase microscope, showing representative fields of control uninfected U251 cells (Uninfected), cells infected with either untreated EV71 (EV71), vimentin-pretreated EV71 (Vim, 20μg ml⁻¹) or control BSA-pretreated cells (BSA, 20μg ml⁻¹), respectively.

**F:** Analysis of the influence of vimentin on the binding of EV71 to U251 cells using...
quantitative RT-PCR. Cells were infected with EV71 at 4°C. Vimentin (20µg ml⁻¹) or BSA (20µg ml⁻¹) was then added to the cell culture at the indicated time postinfection. All cells were harvested at 60min postinfection and the binding of EV71 was then analyzed using quantitative RT-PCR.

**Figure 5.** Analysis of the role of vimentin in the attachment of EV71 to RD, HeLa and Vero cells.

A: Western blot analysis of the inhibition of EV71 infection after pretreating the virus inoculum with vimentin (vim) or BSA. Cells were infected with EV71 at 4°C for 1h, lysed and western blotted with antibody to EV71 and β-actin (internal control) as described in Materials and Methods. Control = cells infected with untreated virus, BSA = cells infected with BSA pretreated virus, Vim = cells infected with vimentin pretreated virus.

B: Analysis of the binding of EV71 to cells using quantitative RT-PCR. Control = cells infected with untreated virus, Mock = cells incubated with no virus. C: Infectivity of vimentin-pretreated EV71 in RD, HeLa and Vero cells. EV71 was pretreated with vimentin or BSA at 4°C for 1 h prior to infecting the cells. The total virus yield at 12, 24 and 48 h post-infection at 37°C was determined. Control = cells infected with untreated virus, BSA = cells infected with BSA treated virus, Vim = cells infected with vimentin treated virus, Mock = cells incubated with no virus.

**Figure 6.** Effect of pretreating host cells with vimentin antibodies on the binding of EV71 to the cells. A: Western blot analysis of EV71 BrCr replication in U251 cells that had been preincubated with vimentin antibodies prior to infection. Cells were pretreated with the indicated concentrations of vimentin antibodies (µg ml⁻¹) or rabbit IgG (Isotype ab, 80µg ml⁻¹) before being infected with...
EV71 for 1h at 4°C. Cells were then lysed and subjected to western blot analysis with antibody to 
EV71 and β-actin (internal control). Control = cells incubated with EV71. B: CPE of EV71 BrCr 
infection viewed under a visible light phase microscope, showing representative fields of the 
uninfected control U251 cells (Uninfected), vimentin antibody-pretreated cells infected with EV71 
(Vim ab (60 μg ml⁻¹) + EV71), and rabbit IgG -pretreated cells infected with EV71 (Isotype ab 
(60 μg ml⁻¹) + EV71), respectively. C: Analysis of the binding of EV71 BrCr to U251 cells using 
quantitative RT-PCR. Cells were pretreated with vimentin antibodies (Vim ab, 60 μg ml⁻¹) or 
rabbit IgG (Isotype ab, 60 μg ml⁻¹) before infection as described above. Control = untreated cells 
incubated with EV71. Mock = incubated cells with no EV71. Isotype ab = isotype 
antibody-pretreated cells infected with EV71. Vim = vimentin antibody-pretreated cells infected 
with EV71. D: Graphs showing virus titers in infected U251 cells and the corresponding culture 
media at 0, 12 and 24 h after infection. The cells were pretreated with vimentin antibody (Vim ab, 
60 μg ml⁻¹) or rabbit IgG (Isotype ab, 60 μg ml⁻¹) before infection. Control = untreated cells 
infected with EV71. Mock = uninfected mock treated cells. E: Analysis of the influence of 
vimentin antibody (Vim ab) and isotype antibody (Isotype ab) on the binding of EV71 Hubei09 
and HeN09 strains to U251 cells using quantitative RT-PCR. Control = untreated cells incubated 
with EV71.

**Figure 7.** Effect of the down-regulation of vimentin expression in U251 cells on the efficiency of 
EV71 binding and replication in host cells. U251, C-U251 and VK-U251 are cells with no vector, 
cells with empty vector and cells with the vimentin knockdown plasmid, respectively. A: Flow 
cytometry analysis of cell surface vimentin expression in U251 (gray hairline), C-U251 (black
hairline) and VK-U251 (gray heavy line) cells. Non-permeabilized cells were fixed and stained
with antibody specific to vimentin and subjected to flow cytometry analysis. Thick black line =
VK-U251 cells stained with mouse IgG. Y axis (Counts) = cell counts. X axis (FL1-H) =
fluorescence density. B: Flow cytometry analysis of the binding of EV71 to VK-U251 and
C-U251 cells. VK-U251 and C-U251 cells were incubated with EV71 for 1h at 4°C and then fixed,
stained with antibody to EV71 and subjected to flow cytometry. Black heavy line = VK-U251
cells with no EV71 infection, gray heavy line = C-U251 cells with no EV71 infection. Gray dotted
line = infected VK-U251 cells stained with EV71 antibody. Gray hairline = infected U251 cells
stained with EV71 antibody. Black hairline = infected C-U251 cells stained with EV71 antibody.
C: Analysis of EV71 binding in U251, VK-U251 and C-U251 cells that had been infected with
virus inoculum preincubated with vimentin before transfection by quantitative RT-PCR. Control =
cells incubated with untreated EV71, BSA = cells infected with virus inoculum preincubated with
BSA. Vim = cells infected with virus inoculum preincubated with vimentin. Mock = cells mock
treated with no virus infection. D: Western blot analysis of EV71 binding and expression of
SCARB2, PSGL-1 and vimentin in U251, C-U251 and VK-U251 cells. The respective cells were
infected with EV71 at 4°C for 1h, then lysed and blotted with antibodies to either EV71, SCARB2,
PSGL-1, vimentin or β-actin (internal control). E: Analysis of CPE of EV71 infection in C-U251
and VK-U251 cells after infection at 37°C for 48h using a visible light phase microscope. F:
Determination of virus titers in infected U251, C-U251 and VK-U251 cells and the corresponding
culture supernatants at 12, 24 and 48h after infection with EV71.

**Figure 8.** Effect of pretreating U251 cells with SCARB2 antibodies on the binding of EV71 to the
A: Flow cytometry analysis of the cell surface expression of U251, RD, Vero, HeLa and Jurkat cells. Cells were fixed and incubated with either mouse IgG (black line) or antibody to vimentin (gray line) and then incubated with the fluorescent secondary antibody and subjected to flow cytometry analysis. Y axis (Counts) = cell counts. X axis = fluorescence density. 

B: Quantitative RT-PCR analysis of the binding of EV71 to VK-U251 cells that were infected after preincubation with antibodies to either vimentin (Vim ab, 60 µg ml⁻¹), PSGL-1 (PSGL-1 ab, 60 µg ml⁻¹), SCARB2 (SCARB2 ab, 60 µg ml⁻¹) or isotype IgG (Isotype ab, 60 µg ml⁻¹). SCARB2 ab + Vim ab = Cells preincubated with SCARB2 antibody (60 µg ml⁻¹) for 30 min at 37°C and then incubated with vimentin antibody for 30 min at 37°C. Vim ab + SCARB2 ab = Cells preincubated with vimentin antibody for 30 min at 37°C followed by incubation with SCARB2 antibody for 30 min at 37°C. Control = EV71 infected cells without preincubation, Mock = mock treated cells with no virus. 

C: Quantitative RT-PCR analysis of the binding of EV71 to U251 cells that were preincubated with antibodies to either vimentin, SCARB2 or isotype IgG as described above. 

D: Determination of EV71 virus titers in infected U251 cells and the corresponding culture media at 12, 24 and 48 h after infection. The cells were preincubated with vimentin antibody (Vim ab, 60 µg ml⁻¹), SCARB2 antibody (SCARB2 ab, 60 µg ml⁻¹) or isotype IgG (Control, 60 µg ml⁻¹) before infection. Mock = uninfected cells. 

E: Determination of EV71 virus titers in infected VK-U251 cells and the corresponding culture supernatants as described in D. Cells were preincubated with vimentin antibody (Vim ab), SCARB2 antibody (SCARB2 ab) or isotype IgG (Isotype ab) before infection. Control = EV71 infected cells without preincubation.

Figure 9. A: Analysis of the binding of EV71 in U251 cells after pretreating the virus inoculum.
with BSA (20 μg ml⁻¹), mouse FC (20 μg ml⁻¹), PSGL-1-FC (20 μg ml⁻¹), SCARB2 (20 μg ml⁻¹) or/and vimentin (20 μg ml⁻¹) by using quantitative RT-PCR. Control = cells infected with EV71.

**Figure 10.** Effect of pretreating RD, HeLa, Vero and Jurkat cells with SCARB2 antibodies on the binding of EV71 BrCr strain. A: Quantitative RT-PCR analysis of the binding of EV71 to RD, HeLa and Vero cells that were preincubated with antibodies to vimentin (Vim ab, 60 μg ml⁻¹), SCARB2 (SCARB2 ab, 60 μg ml⁻¹) or isotype IgG (Isotype ab, 60 μg ml⁻¹). Control = EV71 infected cells without preincubation, Mock = uninfected cells. B: Determination of virus titers in EV71 infected RD, HeLa and Vero cells and the corresponding culture supernatants. The cells were preincubated with vimentin antibody, SCARB2 antibody or isotype IgG (Control) before infection. C: Quantitative RT-PCR analysis of the effect of pretreating U251 cells with SCARB2 antibodies (SCARB2 ab, 60 μg ml⁻¹) on the binding of EV71 Hubei 09 and HeN09 strains to U251 cells. Control = EV71 infected cells with no preincubation, Mock = uninfected cells.

**Figure 11.** Experiments on the effect of pretreatment of RD, HeLa, Vero and Jurkat cells with PSGL-1 antibodies on the binding of EV71 Hubei 09 strain. A: Analysis of the binding of EV71...
to RD, HeLa and Vero that preincubated with antibodies to vimentin, PSGL-1 or isotype IgG (60 μg ml⁻¹) by using quantitative RT-PCR as described in materials and methods. Control = cells infected with EV71. B: Quantitative RT-PCR analysis of the effect of pretreatment of Jurkat cells with PSGL-1 antibodies on the binding of EV71. Control = cells infected with virus, Mock = cells no virus infection. C: Virus titers in the supernatants and infected Jurkat cells that were preincubated with vimentin antibody, PSGL-1 antibody or isotype IgG (Control, 60 μg ml⁻¹) before infection. The data are shown as mean virus titers ±SD based on three independent experiments. 

**Figure 12.** Analysis of the role of vimentin in EV71 binding in mouse 3T3 cells. A: Detection of vimentin expression on the cell surface of mouse 3T3 cells. Cells were fixed and incubated with either mouse IgG (black line) or antibody to vimentin (gray line). The cells were then incubated with the fluorescent secondary antibody and subjected to flow cytometry analysis as described in Materials and Methods. Y axis (Counts) = cell counts. X axis = fluorescence density. B: Flow cytometry analysis of the binding of EV71 to 3T3 cells. Cells were incubated with EV71 at a MOI of 20 at 4°C for 1h, fixed and incubated with either mouse IgG (black line) or antibody to EV71 (gray line). C: Quantitative RT-PCR analysis of the binding of EV71 to 3T3 cells that were preincubated with antibodies to vimentin, SCARB2, PSGL-1 or isotype IgG (60 μg ml⁻¹) and then infected with EV71. Control = EV71 infected cells without preincubation, Mock = uninfected cells. D: Determination of virus titers in EV71 infected 3T3 cells and the corresponding culture supernatants at 12, 24 and 48 h postinfection. The cells were preincubated with vimentin antibody, SCARB2 antibody or isotype IgG (Control) before infection. Mock = uninfected cells. E: Co-immunoprecipitation and western blot analysis of the interaction between mouse vimentin and
EV71 VP1 as described in Materials and Methods. 3T3 cells were transfected with plasmids expressing VP1. Co-immunoprecipitation was performed with Flag antibody. The precipitated proteins were analysed by western blotting using antibodies to vimentin 1 and Flag. Lane Control = proteins from cell lysate incubated with agarose beads. Lane VP1 = proteins from cell lysate incubated with Flag-conjugated agarose beads. Lane IgG = proteins from cell lysate incubated with IgG-conjugated agarose beads.

F: Detection of vimentin expression on the cell surface of mouse 3T3 (black dotted line), siC-3T3 (gray line) and siVim-3T3 (black line) cells. Cells were fixed and incubated with either mouse IgG (shaded area) or antibody to vimentin. The cells were then incubated with the fluorescent secondary antibody and subjected to flow cytometry analysis as described in Materials and Methods.

G: Analysis of the binding of EV71 to 3T3, siC-3T3 and siVim-3T3 cells using quantitative RT-PCR.

Figure 13. Identification of the domain of vimentin involved in the interaction with EV71 VP1. A: Analysis of various truncated vimentin fragments for interaction with VP1. The His tagged vimentin and its fragments fragments (aa 1-466, 1-230, 231-466, 1-115, 116-230, 1-56 and 57-115) were subjected to a pull-down assay using the GST-VP1 coupled glutathione-Sepharose beads or GST beads as described in Materials and Methods. Proteins co-precipitating with the beads were analyzed by immunoblotting using anti-His antibody or anti-GST antibody. B: Effect of pretreating EV71 inoculum with full length vimentin and truncated vimentin on the binding of the virus to U251 cells. EV71 were pretreated with vimentin fragments at a concentration of 20μg ml⁻¹ at 37°C for 1 h prior to infecting U251 cells. The cells were then infected with EV71 at 4°C for 1 h. Quantitative RT-PCR analysis were then performed to determine the EV71 RNA levels as
described in Materials and Methods. Control = cells infected with untreated EV71.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 6
Figure 7
Figure 8
Figure 9
Figure 10
Figure 12
Figure 13