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Development of *Rous sarcoma* Virus-like Particles Displaying hCC49 scFv for Specific Targeted Drug Delivery to Human Colon Carcinoma Cells

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Running Head: Specific drug delivery to LS174T cells using RSV VLPs

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1 **ABSTRACT**

2 **Purpose:** Virus-like particles (VLPs) have been used as drug carriers for drug delivery
3 systems. In this study, hCC49 single chain fragment variable (scFv)-displaying *Rous*
4 *sarcoma* virus-like particles (RSV VLPs) were produced in silkworm larvae to be a
5 specific carrier of an anti-cancer drug.

6 **Method:** RSV VLPs displaying hCC49 scFv were created by the fusion of the
7 transmembrane and cytoplasmic domains of hemagglutinin from influenza A (H1N1)
8 virus and produced in silkworm larvae. The display of hCC49 scFv on the surface of
9 RSV VLPs was confirmed by enzyme-linked immunosorbent assay using tumor-
10 associated glycoprotein-72 (TAG-72), fluorescent microscopy, and immunoelectron
11 microscopy. Fluorescein isothiocyanate (FITC) or doxorubicin (DOX) was incorporated
12 into hCC49 scFv-displaying RSV VLPs by electroporation and specific targeting of
13 these VLPs was investigated by fluorescent microscopy and cytotoxicity assay using
14 LS174T cells.

15 **Results:** FITC was delivered to LS174T human colon adenocarcinoma cells by
16 hCC49 scFv-displaying RSV VLPs, but not by RSV VLPs. This indicated that hCC49
17 scFv allowed FITC-loaded RSV VLPs to be delivered to LS174T cells. DOX, which is
18 an anti-cancer drug with intrinsic red fluorescence, was also loaded into hCC49 scFv-
19 displaying RSV VLPs by electroporation; the DOX-loaded hCC49 scFv-displaying
20 RSV VLPs killed LS174T cells via the specific delivery of DOX that was mediated by
21 hCC49 scFv. HEK293 cells were alive even though in the presence of DOX-loaded
22 hCC49 scFv-displaying RSV VLPs.

23 **Conclusion:** These results showed that hCC49 scFv-displaying RSV VLPs from
24 silkworm larvae offered specific drug delivery to colon carcinoma cells in vitro. This
25 scFv-displaying enveloped VLP system could be applied to drug and gene delivery to

26 other target cells.

27 **KEY WORDS:** drug delivery • colon carcinoma • *Rous sarcoma* virus-like particles •

28 silkworm • doxorubicin

29

30 **ABBREVIATIONS**

| | | |
|----|-------------------|--|
| 31 | CLSM | confocal laser scanning microscope |
| 32 | BmNPV | <i>Bombyx mori</i> nucleopolyhedrovirus |
| 33 | BmNPV/RSV-gag-577 | BmNPV bacmid encoding the RSV gag protein gene |
| 34 | BSA | Bovine serum albumin |
| 35 | DOX | doxorubicin |
| 36 | DLS | dynamic light scattering |
| 37 | ELISA | enzyme-linked immunosorbent assay |
| 38 | FITC | fluorescein isothiocyanate |
| 39 | gag | group antigen protein |
| 40 | GPI | glycosylphosphatidylinositol |
| 41 | HA | hemagglutinin |
| 42 | hCC49 | humanized CC49 antibody |
| 43 | HRP | horseradish peroxidase |
| 44 | MTT | 3-(4,5-di-methylthiazol-2-2yl)-2,5-diphenyltetrazolium bromide |
| 45 | PBS | phosphate-buffered saline |
| 46 | RSV | <i>Rous sarcoma</i> virus |
| 47 | RSV VLPs | <i>Rous sarcoma</i> virus-like particles |
| 48 | scFv | single-chain variable fragment |
| 49 | TAG-72 | tumor associated glycoparticle-72 |
| 50 | VLPs | virus-like particles |

51 **INTRODUCTION**

52 Virus-like particles (VLPs) derived from various viruses have been utilized for vaccines, as
53 well as gene and drug delivery systems. VLPs have nearly the same properties as intact viruses,
54 but they have no genomic DNA or RNA that encodes viral proteins. Therefore, safety concerns
55 related to the use of inactivated or attenuated viruses can be mitigated, especially for in vivo
56 applications of VLPs. Additionally, VLPs have empty interior space in which various materials,
57 drugs, nucleic acids, and nanoparticles can be loaded for gene and drug delivery (1, 2). Non-
58 enveloped VLPs can be produced through the expression of a viral capsid protein. Expressed
59 capsids are self-assembled and form VLPs. These VLPs sometimes have nucleic acids derived
60 from host cells, but the nucleic acids can be removed by the disassembly of VLPs. The
61 disassembly and reassembly of VLPs ensures a uniform size of VLPs (3). Enveloped VLPs
62 have a lipid membrane and an envelope that are derived from host cells when the VLPs self-
63 assemble and bud from host cells. When enveloped VLPs are expressed simultaneously with
64 membrane proteins, the membrane proteins are embedded into the envelope of VLPs during
65 the budding process (2).

66 VLPs can be functionalized through various methods to provide specificity as nanoparticles.
67 The surface and the interior of VLPs can be specialized for functions including cell specificity,
68 display of immunological antigens, and stabilization by chemical and genetic modification.
69 The surface of non-enveloped VLPs can be functionalized by a covalent approach through
70 amino acid residues on the surface, including lysine, cysteine, and others (4). This covalent
71 modification is an irreversible reaction, which is favorable for long-term binding to targets.
72 Alternatively, peptides and proteins can be displayed on the surface of non-enveloped VLPs by
73 using a fusion technique with virus capsid proteins. Enveloped VLPs can also be functionalized
74 chemically and genetically through the modification of envelope proteins. The fusion of a
75 foreign protein with a full-length or transmembrane domain of a viral envelope protein enables

76 its protein to be displayed on the surface of enveloped VLPs (5, 6). Additionally, foreign
77 transmembrane proteins can be displayed on enveloped VLPs by their co-expression in hosts,
78 which allows for multifunctional VLPs (1, 7).

79 Recently, *Rous sarcoma virus* (RSV) VLPs displaying a scFv of humanized CC49 antibody
80 (hCC49) were produced in silkworm larvae in order to target the specific delivery of drugs to
81 colon carcinoma cells (8). In this case, hCC49 scFv was linked by glycosylphosphatidylinositol
82 (GPI) anchor on the surface of RSV VLPs. These particles specifically delivered
83 sulforhodamine B to colon carcinoma cells, LS174T cells. In other paper, using RSV gag
84 protein and M1 protein from influenza A virus, chimeric VLPs were produced in silkworm
85 larvae and applied to drug delivery system and vaccine production (9). Modification of RSV
86 VLPs can provide these particles various capacities.

87 In this study, RSV VLPs displaying hCC49 scFv by using transmembrane and cytoplasmic
88 domains of hemmagglutinin (HA) from influenza A virus were produced in silkworm larvae for
89 the application to drug delivery system, instead of the use of GPI anchor reported previously
90 (8). The hCC49 scFv binds specifically to tumor-associated glycoprotein-72 (TAG-72) on the
91 surface of colon carcinoma cells (10, 11). TAG-72 is also expressed very low level in human
92 adenocarcinomas of the colon, pancreas, and breast, but it is not expressed in normal tissues
93 (12). Doxorubicin (DOX) was used as an anti-cancer drug to be delivered to colon carcinoma
94 cells by hCC49 scFv-displaying RSV VLPs.

95

96 **MATERIALS AND METHODS**

97 *Cell Lines, Media, and Silkworms*

98 LS174T human colon adenocarcinoma (ATCC CL-188) and HEK293 (RCB1637) cell lines
99 were purchased from ATCC (Manassas, VA, USA) and Riken Bio Resource Center (Tsukuba,

100 Ibaraki, Japan), respectively. LS174T cells were cultured in 25-cm² T-flasks with MEM-Eagle
101 medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% (v/v) fetal bovine serum
102 (Invitrogen, Carlsbad, CA, USA) and supplemented with 1% (v/v) antibiotic solution
103 containing penicillin, streptomycin, and fungizone (Sigma-Aldrich). The cultures were placed
104 in an incubator (MCO-175 Sanyo, Osaka, Japan) maintained at 37°C with 5% CO₂. HEK293
105 cells were cultured in 25-cm² T-flasks with MEM/EBSS medium (HyClone Laboratories Inc.,
106 Utah, USA) containing 2 mM L-glutamine, 1% non-essential amino acid solution (Invitrogen),
107 and 10% fetal bovine serum and supplemented with 1% (v/v) antibiotic solution containing
108 penicillin, streptomycin, and fungizone. The cultures were placed in an incubator maintained
109 at 37°C with 5% CO₂.

110 Molting fourth instars of silkworm larvae were purchased from Ehimesansyu (Yahatahama,
111 Ehime, Japan). Silkworm larvae were reared on an artificial diet of Silkmate 2S (Nihon Nosan
112 Kogyo, Yokohama, Japan) in a 60% humidity chamber (MLR-351H, Sanyo, Tokyo, Japan)
113 maintained at 25°C.

114 *Construction of Recombinant Bacmids*

115 The construction of the *Bombyx mori* nucleopolyhedrovirus (BmNPV) bacmid for the
116 expression of the RSV gag protein (BmNPV/RSV-gag-577) was described previously (13). The
117 scFv of hCC49 was amplified by PCR using Eco-bx-FLAG-hCC49scFv and scFv-spe primers
118 (Table 1) with pROX-FL92amber (hCC49) (kindly donated by Professor Hiroshi Ueda, Tokyo
119 Institute of Technology) as a template. Also, the C-terminal domain of HA was amplified by
120 PCR using Spe-H1N1 and H1N1-Hind primers (Table 1) with Influenza A (H1N1, A/New
121 Caledonia/20/99) HA cDNA clone (Sino Biological Inc., Beijing, China) as a template. The
122 scFv fragments were digested by *EcoRI* and *SpeI*, and the C-terminal domain of HA was
123 digested by *SpeI* and *HindIII*. The digested fragments were ligated into the *EcoRI* – *HindIII*

124 site in the pFastBac1 vector. The constructed vector was transformed into *E. coli* BmDH10Bac
125 (14). The recombinant BmNPV (BmNPV/hCC49-scFv) bacmid was extracted from a white
126 transformant.

127 *Production and Purification of RSV VLPs in Silkworm Larvae*

128 Each recombinant BmNPV bacmid was mixed in a 1:1 ratio with a total of 10 µg DNA. The
129 bacmid mixture was then mixed with 1/10 volume of DMRIE-C reagent (Life Technologies
130 Japan, Tokyo, Japan) and incubated at room temperature for 30 min. This mixture was injected
131 into the fifth instars of silkworm larvae and these larvae were reared for 6 to 7 days.
132 Hemolymph was collected from bacmid-injected larvae and 1-phenyl-2-thiourea (5 mM) was
133 added to the collected hemolymph. The hemolymph was diluted with phosphate-buffered
134 saline (PBS, pH 7.4) and loaded onto a 25% sucrose cushion; it then was centrifuged to collect
135 VLPs. Pelleted VLPs were suspended with PBS by brief sonication. Sucrose density gradient
136 centrifugation (25–60%) was performed to obtain the VLPs. The top (0.5 ml) fraction was
137 collected from each sample; a total of 10 fractions were collected. These fractions containing
138 VLPs were dialyzed with PBS using a dialysis membrane with a molecular weight cutoff of
139 300 kDa (Spectrum Japan, Shiga, Japan).

140 *Protein Concentration Measurement and Western Blot*

141 Protein concentration was measured using the Reducing Agent Compatible version of the BCA
142 Protein Assay (Thermo Fisher Scientific, Rockford, IL, USA). SDS-PAGE and western blot
143 were conducted as described in previous papers (13). For the western blot, mouse anti-FLAG
144 M2 antibody (Sigma-Aldrich) or mouse anti-DYKDDDDK tag antibody (Wako Pure Chemical
145 Industries, Osaka, Japan) was used as the primary antibody to detect hCC49 scFv. Sheep anti-
146 mouse IgG antibody (GE healthcare Japan, Tokyo, Japan) was also used as a secondary

147 antibody.

148 *Enzyme-linked Immunosorbent Assay*

149 For the enzyme-linked immunosorbent assay (ELISA), TAG-72 antigen from human fluids
150 (Sigma-Aldrich) diluted to 10 U/ml with PBS was loaded into each well of a 96-well plate.
151 Bovine serum albumin (BSA) dissolved with PBS to a final concentration of 10 µg/ml was
152 used as a negative control. The plates were blocked with 2% skimmed milk dissolved with PBS
153 at room temperature for 2 h. Each well was washed three times with PBS and then samples
154 diluted with 2% skimmed milk were added into each well; the plate was then incubated at room
155 temperature for 1 h. Each well was washed three times with PBS and then horseradish
156 peroxidase (HRP)-conjugated anti-FLAG M2 antibody (Sigma-Aldrich) diluted 5000-fold with
157 2% skimmed milk was added. The plate was incubated at room temperature for 1 h and washed
158 with PBS. Then, the HRP reaction was performed to measure absorbance at 450 nm in each
159 well.

160 *Immunogold-labeling in Transmission Electron Microscopy and Dynamic Light* 161 *scattering (DLS) measurement*

162 The hCC49 scFv-displaying RSV VLPs were placed on carbon grids (Okenshoji, Tokyo, Japan)
163 and dried at room temperature. The grids were blocked with 4% BSA for 1 h and washed with
164 PBS. The grids were incubated with 100 fold-diluted mouse anti-DYKDDDDK monoclonal
165 antibody (Wako) for 2 h and then washed with PBS. The grids were then incubated with 200-
166 fold diluted goat polyclonal anti-mouse IgG+IgM (H+L) conjugated with 10-nm gold particles
167 (BB International, Cardiff, UK) for 2 h and washed with PBS. Negative staining was performed
168 using 2% phosphotungstic acid and VLPs were observed using a transmission electron
169 microscope (JEM2100F-TEM, JEOL, Tokyo, Japan).

170 For DLS measurement, the samples were loaded onto disposable cuvettes (DTS-1061) for
171 the measurement of size with the Zetasizer Nano series (Malvern, Worcestershire, United
172 Kingdom).

173 *Loading Fluorescein isothiocyanate (FITC) and DOX into RSV VLPs*

174 FITC, a fluorescent material, and DOX, an anti-cancer drug, were used as loading compounds.
175 To load these materials into RSV VLPs, 100 µg/ml FITC or 50 µg/ml DOX were mixed with
176 0.5 mg of protein/ml RSV VLPs and this mixture was electroporated using the Gene Pulser
177 Xcell system (BIO-RAD, Hercules, CA, USA) (250 V, 750 µF). After electroporation, the
178 mixture was cooled on ice, dialyzed, and concentrated with Amicon Ultra centrifugal filters
179 with a membrane nominal molecular weight limit of 30 kDa (Merck Millipore, Billerica, MA,
180 USA).

181 *Fluorescence Microscopy*

182 For immunofluorescence microscopy, 2000 cells of human LS174T and HEK293 cell lines
183 were seeded onto a slide glass coated with polylysine. Cells were fixed with 10% formaldehyde
184 for 20 min. The slide glass was washed four times with PBS and the remaining formaldehyde
185 was removed using 50 mM NH₄Cl. The slide glass was again washed four times with PBS and
186 then the slide glass was blocked with 4% BSA at room temperature for 2 h. The slide glass was
187 washed four times with PBS and then VLPs were added to cells on the slide glass and incubated
188 at room temperature for 2 h. The slide glass was washed four times with PBS and then
189 incubated with 5000-fold diluted mouse anti-DYKDDDDK tag monoclonal antibody (Wako)
190 at room temperature for 1 h. The slide glass was washed four times and then 1000-fold diluted
191 goat polyclonal anti-mouse IgG (H & L) conjugated with Alexa597 (Abcam, Tokyo, Japan)
192 was added to the cells; the slide glass was incubated at room temperature for 1 h. Finally, the

193 slide glass was washed four times with PBS and the cells were observed using a confocal laser
194 scanning microscope (CLSM) (LSM 700, Carl Zeiss, Oberkochen, Germany).

195 For the analysis of chemical delivery, 2000 cells of human LS174T and HEK293 cell lines
196 were seeded onto an aminosilane-coated slide glass with chambers and cultivated at 37°C for
197 24 h. Culture media in each chamber was discarded and VLP solution was added into each
198 chamber. The slide glass was incubated at 37°C for 24 h and the cells were fixed with 10%
199 formaldehyde for 20 min. The slide glass was washed three times and the remaining
200 formaldehyde was removed with 50 mM NH₄Cl. Finally, 4',6-diamidino-2-phenylindole was
201 added to the cells and the stained cells were observed using CLSM.

202 *3-(4,5-di-Methylthiazol-2-2yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay*

203 2000 cells of human LS174T and HEK293 cell lines were seeded into each well of a 96-well
204 plate and cultivated at 37°C for 48 h. Culture medium was replaced with fresh medium and 10
205 µl of 0.2 mg of protein/ml VLP were added into each well. After 24 h of cultivation, the MTT
206 assay was conducted using the MTT Cell Proliferation/Viability Assay Kit (Trevigen,
207 Gaithersburg, MD, USA). Viability was calculated by the following formula:

$$208 \text{ Cell viability (\%)} = \frac{(A_s - A_b)}{(A_c - A_b)} \times 100$$

209 where A_s , A_b , and A_c denote absorbances of sample, blank, and negative control (cell only).

210

211 **RESULTS**

212 *Co-expression of RSV gag Proteins and hCC49 scFv in Silkworm Larvae*

213 RSV gag protein was expressed in silkworm larvae and formed successfully enveloped VLPs
214 in the hemolymph of silkworm larvae (13). The hCC49 scFv was displayed on the surface of

215 RSV VLPs that specifically targeted colon adenocarcinoma cells. To display this scFv on the
216 VLPs, the C-terminal domain of HA from Influenza A (H1N1), which contains its
217 transmembrane and cytoplasmic domains, was fused at the C-terminus of scFv (Fig. 1). The
218 recombinant BmNPV bacmid harboring these genes under the polyhedrin promoter was
219 constructed. Using BmNPV/RSV-gag-577 and BmNPV/hCC49-scFv bacmids, the gag protein
220 of RSV and hCC49 scFv were co-expressed in silkworm larvae. The gag protein of RSV has
221 been expressed and processed in various forms (13, 15). Most of the RSV gag protein and
222 hCC49 scFv were expressed in the fat body of silkworm larvae (Fig. 2A). After sucrose density
223 gradient centrifugation, RSV gag protein and hCC49 scFv were observed in the purified sample
224 (Fig. 2B).

225 *Characterization of hCC49 scFv-displaying RSV VLPs*

226 To confirm the display of hCC49 scFv on the surface of these VLPs, ELISA,
227 immunoelectron microscopy, and immunofluorescence microscopy were performed using
228 LS174T cells. Incubation of purified hCC49 scFv-displaying RSV VLPs with LS174T cells
229 yielded specific fluorescence around the cells (upper panel of Fig. 3A). However, purified RSV
230 VLPs to LS174 cells did not provided red fluorescence around LS174T cells (lower panel of
231 Fig. 3A). It indicated that hCC49scFv allowed RSV VLPs to bind specifically to TAG-72 on
232 the surface of LS174T cells. The TAG-72 expression was confirmed by western blot
233 (Supplementary Figure 1). In addition, ELISA revealed specific binding of these purified VLPs
234 to TAG-72; the purified VLPs were compared to a negative control using BSA (Fig. 3B). Gold
235 particles were observed on the surface of purified RSV VLPs (Fig. 3C). The average diameters
236 of RSV VLPs and hCC49 scFv-displaying RSV VLPs were approximately 50 nm and 90 nm,
237 respectively (Fig. 3D). These results indicated that hCC49 scFv was displayed on the surface
238 of hCC49 scFv-displaying RSV VLPs.

239 *Fluorescent Compound Loading and Delivery*

240 FITC was loaded into hCC49 scFv-displaying RSV VLPs and used to model delivery to target
241 cells. FITC was loaded into VLPs by electroporation. LS174T cells treated with FITC-loaded
242 hCC49 scFv-displaying RSV VLPs were observed using CLSM and green fluorescence of
243 FITC was observed inside the cells (Fig. 4). However, only slight green fluorescence of FITC
244 was observed in LS174T cells treated with FITC-loaded RSV VLPs. This indicated that the
245 hCC49 scFv allowed RSV VLPs to specifically target LS174T cells and FITC was specifically
246 delivered to these cells.

247 To further investigate the possibility to use hCC49 scFv-displaying RSV VLPs for drug
248 delivery system, DOX-loaded hCC49 scFv-displaying RSV VLPs was prepared. DOX was
249 used as a model of an anti-cancer drug in this study. DOX causes intercalation of DNA in cancer
250 cells, which leads to cancer cell death, but DOX has several adverse effects, including serious
251 heart damage. Specific delivery of DOX to target cells would reduce its adverse effects. DOX
252 can be easily measured by either fluorescent microscope or fluorescence spectrophotometer
253 because it exhibits red fluorescence (ex. 480 nm, em. 575 nm). Approximately 14 µg/ml of
254 DOX (10 µl) was loaded in 200 µg of protein of hCC49 scFv-displaying RSV VLPs by
255 electroporation (incorporation efficiency 0.7%, data not shown). LS174T cells were treated
256 with DOX-loaded hCC49 scFv-displaying RSV VLPs and DOX-loaded RSV VLPs. DOX-
257 loaded RSV VLPs were used as a control. The cytotoxicity of these VLPs was investigated
258 using LS174T cells and HEK293 cells. The viability of LST174T cells treated with DOX-
259 loaded hCC49 scFv-displaying RSV VLPs decreased by 35%, but the viability of HEK293
260 cells treated with the same VLPs remained at 100% (Fig. 5). This indicated that hCC49 scFv-
261 displaying RSV VLPs specifically recognized LS174T cells and delivered DOX to LS174T
262 cells. Non-specific delivery of DOX to HEK293 cells was not observed. In addition, the
263 cytotoxicity of DOX-loaded RSV VLPs to LS174T cells and HEK293 cells was not observed,

264 which indicated that hCC49 scFv displayed on the surface of RSV VLPs recognized its antigen,
265 TAG-72, on the surface of LS174T cells. Free DOX (10 µg/ml, total amount 0.1 µg) killed 18%
266 LS174T cells, but DOX-loaded hCC49 scFv-displaying RSV VLPs (amount of DOX loading:
267 0.14 µg) have the higher cytotoxicity specific to LS174T cells (35%). It indicated that DOX-
268 loaded hCC49 scFv-displaying RSV VLPs was more efficient to kill LS174T cells than free
269 DOX even if the same amount of DOX loaded into hCC49 scFv-displaying RSV VLPs was
270 used as free DOX. In this experiment, it is possible that DOX is adhered to these VLPs on the
271 surface, not loaded inside these VLPs, and delivered to LS174T cells. To exclude this
272 possibility, hCC49 scFv-displaying RSV VLPs, which was purified after just mixing with 50
273 µg/ml DOX, were used as a control. The hCC49 scFv-displaying RSV VLPs mixed with DOX
274 slightly killed LS174T cells (8%), but DOX-loaded hCC49 scFv-displaying RSV VLPs killed
275 more LS174T cells (35%). This result indicated DOX was not adhered to the surface of hCC49
276 scFv-displaying RSV VLPs by mixing with free DOX and was loaded to these VLPs by
277 electroporation.

278

279 **DISCUSSION**

280 In this study, hCC49 scFv-displaying RSV VLPs were produced by the co-expression of RSV
281 gag protein and hCC49 scFv fused with the C-terminal domain of HA in silkworm larvae.
282 Specific delivery of DOX to LS174T cells was completed using these RSV VLPs. When RSV
283 VLPs are prepared using BmNPV bacmid in silkworm larvae, GP64, which is a major
284 baculoviral envelope protein, is displayed on the surface of RSV VLPs (16). GP64 is essential
285 for the transduction of baculoviruses into mammalian cells and is attached to the cell surface
286 and internalized by receptor-mediated endocytosis followed by low pH-triggered membrane
287 fusion (17–19). The interaction of GP64 with phospholipids and cholesterol heparin mediates

288 baculovirus internalization to mammalian cells (20–22). In this study, hCC49 scFv displayed
289 on the surface of RSV VLPs allowed DOX to be specifically delivered to LS174T cells (Figs.
290 4 and 5). DOX was not specifically delivered to LS174T cells using RSV VLPs, even though
291 RSV VLPs have GP64 on the surface (Figs. 4 and 5). These results suggest that the specificity
292 of hCC49 scFv to LS174T cells is high, which enables specific delivery of DOX to LS174T
293 cells without its non-specific delivery via GP64. Antibodies can be also conjugated with VLPs
294 and various nanoparticles, but, sometimes, antibodies lose ligand capacity by promiscuous
295 conjugation with VLPs and nanoparticles.

296 When RSV gag protein was co-expressed with hCC49 scFv fused with the C-terminal region
297 of HA from influenza A virus, no modification steps were needed to obtain hCC49 scFv-
298 displaying RSV VLPs in silkworms. The fusion of hCC49 scFv with the C-terminal domain of
299 HA did not disturb its binding capacity to the antigen and its active scFv on the surface of RSV
300 VLPs specifically bound to colon carcinoma cells, LS174T cells (Fig. 3A and 3B). Antibody-
301 display system on the surface of envelope VLPs using a heterologous transmembrane domain
302 is useful for drug delivery to target cells. This system can be applied to the other expression
303 systems using yeasts, insect cells and mammalian cells instead of silkworms. Especially,
304 monoclonal antibodies against antigens specific to cancer cells have been utilized for drug
305 delivery and tumor cell imaging because of the high specificities to ligands (23). Monoclonal
306 antibodies have the potential to specifically deliver drugs to targeted cells, which could
307 minimize side effects and reduce drug doses. Among monoclonal antibodies, monoclonal scFv,
308 which was also used in this study, is the best choice to be displayed on the surface of
309 nanomaterials including VLPs because of its size and structure.

310 Several methods of loading drugs into enveloped viruses and VLPs have been reported.
311 Electroporation enables carboxylated quantum dots to be loaded into enveloped VLPs (24). In
312 the case of human hepatitis B virus (HBV) particles composed of L protein, the fusion of HBV

313 particles with liposomes containing DNA is more efficient for loading DNA than
314 electroporation (2, 25). In the case of hemagglutinating virus of Japan (HVJ, Sendai virus),
315 detergent treatment, liposome fusion, and electroporation have been applied for the load of
316 drugs, nucleic acids, and nanoparticles (24, 26, 27). For example, detergent treatment of HVJ
317 with centrifugation for plasmid DNA incorporation provides high loading efficiency
318 (approximately 20%) of plasmid DNA into HVJ (28). The loading efficiency of electroporation
319 was 0.7% in this study, but detergent treatment with centrifugation would also be an efficient
320 method for loading various materials, including DOX, into RSV VLPs. In addition, large
321 unilamellar vesicles (LUV) also help DOX and anti-cancer drugs to be loaded into RSV VLPs
322 efficiently (8, 9). However, the use of LUV has more tedious steps than electroporation and
323 detergent treatment.

324 Specific delivery of FITC and DOX to LS174T cells was shown by its fluorescence inside
325 the cells. However, the behavior of these fluorescent materials inside the cells has been still
326 unclear. Viruses can enter into host cells by endocytosis or membrane fusion (29, 30). VLPs
327 can also enter host cells through the same way as viruses. Behaviors of hCC49 scFv-displaying
328 RSV VLPs in LS174T cells and loaded DOX were not determined yet, but DOX seems to reach
329 to nucleus in LS174T cells because DOX-loaded hCC49 scFv-displaying RSV VLPs killed
330 LS174 cells specifically (Fig. 5). However, we have to analyze the internalization pathway of
331 FITC via hCC49 scFv-displaying RSV VLPs or passive transport through lipid bilayer after its
332 release outside the cells from these VLPs. Various endocytosis inhibitors would give the
333 opportunities to reveal the internalization pathway. Alternatively, programmed release system
334 of drugs in cells can carry and release drugs to specific sites in cells and provide more efficient
335 drug delivery to target cells (31, 32).

336 **CONCLUSION**

337 In this study, hCC49 scFv-displaying RSV VLPs were prepared in silkworm larvae using the
338 BmNPV bacmid system. The C-terminal domain of HA from influenza A (H1N1) virus enabled
339 hCC49 scFv to be anchored into the envelope of RSV VLPs. FITC or DOX was loaded into
340 hCC49 scFv-displaying RSV VLPs by electroporation. DOX were delivered to colon
341 carcinoma cells (LS174T cells) by hCC49 scFv-displaying RSV VLPs and killed LS174T cells,
342 but delivery of DOX to the LS174T cells using DOX-loaded RSV VLPs did not confirm.

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432 507.
- 433

434 **Table 1. Primers used**

| Name | 5'-3' |
|-----------------------|--|
| Eco-bx-FLAG-hCC49scFv | CACCATGAAGATACTCCTTGCTATTGCATTAAT GTTGTCAACAGTAATGTGGGTGTCAACAGACT ACAAGGATGACGATGACAAGCAGGTGCAGCT GGTG |
| scFv-spe | CGACTAGTGGATGATATGATGATG |
| Spe-H1N1 | AGACTAGTGAACAATGCCAAGGAGATTG |
| H1N1-Hind | ATAAGCTTTTAATGGTGATGATGGTG |

435

436 **FIGURE LEGENDS**

437 **Figure 1.** Schematic presentation of this study. RSV gag protein and hCC49 scFV
438 fused with the C-terminal region of hemagglutinin from influenza A (H1N1) virus
439 (A/duck/NY/191255-59/02) were simultaneously expressed in silkworm larvae using
440 the BmNPV bacmid system. The hCC49 scFv-displaying RSV VLPs were obtained
441 from collected hemolymph by sucrose density gradient centrifugation. FITC or DOX
442 was loaded into the VLPs by electroporation. Drug delivery to colon carcinoma cells
443 (LS174T cells) was performed using FITC- or DOX-loaded hCC49 scFv-displaying
444 RSV VLPs.

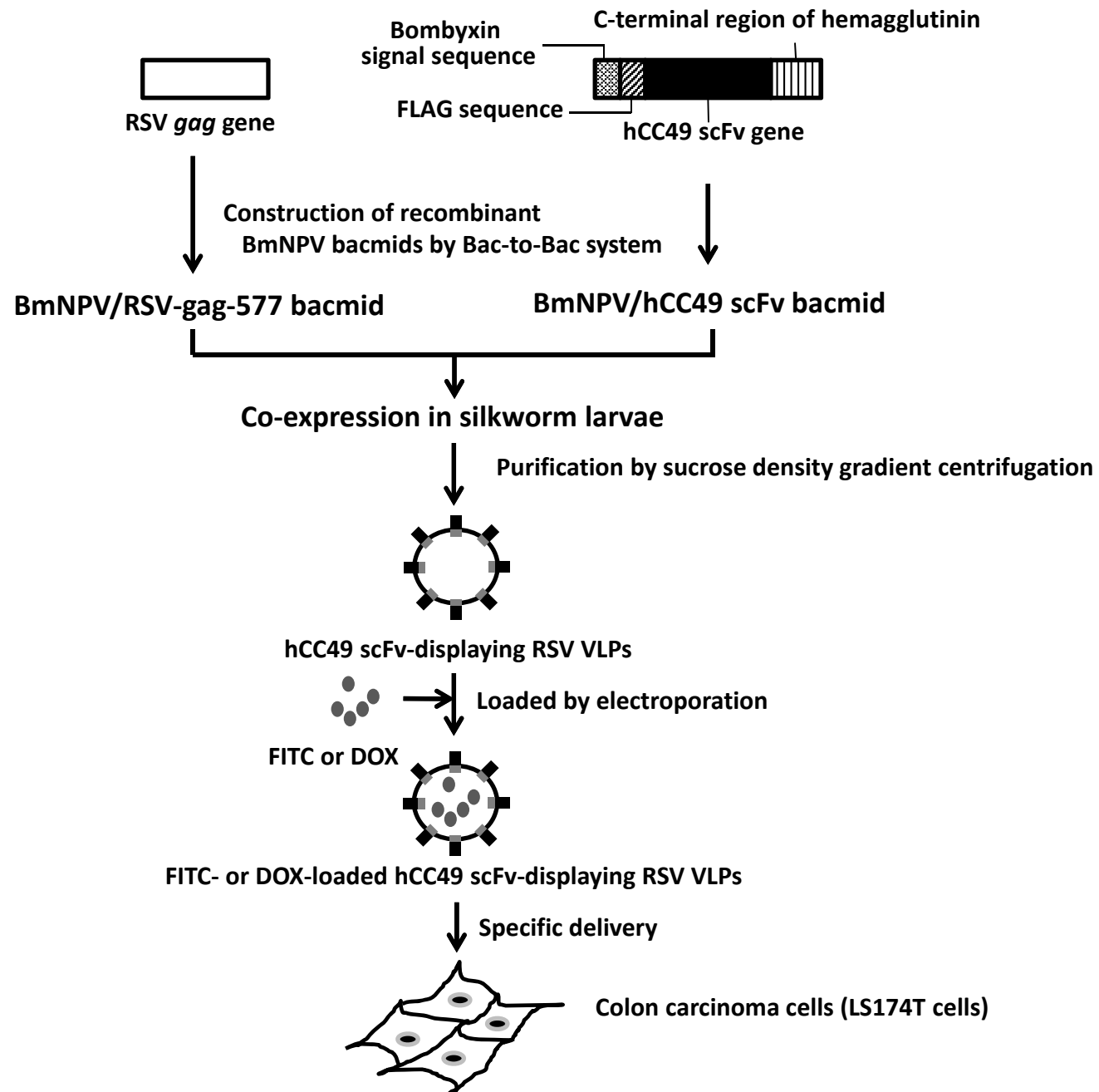
445 **Figure 2.** Expression and purification of hCC49 scFv-displaying RSV VLPs. (A)
446 Detection of hCC49 scFv and RSV gag protein in hemolymph and fat body of silkworm
447 larvae by western blot. Lanes 1–3 denote molecular weight, hemolymph, and fat body
448 samples, respectively. (B) Detection of hCC49 scFv and RSV gag protein in fractions
449 by sucrose density gradient centrifugation of concentrated RSV VLPs. Lane 1:
450 fractions 1 & 2; lane 2: fractions 3 & 4; lane 3: fractions 5 & 6; lane 4: fractions 7 & 8;
451 lane 5: fractions 9 & 10. Open and closed triangles denote scFv fusion protein and
452 Gag protein, respectively.

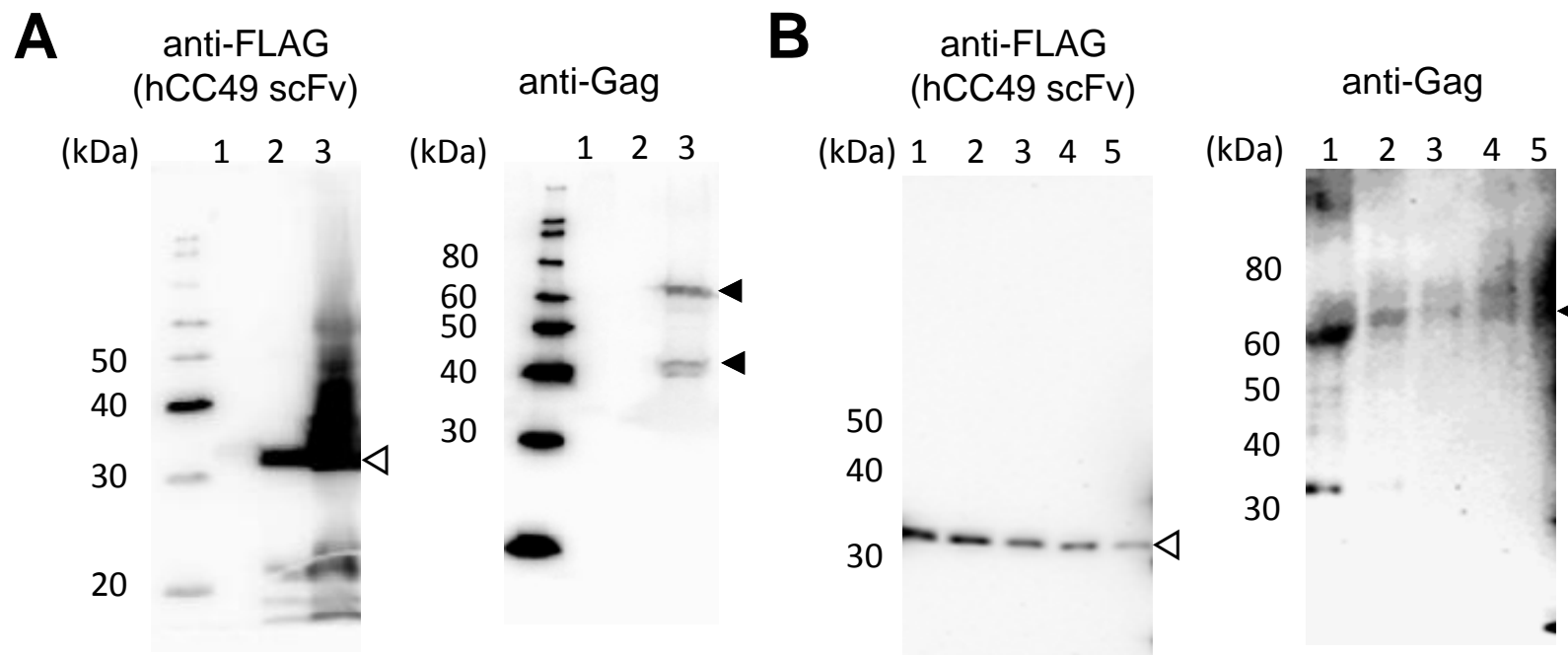
453 **Figure 3.** Characterization of hCC49 scFv-displaying RSV VLPs. (A)
454 Immunofluorescence microscopy of LS174T cells treated with hCC49 scFv-displaying
455 RSV VLPs and RSV VLPs. LS174T cells were treated with hCC49 scFv-displaying
456 RSV VLPs and immunofluorescence microscopy was performed, as described in the
457 Materials and Methods section. The nuclei of LS174T cells were stained with DAPI.
458 (B) Binding assay of hCC49 scFv-displaying RSV VLPs to TAG-72 using enzyme-
459 linked immunosorbent assay (ELISA). TAG-72 was immobilized into each well of a 96-

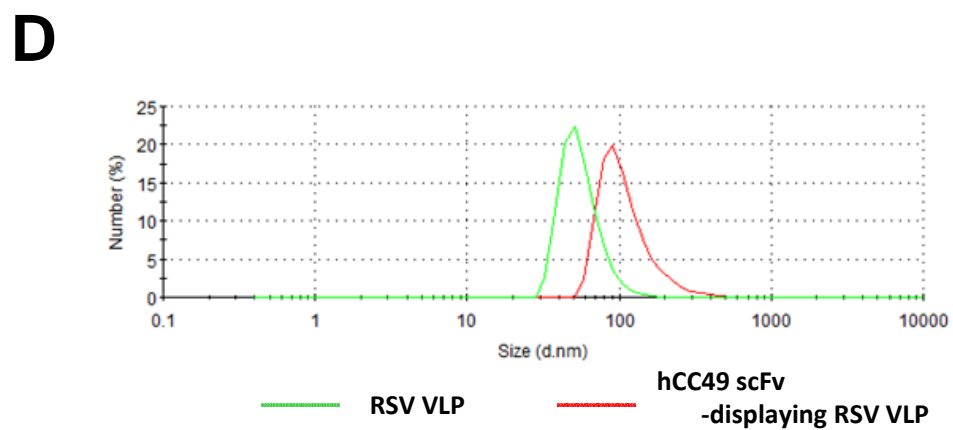
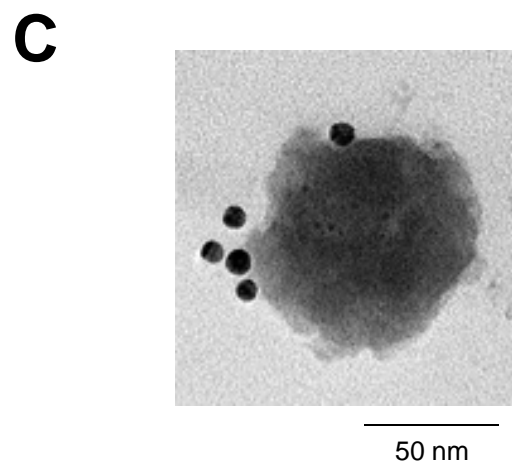
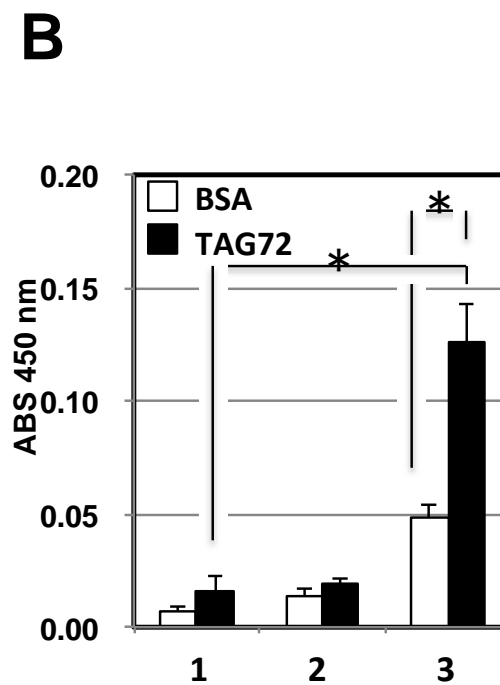
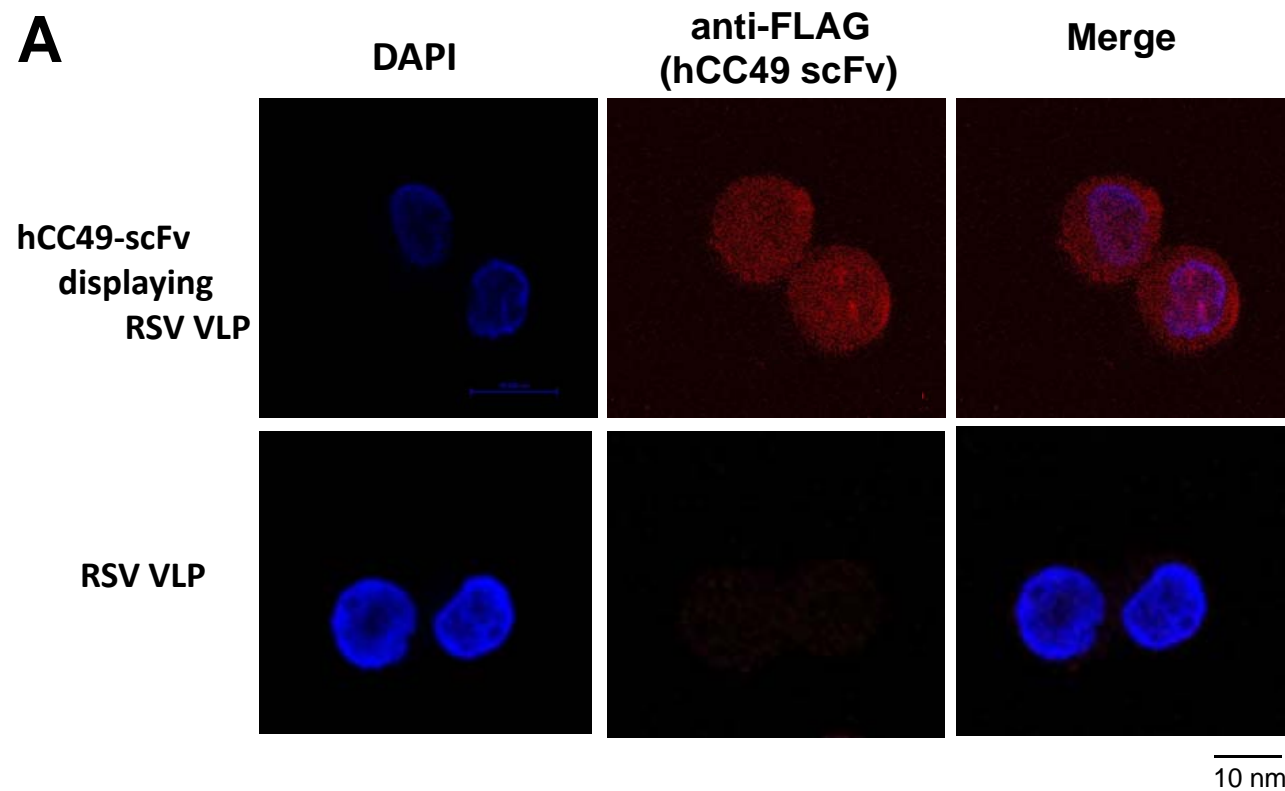
460 well ELISA plate and ELISA was performed, as described in the Materials and Methods
461 section. 1: Hemolymph of mock, 2: Hemolymph of hCC49 scFv-displaying RSV VLPs-
462 expressing silkworms, 3: Purified hCC49 scFv-displaying RSV VLP. * $p < 0.01$ (C)
463 Immunoelectron microscopy of hCC49 scFv-displaying RSV VLPs. Immunoelectron
464 microscopy was performed using mouse monoclonal anti-DYKDDDDK tag antibody
465 and gold nanoparticle-conjugated goat polyclonal anti-mouse IgG+IgM (H+L). (D)
466 Analysis of each VLP size by DLS

467 **Figure 4.** Fluorescence microscopy of LS174T cells treated with FITC-loaded RSV
468 VLPs. FITC was loaded into hCC49 scFv-displaying RSV VLPs and RSV VLPs by
469 electroporation. LS174T cells were incubated with each of the FITC-loaded RSV VLPs.
470 The nuclei of LS174T cells were stained with DAPI. Green fluorescence of FITC and
471 blue fluorescence of DAPI were observed by confocal laser scanning microscope.

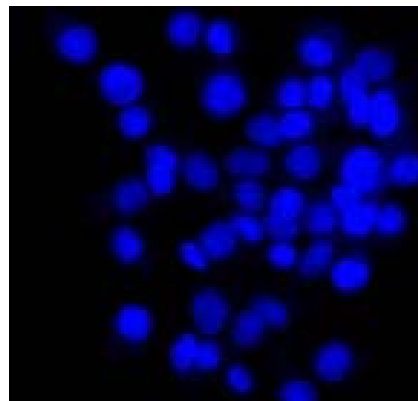
472 **Figure 5.** Cell viability of LS174T and HEK293 cells treated with each of the VLPs.
473 Gag-DOX: DOX-loaded RSV VLPs, SHG-DOX: DOX-loaded hCC49 scFv-displaying
474 RSV VLPs, Mix: mixture of hCC49 scFv-displaying RSV VLPs with DOX (1:1), VLPs:
475 RSV VLPs. DOX10 and DOX50 denote DOX concentration of 10 and 50 $\mu\text{g/ml}$,
476 respectively. Grey and white bars denote LS174T and HEK293 cells, respectively.



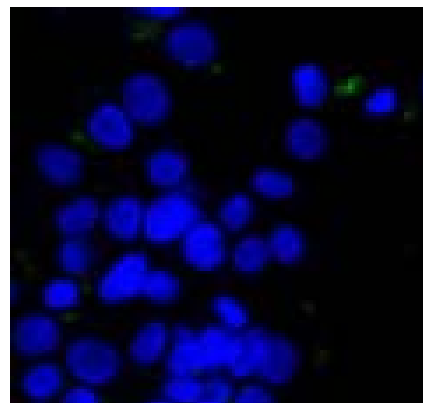




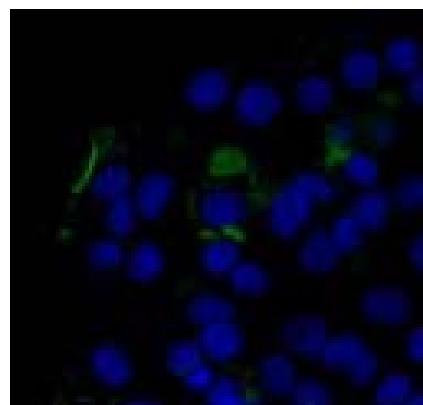
FITC



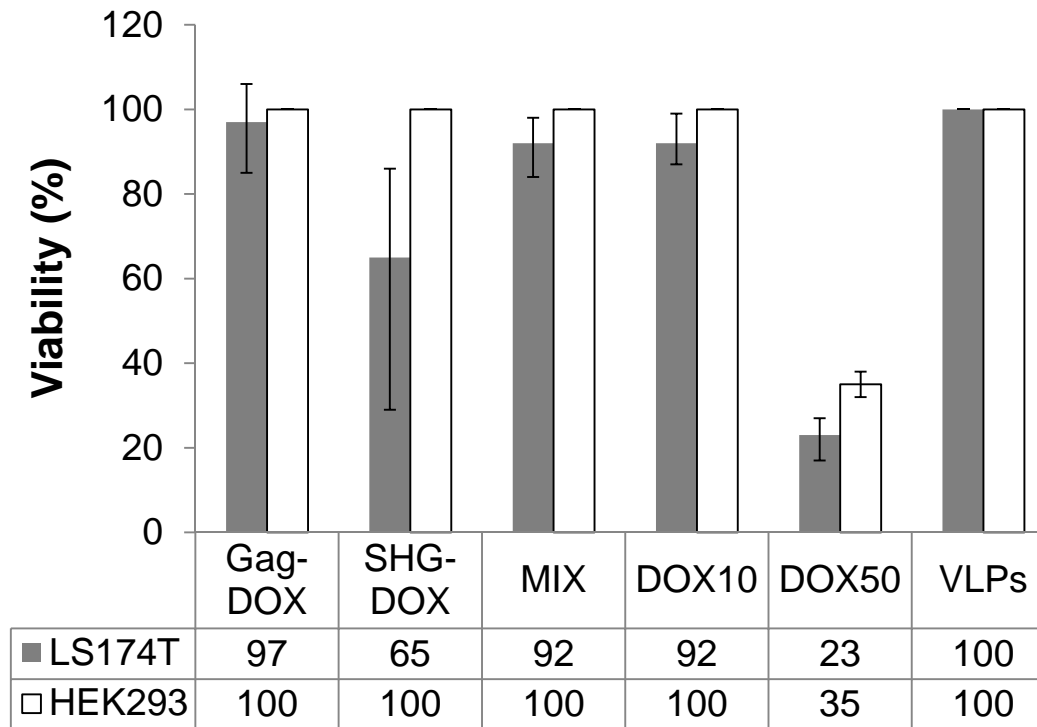
**FITC-loaded
RSV VLP**

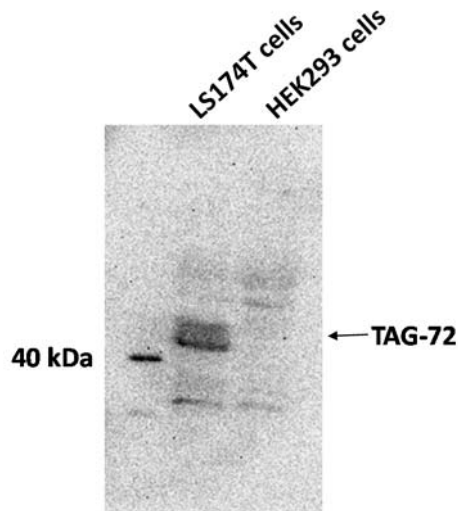


**FITC-loaded
HCC 49 scFv
-displaying RSV VLPs**



20 μ m





Supplementary Figure 1. TAG-72 was observed specifically by mouse anti-TAG-72 antibody (CC49, Santa Cruz Biotechnol., Cat. No. sc-20043) in LS174T cell extract (3×10^6 cells) at around 40 kDa. Mouse anti-TAG-72 antibody was diluted with TBST at 1:500 and HRP-conjugated anti-mouse IgG antibody was diluted with TBST at 1:15000. HEK293 cells extract (3×10^6 cells) was provided to confirm TAG-72 expression investigated, but molecular band of TAG-72 was not shown. It indicates that LS174T cells express TAG-72, which is targeted specifically by hCC49 scFv-displaying RSV VLPs. In the previous paper, TAG-72 was observed in LS174T cell extract at around 40 kDa, unlikely to purified TAG-72 (Cancer Res. 48, 6811-6816, 1988). Our result corresponds to the previously reported result. Specific DOX delivery to LS174T cells by hCC49 scFv displaying RSV VLPs is supported by this data and also Figure 5.