

1 **Seneca Valley virus intercellular transmission mediated by exosomes**

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20 **ABSTRACT**

21 Exosomes are cup-shaped vesicles that are secreted by cells and are involved in the
22 intercellular transport of a variety of substances, including proteins, RNA, and
23 liposomes. Studies have shown that pathogenic microorganisms are contained in
24 exosomes extracted from pathogenic micro-infected cells. The Seneca Valley virus
25 (SVV) is a non-encapsulated single-stranded positive-strand RNA virus that causes
26 ulceration in the pig's nose, the appearance of blisters, and other clinical symptoms
27 similar to foot-and-mouth disease (FMD). Whether exosomes from SVV-infected cells
28 can mediate SVV intercellular transmission is of great significance. There have been
29 no studies showing whether exosomes can carry SVV in susceptible and
30 non-susceptible cells. Here, we first extracted and identified exosomes from
31 SVV-infected IBRS-2 cells. It was confirmed that replication of SVV can be inhibited
32 when IBRS-2 cells treated with exosomes inhibitor GW4869. Furthermore, laser
33 confocal microscopy and qRT-PCR experiments were performed to investigate whether
34 exosomes can carry SVV and enable the virus to proliferate in susceptible and
35 non-susceptible cells. Finally, exosome-mediated intercellular transmission can not be
36 completely blocked by SVV-specific neutralizing antibodies. Taken together, this study
37 showed that exosomes extracted from the SVV-infected IBRS-2 cells can carry SVV
38 and transmit productive SVV infection between SVV susceptible and non-susceptible
39 cells, this transmit infection is resistant to SVV specific neutralization antibody.

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45 **IMPORTANCE**

46 Exosomes participate in intercellular communication between cells. Exosomes derived from
47 virus-infected cells can mediate virus transmission or/and regulate immune response. However,
48 the function of exosomes that from SVV-infected host cells during SVV transmission is unclear.
49 Here, we demonstrate SVV can utilize host exosomes to establish productive infection in
50 intercellular transmission. Furthermore, exosome-mediated SVV transmission is resistant to
51 SVVV-specific neutralizing antibodies. This discovery sheds light on neutralizing antibodies
52 resistant to SVVV transmission by exosomes as a potential immune evasion mechanism.

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65 INTRODUCTION

66 The Seneca Valley virus (SVV) is a single-stranded positive-strand RNA virus that
67 belongs to the genus *Senecavirus* and family *Picornaviridae*. SVV has a typical
68 icosahedral symmetry and a genome of 7.2 kb (1). In 2002, SVV was first discovered in
69 the PER.C6 cell line in Maryland, USA (2). SVV mainly infects pigs, newborn piglets,
70 fattening pigs, and other pigs of all ages, but neutralizing antibodies are also found in
71 other animal bodies, such as cattle and sheep (3, 4). The clinical symptoms after SVV
72 infection are very similar to the clinical symptoms of FMD. The main clinical
73 symptoms are blisters and ulceration in the hoof and nose, as well as fever and anorexia
74 (5). In recent years, the newly discovered SVV-induced blisters in pigs have brought
75 great harm to China's pig industry (6).

76 Exosomes are small vesicles with a diameter of 40–150 nm (7). Most of the model
77 cells can secrete exosomes, which contain multiple substances, including large amounts
78 of proteins and nucleic acids, and carry substances to a variety of cells (8-10). The virus
79 enters the cell through endocytic pathways during the process of exosome formation
80 and completes its own assembly and release (11). It has been reported in the literature
81 that hepatitis A virus (a picornavirus) and hepatitis C virus can use exosomes to spread
82 their DNA and escape the body's immune response (12). However, the pathogenesis of
83 SVV is not yet fully understood. In the view of the aforementioned background
84 research, we suspect that exosomes may be an important mediator of SVV transmission
85 between cells. Therefore, we aim to explore whether exosomes carry SVV for
86 transmission and if the inhibition of exosome secretion and production inhibits the
87 proliferation of SVV in cells.

88 In the present study, we extracted exosomes from SVV-infected IBRS-2 cells and
89 identified them. We then introduced the extracted exosomes into 293T and IBRS-2 cells
90 and found that SVV carried by exosomes can proliferate in these cells. We also
91 inhibited IBRS-2 secretion and production of exosomes, which resulted in the

92 inhibition of SVV proliferation. Interestingly, we also found that SVV carried by
93 exosomes cannot be neutralized by SVV neutralizing antibodies. This study can
94 provide an important reference for the pathogenesis of SVV and the antiviral
95 mechanisms of the body.

96 **RESULTS**

97 **Identification of exosomes extracted from SVV-infected IBRS-2 cells**

98 Exosomes were extracted from SVV-infected IB-IR-2 cells, and the morphology
99 of exosomes was identified by TEM. Further purification was performed with the use
100 of a CD63-immunoaffinity kit. Cup-shaped lipid bilayer vesicles of representative
101 exosome images were observed by TEM (Fig. 1a). To further identify exosomes by the
102 exosome-associated protein markers, including Alix, CD63, and CD9, a WB
103 experiment was performed. The exosomes extracted from SVV-infected IBRS-2 cells
104 contained the exosome-associated protein markers Alix, CD9, and CD63 (Fig. 1b). At
105 the same time, the size of exosomes was evaluated with the NTA method, and it was
106 found that the particle size of the exosomes was mainly distributed in the range of
107 50-150 nm (Fig. 1c). To verify whether the extracted exosomes contained SVV, we
108 identified the SVV gene sequence in exosomes by qRT-PCR. SVV gene sequences
109 were found in SVV-infected IBRS-2 cells and exosomes extracted from SVV-infected
110 IBRS-2 cells (Fig. 1d). These results indicate that the morphology and particle size of
111 the exosomes extracted in this study are consistent with those reported in the literature,
112 and exosome-associated proteins are detected in exosomes. It is important that SVV is
113 included in the exosomes.

114 **Exosomes mediate the spread of SVV in susceptible and non-susceptible cells**

115 In the aforementioned experiments, we found that the extracted exosomes
116 contained SVV. It has been reported in the literature that exosomes can mediate the
117 spread of substances in cells. Therefore, this study aimed to further explore whether

118 exosomes mediate the spread of SVV. The DIL-stained exosomes were introduced into
119 IBRS-2 and 293T cells, and at the same time, mock-exosomes (mock-exo) and
120 SVV-GFP were introduced as the control. The fluorescence was observed under a laser
121 confocal microscope. According to the experimental results, red fluorescence (DIL)
122 was observed in both IBRS-2 and 293T cell membranes, and the GFP carried by SVV
123 co-localized with DIL. In addition, only red fluorescence was observed in the
124 mock-exo group (Fig. 2a). To further investigate whether exosomes mediate the spread
125 of SVV, SVV-exosomes (SVV-exo) was added to IBRS-2 and 293T cells, and
126 mock-exo and SVV-GFP were added as the control group. Finally, the copy number of
127 SVV was detected by qRT-PCR. According to the experimental results, the copy
128 number of SVV in the SVV-exo group was increased in both 293T and IBRS-2 cells
129 compared with that in the mock group, and the copy number in IBRS-2 cells was higher
130 than that in 293T cells (Fig. 2b). These results demonstrate that exosomes carry SVV to
131 293T and IBRS-2 cells, and SVV can proliferate in recipient cells.

132 **Inhibition of exosome secretion can inhibit the proliferation of SVV in IBRS-2** 133 **cells**

134 In aforementioned experiments, we found that exosomes carried SVV and that
135 SVV proliferated in 293T and IBRS-2 cells. Therefore, we next investigated whether
136 inhibiting the secretion of exosomes affected the replication of SVV. To verify whether
137 intracellular RAB27a expression was downregulated after si-RAN27a transfection, we
138 transfected RBA27a into IB cells and detected RAB27a miRNA levels by qRT-PCR.
139 The results showed that the miRNA expression of intracellular RAB27a was
140 significantly downregulated in si-RBA27a-transfected cells compared with that in the
141 control group (Fig. 3a). Then, in order to investigate whether the number of exosomes
142 in the transfected cells was changed, we examined the miRNA expression level of the
143 exosome protein marker ALIX. The results showed that the expression of ALIX was
144 significantly upregulated in RAB27a-transfected IBRS-2 cells compared with that in

145 the control group (Fig. 3b). In addition, the miRNA expression of ALIX was
146 significantly upregulated in si-RAB27a-transfected cells. Therefore, we next explored
147 whether the observed changes in the number of exosomes affected the copy number of
148 SVV in cells. qRT-PCR was performed to detect the copy number of intracellular and
149 extracellular SVV in si-RAB27a-transfected cells infected with SVV. The results
150 showed that the intracellular SVV copy number was significantly upregulated (Fig. 3c)
151 and the extracellular SVV copy number was significantly downregulated (Fig. 3d)
152 compared with those in the control group. To re-validate the secretion of extracellular
153 exosomes after the inhibition of extracellular secretion, we measured the number of
154 extracellular exosomes in si-RAB27a-transfected cells by NTA. The results showed
155 that after RAB27a transfection, the number of exosomes decreased significantly
156 relative to the control group (Fig. 4). The aforementioned results indicate that the
157 inhibition of RAB27a expression leads to a significant increase in the number of
158 exosomes, and the copy number of intracellular SVV is also significantly upregulated.

159 **Inhibition of SVV proliferation by inhibiting the production of exosomes**

160 In order to further verify the aforementioned experimental results, we determined
161 whether the inhibition of exosome production inhibits the proliferation of SVV. IB-IS-2
162 cells were infected with SVV, the infected cells were treated with exosomes secretion
163 inhibitor GW4869, and the copy number of intracellular and extracellular SVV was
164 detected by qRT-PCR. According to the experimental results, the copy number of
165 intracellular and extracellular SVV was significantly decreased after GW4869
166 treatment compared with that of the control group. The aforementioned results indicate
167 that the inhibition of exosome production can inhibit the replication of SVV (Fig. 5).

168 **Exosomes promote the proliferation of SVV in susceptible cells**

169 To further investigate whether exosomes extracted from IBRS-2 cells can promote
170 SVV proliferation, we measured the proliferation of SVV in IBRS-2 cells with

171 exosomes by qRT-PCR. The results showed that the SVV copy number of the
172 mock-exo and SVV-exo groups was significantly higher than that of the SVV control
173 group at 24 h after SVV infection (Fig. 6a), whereas 48 h after SVV infection, the copy
174 number was higher only in the SVV-exo group (Fig. 6b). When the exocytosis dose was
175 50 ng, the SVV copy number of the mock-exo and SVV-exo groups was higher than
176 that of the SVV control group at 24 and 48 h after SVV infection, but the number of
177 SVV copies in the SVV-exo group was only significantly higher than that in the SVV
178 control group 48 h after SVV infection (Fig. 6c and 6d).

179 **Exosome-mediated SVV infection is not inhibited by SVV NABs**

180 Because SVV is carried in exosomes, we investigated if the SVV contained in
181 exosomes can be neutralized by SVV neutralizing antibodies. Exosomes extracted from
182 SVV-infected IBRS-2 cells and SVV were diluted 10 times and then incubated with an
183 SVV neutralizing antibody for 1.5 h. Exosomes and SVV were inoculated into IBRS-2
184 cells, and the copy number of SVV in the inoculated cells was detected by qRT-PCR
185 (Fig. 7).

186 **DISCUSSION**

187 Exosomes are small vesicles that are secreted by cells (13). At present, there is no
188 fixed standard for the separation and identification of exosomes. Many different
189 methods are used for the isolation and identification of exosomes (14), such as UC,
190 gradient UC, co-precipitation, size-exclusion chromatography, NTA electron
191 microscopy (EM) analysis, and protein identification by CD63, CD9, CD81, and ALIX
192 expression. However, UC combined with exosome marker protein-labeled magnetic
193 bead purification is considered the gold standard method for exogenous separation and
194 purification (7, 14, 15).

195 In this study, exosomes were extracted from SVV-infected and normal IBRS-2
196 cells, and the exosomes were identified by WB, NTA, TEM, and qPCR. According to

197 the results, the extracted exosomes contained CD63, CD9, and ALIX, which are protein
198 markers of exosomes. In addition, their diameters were about 40–150 nm, and the
199 cup-like structure of the membrane was observed using TEM. The identification of
200 exosomes extracted from the IBRS-2 cells was consistent with the results that are
201 reported in the literature. Furthermore, the exosomes extracted from the cells of the
202 SVV-infected group contained the SVV nucleic acid sequence, which was determined
203 by qPCR.

204 In the current study, we suggest that exosomes can serve as a delivery vector for
205 pathogen-associated molecules, which help pathogenic microorganisms to spread
206 infections throughout the body's microenvironment (16). In addition, their role in viral
207 infections is receiving more and more attention (17, 18). Some viruses are included in
208 the exosomes for transmission, which may be an important way to escape the immune
209 response (19). During the process of exosome formation, some viruses enter the cell
210 through exosomes, mainly in the endocytic pathway, which can deliver the virus
211 directly into the cell without the need for cell membrane receptors (12, 19, 20). In our
212 study, we isolated exosomes with DIL staining and introduced them into IBRS-2 and
213 293T cells. We found that the exosomes were able to re-enter the cells, validating the
214 activity of the exosomes. Then, we inoculated SVV and exosomes with the same copy
215 number of SVV into IBRS-2 and 293T cells and found that SVV contained within
216 exosomes proliferated in IBRS-2 and 293T cells. However, the copy number in 293T
217 cells was lower than that in IBRS-2 cells. Therefore, exosomes extracted from IB cells
218 can enter IB and 293T cells and mediate the spread of SVV.

219 The aforementioned results suggest that exosomes can mediate the spread of SVV,
220 but do exosomes play an important role in the spread of SVV in host cells?
221 Furthermore, does the inhibition of exosome secretion affect the proliferation of
222 exosomes? Studies have shown that silencing the expression of Rab27a and Rab27b
223 reduces exosome secretion of CD63, CD81, and MHC class II, and the downregulation

224 of Rab27 effector Slp4 and Slac2b also inhibits exosome secretion (21, 22). We
225 synthesized RAB27a siRNA and transfected it into IBRS-2 cells and then detected the
226 expression level of RAB27a miRNA and ALIX. The number of intracellular
227 exosomes was significantly upregulated after the downregulation of RAB27a
228 expression. The copy number of intracellular SVV increased significantly, whereas the
229 copy number of extracellular SVV decreased significantly after RAB27a interference.
230 GW4869 inhibits exosome formation by inhibiting neutral sphingomyelinase 2 (23).
231 We treated SVV-infected IBRS-2 cells with different doses of GW4869 and measured
232 the copy number of SVV *in vitro* and *in vivo*. Unlike with si-RAB27a, the number of
233 copies of intracellular SVV decreased in a dose-dependent manner after the treatment
234 of cells with GW4869. According to our current experimental results, it cannot be
235 explained why the intracellular SVV copy number was significantly increased after
236 RAB27a interference but decreased after GW4869 treatment. We hypothesize that this
237 may be related to the different mechanisms by which these two inhibitors block
238 exosome formation and secretion. In any case, the copy number of extracellular SVV
239 was decreased following the treatment with these two different methods.

240 *In vitro* infected EV71-isolated exosomes are rich in miRNA-146a, and
241 miRNA-146a can inhibit type I interferon and promote EV71 replication (24). In our
242 study, exosomes were found to promote SVV replication. We treated IB-RS cells with
243 SVV-exosomes and mock-exo at the same protein dose and then inoculated SVV. We
244 found that after treatment with SVV-exosomes and mock-exosomes at 24 and 48 h of
245 SVV infection, the copy number of SVV was higher than that of SVV infection, and it
246 was more significant at 24 h. Our current research is insufficient to explain how
247 exosomes promote the proliferation of SVV.

248 Studies have shown that viruses carried by exosomes, such as PRRSV, HCV, and
249 EV71, cannot be neutralized by neutralizing antibodies (25-30). It is well established
250 that some infected pigs have high levels of antibodies, but the virus in the body can be

251 detected, which may be related to the protection of exosomes. According to our current
252 research, SVV carried in exosomes was not neutralized by SVV neutralizing antibodies,
253 which is consistent with the results reported in related literature. This also suggests an
254 important way for the virus to enter into exosomes, thereby evading the body's immune
255 response.

256 In conclusion, we successfully extracted, purified, and identified exosomes from
257 SVV-infected IBRS-2 cells and determined that exosomes can carry SVV, which allows
258 the proliferation of the virus in susceptible and non-susceptible cells. The inhibition of
259 exosome secretion and production inhibits the replication of SVV. Moreover, exosomes
260 extracted from IBRS-2 cells can promote the proliferation of SVV. SVV carried in
261 exosomes cannot be neutralized by SVV neutralizing antibodies. Taken together, these
262 datas revealed an advanced and novel mechanism for better understanding that viral
263 transmission through exosomes contributes to the known immune evasive properties
264 of SVV.

265 **MATERIALS AND METHODS**

266 **Cell culture**

267 To obtain a cell culture supernatant for exosome extraction, we used IBRS-2
268 cells as a model. IBRS-2 cells were cultured in Dulbecco's modified Eagle's medium
269 (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin,
270 and 100 mg/mL streptomycin. The cells were cultured in an incubator maintained at
271 37 °C and with a CO₂ concentration of 5%.

272 **SVV infection and cell culture supernatant collection**

273 In order to obtain exosomes secreted by SVV-infected cells, we inoculated SVV
274 into IBRS-2 cells and collected the supernatants at specific times after infection. SVV
275 was isolated previously, as described later in the text, and preserved by our lab (China
276 Reference Laboratory Network for FMD) (31). IBRS-2 cells were incubated in a

277 150-mm culture dish until they became confluent (Corning, New York, USA). The
278 culture supernatant was then discarded, the cells were washed with PBS, and FBS-free
279 DMEM was added. SVV (0.05 TCID₅₀) was inoculated, and PBS was used as a control.
280 After 1 h of incubation, SVV was discarded and replaced with DMEM containing 2%
281 exosome-depleted FBS. The cell culture supernatant was collected after 36 h of culture.

282 **Exosome isolation and purification**

283 In order to further separate and purify the collected supernatant, we performed
284 differential centrifugation with the collected supernatant. All of the following
285 centrifugation processes were carried out in a 4 °C environment. The collected
286 supernatant was centrifuged at 500 × g for 5 min to remove larger fragments and cells,
287 and then the supernatant was collected and centrifuged at 2,000 × g for 10 min to
288 further remove cell debris. To remove any cells, the collected supernatant was
289 centrifuged at 12,000 × g for 45 min. The large vesicles were collected and filtered
290 through a 0.22-µm filter. Finally, the collected supernatant was centrifuged at
291 120,000 × g for 2 h with an ultracentrifuge (Thermo Scientific Sorvall WX100), and
292 the contents at the bottom of the centrifuge tube were resuspended in 500 µL of PBS.
293 To further purify the extracted exosomes, we used a CD63 antibody-labeled exosomes
294 isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany).

295 **Transmission electron microscopy (TEM)**

296 Direct morphological observation of the characteristics of exosomes is an
297 important method for exosome identification (Shao et al., 2018). Therefore, we
298 analyzed the extracted exosomes using TEM (Hitachi H-7000FA, Tokyo, Japan). After
299 observation, we first extracted the exosomes with a TEM 200 copper mesh (EMS
300 80100-Cu US) and then stained the exosomes with phosphoric acid dock for 2 min.
301 After drying under an incandescent lamp, the electron microscope was used to observe
302 the extracted exosomes, and the observed voltage was 80 kV.

303 **Western blot analysis**

304 Western blot (WB) analysis was performed using the following protocols. Briefly,
305 purified exosomes were lysed with a radio-immunoprecipitation assay buffer (Santa
306 Cruz Biotechnology, Dallas, TX, USA), and the cleared lysate was collected by
307 centrifugation for protein separation on 12% sodium dodecyl sulfate-polyacrylamide
308 gel electrophoresis gels. After electrophoresis, the separated proteins were transferred
309 onto 0.45 μm polyvinylidene difluoride membranes (Millipore, USA). Next, the
310 membranes were blocked for 1 h with 10% fat-free milk in Tris-buffered saline
311 containing Tween 20 (TBST). The blots were then incubated with primary antibodies at
312 4 °C overnight. Primary antibodies for CD63 (Abcam, Cambridge, UK), CD9 (Abcam,
313 Cambridge, UK), and Alix (Cell Signaling Technology, Waltham, MA, USA) were
314 used. After washing three times with TBST, the membranes were incubated with
315 horseradish peroxidase-labeled secondary antibodies (Proteintech, Chicago, IL, USA)
316 for 2 h at room temperature. Finally, the proteins were visualized with a clarity
317 enhanced chemiluminescence WB substrate (Bio-Rad Laboratories, Hercules, CA,
318 USA).

319 **Analysis and quantification of SVV RNA**

320 For the PCR detection of SVV RNA, total RNA from exosomes and cells was
321 extracted with a total exosome RNA and protein isolation kit (Life Technologies, USA)
322 according to the manufacturer's instructions. To quantify the RNA copies of SVV in
323 SVV-infected or exosome-treated cells, total RNAs from cell culture samples were
324 isolated with the E.Z.N.A. total RNA kit I (Omega Bio-tek). Detection of the number of
325 copies of extracted RNA was performed using the Real-Time One-Step RT-PCR
326 reagent (Takara). The following was the reaction system: 2X One-Step RT-PCR Buffer
327 III 10 μL , TaKaRa Ex Taq HS (5 U/ μL) 0.4 μL , Prime Script RT Enzyme Mix II
328 0.4 μL , PCR forward primer (10 μM) 0.4 μL , PCR reverse primer (10 μM) 0.4 μL ,
329 SVV-3D probe 0.8 μL , total RNA 2 μL , and RNase-free dH₂O 5.2 μL (PCR primers

330 and the SVV-3D probe were provided by our laboratory). The reaction times and
331 temperatures of the PCR were 42 °C for 15 min (1 cycle) and 40 cycles of 94 °C for
332 10 s, 57 °C for 30 s, and 72 °C for 30 s. The Applied Biosystems 7300 Real-Time
333 PCR System (Thermo Fisher) was used.

334 **Nanoparticle tracking analysis (NTA)**

335 The mean size and size distribution profile of exosomes that were isolated and
336 purified from SVV-infected or control-treated IBRS-2 cell culture supernatants were
337 analyzed, as described previously (26, 32). In brief, exosomes were diluted 100-fold
338 with PBS prior to analysis, and the relative concentration was calculated on the basis of
339 the dilution factor. Data analysis was performed with NTA 3.2 software (Malvern
340 Panalytical Ltd., Malvern, Worcestershire, UK), and the samples evaluated using a
341 Nanosight NS300 instrument (Malvern Panalytical Ltd., Malvern, Worcestershire, UK).
342 Each sample was analyzed five times, and the counts were averaged.

343 **Fluorescence localization of exosomes**

344 The exosomes extracted from SVV- green fluorescence protein (GFP)-infected cells
345 and normal cells were purified using an exosome purification kit (Miltenyi Biotec,
346 Bergisch Gladbach, Germany), and the copy number of SVV was detected. The
347 exosomes were stained with DIL and washed twice by ultracentrifugation (UC).
348 IBRS-2 and 293T cells were plated in a 20 mm laser confocal dedicated cell culture
349 dish (Thermo Scientific Nunc). Exosomes and SVV-GFP with the same number of
350 SVV copy numbers were inoculated into cells and incubated for 8 h. The cells were
351 then fixed with paraformaldehyde, stained with DAPI, and sealed to avoid light during
352 the aforementioned experimental process. The uptake of the fluorescently labeled SVV
353 in IBRS-2 and 293T cells was visualized with a fluorescence microscope (Leica,
354 Germany).

355 **Si-RAB27a transfection and quantification of RAB27a and Alix miRNA**

356 RAB27a interfering RNA (100 pmol) was transfected into IBRS-2 cells using
357 liposome 2000. SVV was inoculated 24 h after transfection, and cells were harvested
358 24 h after SVV inoculation. Total RNAs from cell culture samples were isolated with
359 the E.Z.N.A. total RNA kit I (Omega Bio-tek). The reverse transcription of RNA into
360 cDNA was performed using a Prime Script™ RT Master Mix (Takara). The following
361 was the reaction system: 5X PrimeScript RT Master Mix (Perfect Real Time) 2 µL,
362 total RNA 5 µL (200 ng), and RNase-free dH₂O 3 µL. The reaction temperature and
363 time of the PCR were 37 °C for 15 min and 85 °C for 5 s (reverse transcription
364 reaction), respectively. Using GAPDH as an internal reference gene, qRT-PCR was
365 performed using TB Green™ Premix Ex Taq™ II (Takara). The following was the
366 reaction system: TB Green Premix Ex Taq II (Tli RNaseH Plus) 10 µL, PCR forward
367 primer (10 µM) 0.8 µL, PCR reverse primer (10 µM) 0.8 µL, DNA (<100 ng) 2 µL, and
368 sterilized water 6.4 µL.

369 **Exosome treatment with a SVV-specific neutralizing antibody**

370 IBRS-2 cells were plated into 12-well cell culture plates, and the cells were
371 replaced with serum-free DMEM once the cells reached 70%–80% confluency.
372 Exosomes extracted from SVV-infected cells and SVV were simultaneously diluted to
373 obtain concentrations ranging from 10⁻¹ to 10⁻⁴. The diluted exosomes and SVV were
374 incubated with the SVV neutralizing antibody for 1.5 h at 37 °C and then added to the
375 prepared IBRS-2 cells. At the same time, exosomes and SVVs that were not incubated
376 with the SVV neutralizing antibody were used as controls. The cells were cultured in a
377 5% CO₂ cell culture incubator at 37 °C for 24 h. The cells and culture supernatants
378 were used to detect the copy number of SVV.

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494 **FIGURE LEGENDS**

495 **Fig 1: Isolation and characterization of exosomes extracted from SVV-infected**

496 **IBRS-2 cells.** (a) Transmission electron microscopic observation of exosomes

497 extracted from SVV-infected IBRS-2 cells after negative staining with phosphotungstic

498 acid. (b) Exosomes were extracted from SVV-infected IBRS-2 cells, purified, and

499 identified by western blot with antibodies directed against Alix, CD9, and CD63. (c)

500 SVV RNA genomic RNAs were identified in exosomes that were extracted from

501 SVV-infected IBRS-2 cells (Exo-svv). At the same time, SVV, SVV-infected IBRS-2

502 cells (Cell-svv), normal IBRS-2 cells (Cell-mock), and exosomes extracted from

503 normal IBRS-2 cells (Exo-mock) were used as controls. (d) Histogram displaying the

504 mean size and size distribution profile of exosome particles isolated from the culture

505 supernatants of SVV-infected IBRS-2 cells by the NTA method.

506

507 **Fig 2: Exosomes mediate the spread of SVV in susceptible and non-susceptible**

508 **cells.** (a) Exosomes extracted from SVV-GFP-infected cells were stained with DIL,

509 washed twice by ultracentrifugation, and incubated in 293T and IBRS-2 cells. Eight

510 hours after incubation, the nuclei were stained with DAPI, and fluorescence was

511 observed. (b) Exosomes extracted from SVV-infected IBRS-2 cells (SVV-exo) were

512 incubated with 293T and IBRS-2 cells, and at the same time, exosomes extracted from

513 normal IBRS-2 cells, SVV venom with the same viral load as SVV-exo, and PBS were

514 used as controls. The virus copy number was detected by qRT-PCR 24 h after

515 incubation.

516

517 **Fig 3: Changes in copy number of SVV.** (a) Si-RAB27a was transfected into IBRS-2
518 cells, which were also infected with SVV, and the expression of RAB27a mRNA in
519 IBRS-2 cells was detected by qRT-PCR. (a) Si-RAB27a was transfected into IBRS-2
520 cells, which were also infected with SVV, and the expression of Alix mRNA was
521 detected by qRT-PCR. (c) Si-RAB27a was transfected into IBRS-2 cells, which were
522 also infected with SVV, and the copy number of intracellular SVV in IBRS-2 cells was
523 detected by qRT-PCR. (c) Si-RAB27a was transfected into IBRS-2 cells, which were
524 also infected with SVV, and the copy number of extracellular SVV in IBRS-2 cells was
525 detected by qRT-PCR. Significance was calculated using a two-tailed t-test and labeled
526 as $*P < 0.05$ and $**P < 0.01$ in graphs.

527

528 **Fig 4: The number of exosomes secreted by IBRS-2 cells was detected after**
529 **interfering with RAB27a.** Si-RAB27a (100 pmol) was transfected into IBRS-2 cells,
530 which were also infected with SVV. Exosomes were extracted from the culture
531 supernatants of SVV-infected IBRS-2 cells, and the number of exosomes was detected
532 by the NTA method.

533

534 **Fig 5: Inhibition of exosome release impairs SVV transmission mediated by**
535 **exosomes.** IBRS-2 cells were infected with SVV for 1.5 h and then incubated with 1, 5,
536 and 10 μmol of GW4869 for 36 h. DMSO was used as a control, and cells without any
537 treatment were used as a negative control. (a) The intracellular SVV copy number was
538 detected. (b) The extracellular SVV copy number was detected.

539

540 **Fig 6: Exosomes promote the proliferation of SVV in IBRS-2.** Exosomes were

541 extracted from SVV-infected IB cells and cells without any treatment, and the protein
542 concentration was measured. The extracted exosomes were divided into a high-dose
543 group and a low-dose group and then re-inoculated into IB-SI-2 cells. After 8 h of
544 incubation, exosomes were replaced with SVV, cells and culture supernatants were
545 collected at 24 and 48 h after inoculation, and the copy number of SVV was detected. (a)
546 The exosome protein concentration was inoculated at a dose of 25 ng, and the viral load
547 was measured 24 h after SVV infection. (b) The exosome protein concentration was
548 inoculated at a dose of 25 ng, and the viral load was measured 48 h after SVV infection.
549 (c) The exosome protein concentration was inoculated at a dose of 50 ng, and the viral
550 load was measured 24 h after SVV infection. (d) The exosome protein concentration
551 was inoculated at a dose of 25 ng, and the viral load was measured 48 h after SVV
552 infection. Significance was calculated using a two-tailed t-test and labeled as $*P < 0.05$
553 and $**P < 0.01$ in graphs.

554

555 **Fig 7: Exosome-mediated SVV infection is not blocked by SVV-specific**
556 **neutralizing antibodies (NAbs).** Purified SVV-positive exosomes and SVV were
557 diluted and then the diluted exosomes and SVV were incubated with SVV-specific
558 NAbs separately (the titer of serum neutralization against SVV was $>1:1024$,
559 determined by VNT) for 1 h. Exosomes and SVV without any treatment were used as a
560 control. Then, PK-15 cells were exposed to the NAbs-treated exosomes or SVV for 2 h.
561 The exosomes or viruses were washed off with PBS at 37 °C, and the medium was
562 replaced with fresh maintenance medium for 24 h. qRT-PCR was used to evaluate SVV
563 replication in IBRS-2 cells.

564

565

566

Fig.1

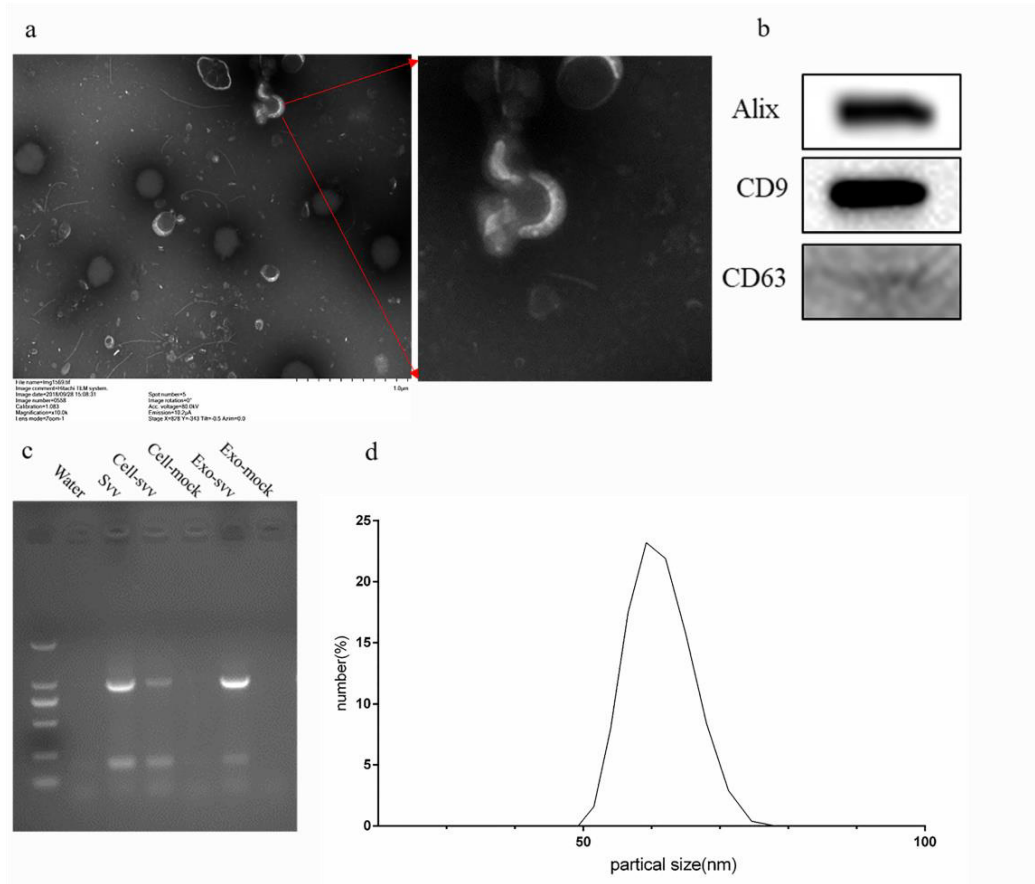


Fig.2

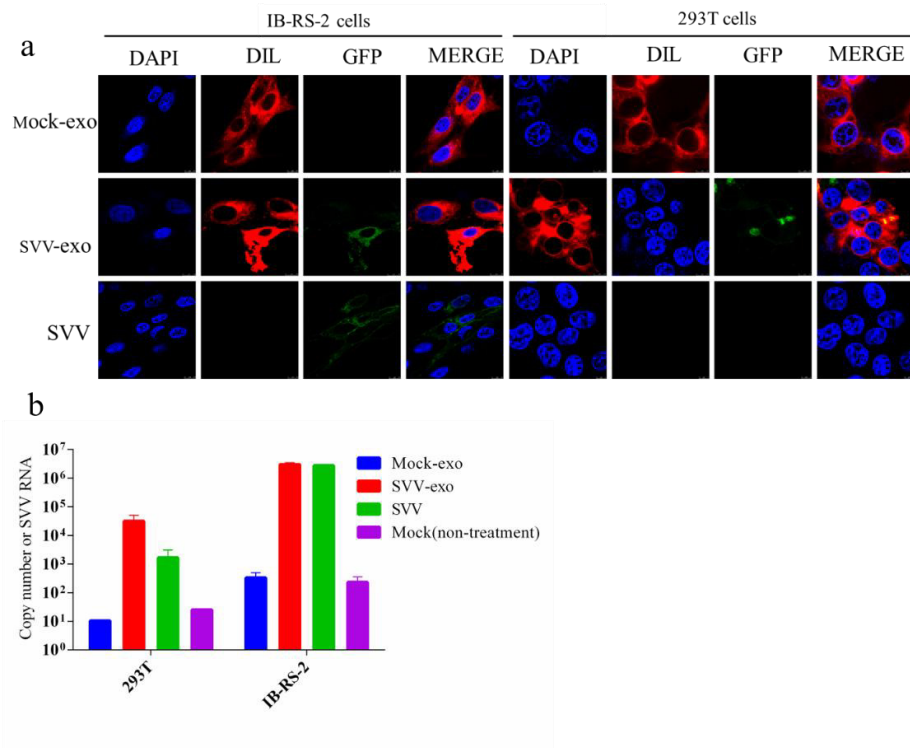


Fig.3

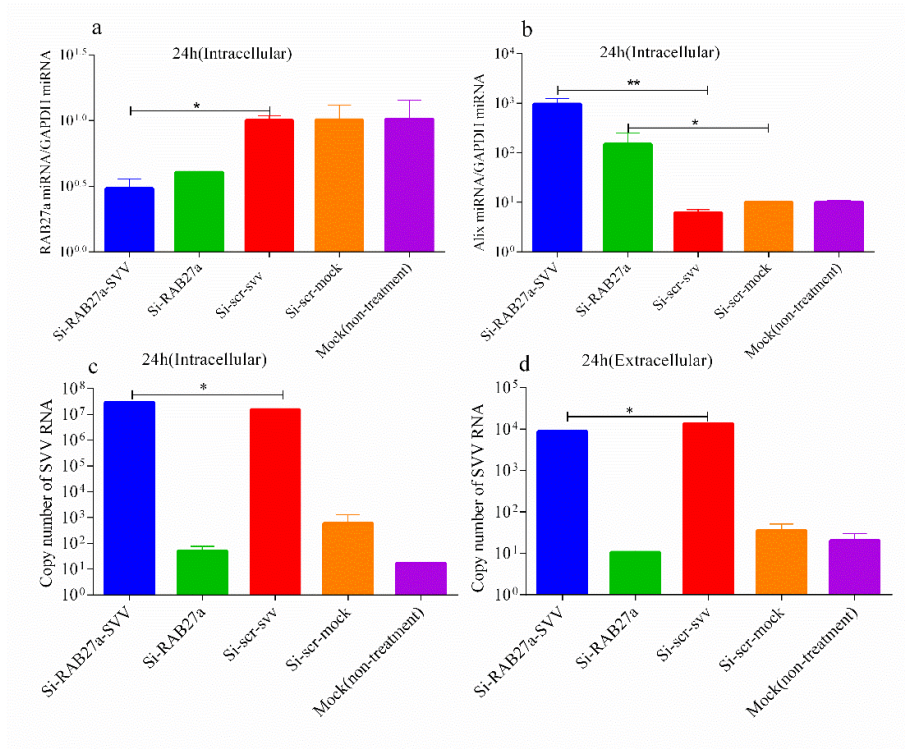


Fig.4

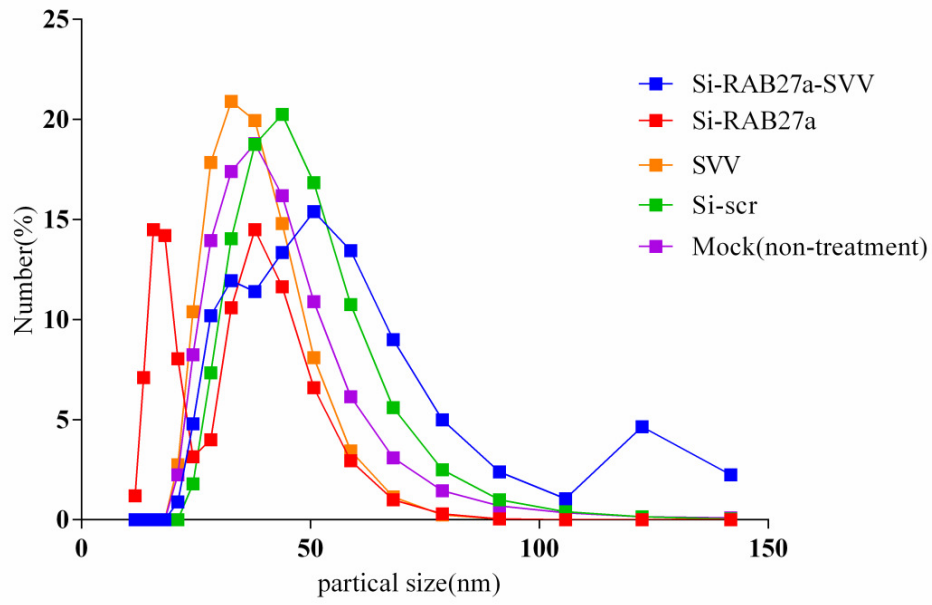


Fig.5

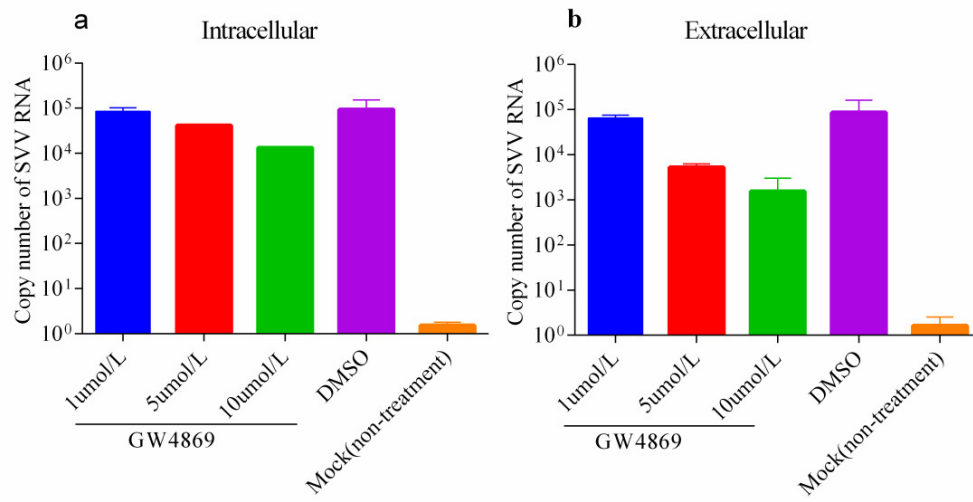


Fig.6

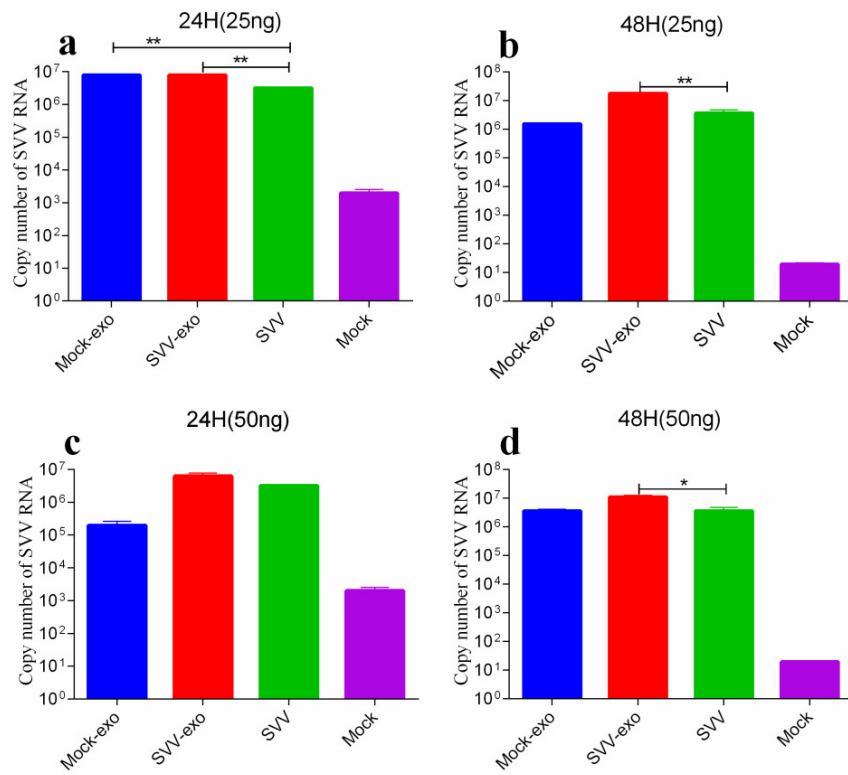


Fig.7

