

## ARTICLE

# Development of new therapy for canine mammary cancer with recombinant measles virus

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Oncolytic virotherapy is a promising treatment strategy for cancer. We previously generated a recombinant measles virus (rMV-SLAMblind) that selectively uses a poliovirus receptor-related 4 (PVRL4/Nectin4) receptor, but not signaling lymphocyte activation molecule (SLAM). We demonstrated that the virus exerts therapeutic effects against human breast cancer cells. Here, we examined the applicability of rMV-SLAMblind to treating canine mammary cancers (CMCs). We found that the susceptibilities of host cells to rMV-SLAMblind were dependent on canine Nectin-4 expression. Nectin-4 was detected in four of nine CMC cell lines. The rMV-SLAMblind efficiently infected those four Nectin-4-positive cell lines and was cytotoxic for three of them (CF33, CHMm, and CTBm). *In vivo* experiment showed that the administration of rMV-SLAMblind greatly suppressed the progression of tumors in mice xenografted with a CMC cell line (CF33). Immunohistochemistry revealed that canine Nectin-4 was expressed in 45% of canine mammary tumors, and the tumor cells derived from one clinical specimen were efficiently infected with rMV-SLAMblind. These results suggest that rMV-SLAMblind infects CMC cells and displays antitumor activity *in vitro*, in xenografts, and *ex vivo*. Therefore, oncolytic virotherapy with rMV-SLAMblind can be a novel method for treating CMCs.

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## INTRODUCTION

Canine mammary tumors (CMTs) are the most frequent canine tumors and account for more than 40% of all tumors in female dogs.<sup>1–3</sup> Approximately 50% of CMTs are malignant, and most are classified as adenocarcinomas.<sup>4</sup> Surgical resection is the standard method of treatment for CMTs.<sup>5</sup> However, surgical resection is not appropriate for dogs diagnosed with metastatic cancer, and approximately half of all dogs with malignant CMTs have metastatic cancer at the time of surgery, resulting in a poor prognosis.<sup>6,7</sup> Classic chemotherapeutic drugs are used to treat metastatic CMTs, but the treatment is accompanied by strong adverse effects.<sup>7</sup> Unlike in human breast cancer, there is little evidence of the effectiveness of molecule-targeted therapies for CMTs, such as anti-estrogen therapy or epidermal growth factor receptor/human epidermal growth factor receptor kinase inhibitors.<sup>7–11</sup> Therefore, the development of a new therapy for CMTs is required.

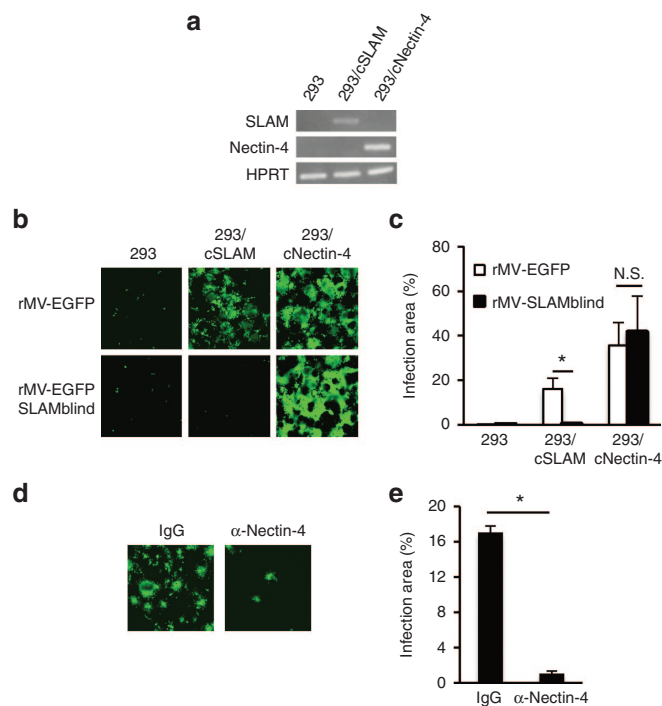
In recent years, oncolytic virotherapy has been proposed as a new strategy for the treatment of cancer, and preclinical research and clinical trials have investigated its potency in the clinical context.<sup>12,13</sup> In the veterinary field, several viruses have been reported as oncolytic agents, including adenovirus, reovirus, vaccinia virus, and canine distemper virus (CDV).<sup>13–18</sup> Adenovirus, vaccinia virus, and reovirus grow readily in tumor cells because of their replication mechanisms, which are regulated by tumor-specific nuclear transcription factors.<sup>13,16–21</sup> However, viral replications have been

observed in the organs and cells of infected mice.<sup>14,16,18</sup> Because replication in normal cells may cause unacceptable toxicity, it is necessary to develop oncolytic agents with greater specificity to tumor cells. CDV is reported to selectively infect and lyse canine lymphoma cells *in vitro*, and its replication is dependent on the expression of its receptors.<sup>15</sup> However, there are obstacles to using CDV in the clinical context that include its pathogenicity, preexisting anti-CDV antibodies in the host, and viral transmission to other dogs.

Instead of the viruses described above, we focused on measles virus (MV), which, like CDV, belongs to the genus *Morbillivirus* in the family *Paramyxoviridae*. MV has been used in oncolytic agents for various types of human cancer.<sup>12,22,23</sup> In our previous study, we found that wild-type MV (HL strain) has high antitumor activity against human breast cancer cells. Wild-type MV infects immune cells through signaling lymphocyte activation molecule (SLAM) as its major receptor, followed by the spread of the virus throughout the entire body by infected immune cells, which dissemination pathway is related with its pathogenicity including severe immunosuppression.<sup>22,24</sup> For infection of human breast cancer cells, however, we found that the cells do not express SLAM, and MV-HL only uses human poliovirus receptor-related protein 4 (PVRL4/Nectin-4), which is an another receptor. Therefore, we generated a recombinant MV (rMV-SLAMblind), which is unable to bind human SLAM, to reduce the pathogenicity. The administration of rMV-SLAMblind caused no symptoms, including immunosuppression,

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**Figure 1** Receptor usage by rMV-EGFP-SLAMblind. **(a)** RT-PCR confirmed the expression of Nectin-4 and SLAM in HEK293/canineNectin-4 (293/cNectin-4) and HEK293/canineSLAM (293/cSLAM) cells. HPRT served as a control. **(b)** and **(c)** Cells were infected with rMV-EGFP or rMV-EGFP-SLAMblind at MOIs of 0.01. **(b)** rMV-EGFP-SLAMblind efficiently infected 293/cNectin-4 but not 293/cSLAM cells nor HEK293 cells. Cells were photographed at 2 dpi. Magnification,  $\times 100$ . **(c)** Area of EGFP fluorescence in a visual field was quantified with Image J 1.48v, by integrating five random fields in **b**. Error bar indicates the SD of three independent experiments.  $*P < 0.05$  when compared with rMV-EGFP. NS, not significant. **(d)** and **(e)** 293/cNectin-4 cells were infected with rMV-SLAMblind at an MOI of 0.01 in the presence of 10  $\mu\text{g/ml}$  anti-Nectin-4 antibody or control IgG. **(d)** rMV-SLAMblind was blocked by an anti-Nectin-4 antibody but not by control IgG. Photograph was taken at 2 dpi. Magnification,  $\times 100$ . **(e)** Area of EGFP fluorescence in the visual field was quantified with Image J 1.48v by integrating five random fields in **d**. Error bar indicates the SD of three independent experiments.  $*P < 0.05$ , when compared with the control IgG. RT-PCR, reverse transcription PCR.

in monkeys.<sup>25,26</sup> In contrast, rMV-SLAMblind maintained an efficient capacity to infect human Nectin-4-positive breast cancer cells.<sup>25</sup>

Nectin-4 is a member of the nectin family, which belongs to the immunoglobulin superfamily and is classified as a type I transmembrane glycoprotein.<sup>27</sup> Nectin-4 expression is upregulated in several types of human cancer: breast, lung, ovarian, pancreatic, and prostate cancers.<sup>28–30</sup> However, its expression in normal human tissues is restricted to the placenta, with slight expression in the trachea.<sup>31</sup> These findings suggest that Nectin-4 is a new tumor-associated antigen and therefore a therapeutic target in tumors.<sup>28,29</sup> Human and dog Nectin-4 proteins share high homology, and the domains critical for binding MV are completely conserved in the two species. However, whether MV is able to use canine Nectin-4 to infect canine cells and grow well within the cells remains unknown. Therefore, in this study, we examined whether rMV-SLAMblind is a good therapeutic candidate for treating canine mammary cancers (CMCs).

## RESULTS

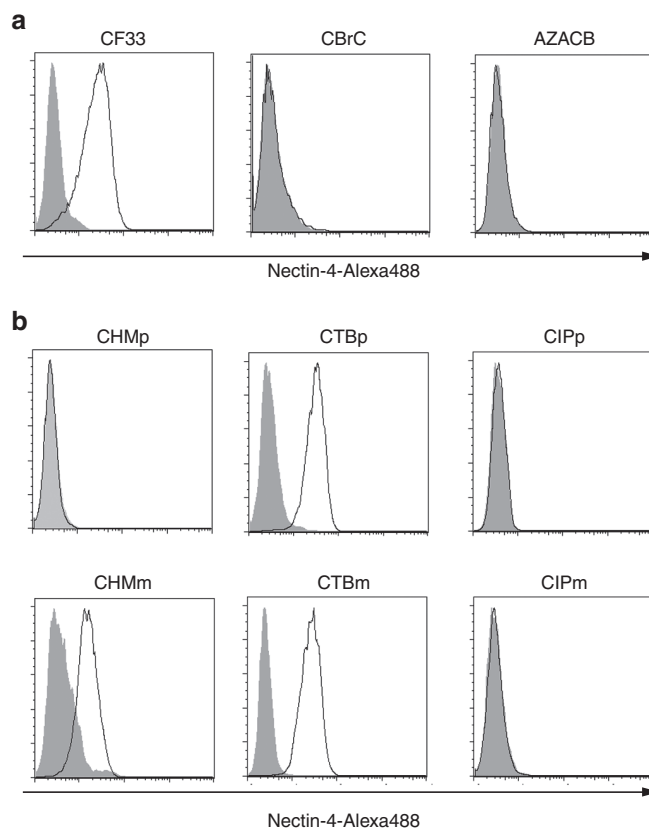
### Receptor usage by rMV-EGFP-SLAMblind

Human PVRL4/Nectin-4 is a receptor for wild-type MV, but the usability of canine Nectin-4 for MV infection is unknown. We first

**Table 1** A list of CMC cells used in this study

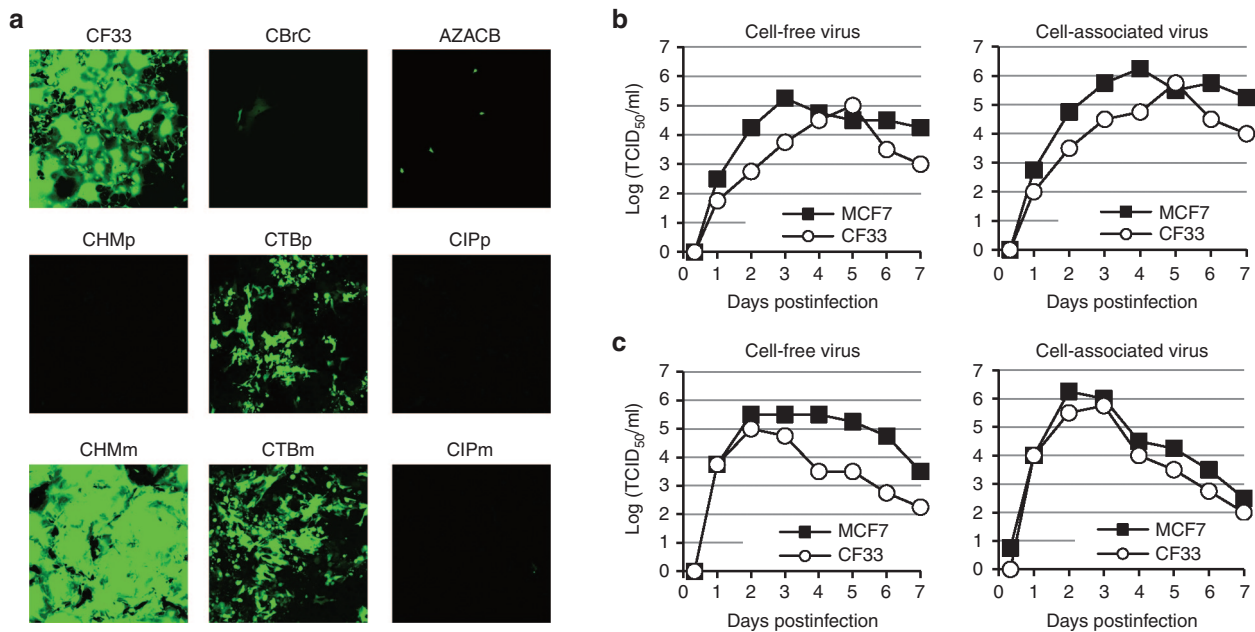
Name of cells	Pathological diagnosis	Source of cells	Expression of Nectin-4
CF33	AC	Primary	+
AZACB	Complex AC	Primary	–
CBrc	Inflammatory AC	Primary	–
CHMp	Inflammatory AC	Primary	–
CHMm	Inflammatory AC	Pleural effusion	+
CTBp	Inflammatory AC	Primary	+
CTBm	Inflammatory AC	Metastatic RLN	+
CIPp	AC	Primary	–
CIPm	AC	Metastatic RLN	–

AC, adenocarcinoma; CMC, canine mammary cancer; RLN, regional lymph node.



**Figure 2** Nectin-4 expression on canine mammary cancer cell lines. **(a)** and **(b)** Cell-surface expression of canine Nectin-4 on canine mammary tumor cells was analyzed with flow cytometry. Cells were incubated with anti-Nectin-4 antibody (white histogram) or control IgG (gray histogram) and then incubated with Alexa-Fluor-488-conjugated rabbit anti-goat antibody. Four of the nine CMC cell lines expressed canine Nectin-4. CHMp and CHMm, CTBp and CTBm, and CIPp and CIPm cells were derived from three different dogs. Data were analyzed with the FlowJo software. CMC, canine mammary cancer.

examined the rMV-SLAMblind infectivity of canine cells using canine Nectin-4. We established canine Nectin-4-expressing HEK293 cells and canine SLAM-expressing HEK293 cells as controls (HEK293/canineNectin-4 and HEK293/canineSLAM, respectively) (Figure 1a).



**Figure 3** Infection of CMC cells with rMV-SLAMblind and its replication in a CMC cell line. (a) Cells derived from CMCs were infected with rMV-EGFP-SLAMblind at MOIs of 2. Consistent with Nectin-4 expression, four canine cell lines were susceptible to infection with rMV-SLAMblind. At 2 dpi, the cells were observed under a fluorescence microscope. Magnification,  $\times 100$ . (b and c) CF33 cells and MCF7 cells were infected with rMV-SLAMblind at an MOI of (b) 0.01 and (c) 0.1. Infection titers of cell-free and cell-associated viruses were determined at various time points. CMC, canine mammary cancer; MOI, multiplicity of infection.

To monitor the viral infection, rMV-SLAMblind expressing EGFP (rMV-EGFP-SLAMblind) was used in this study. Cells were inoculated with rMV-EGFP-SLAMblind at a multiplicity of infection (MOI) of 0.1. At 2 days postinfection (dpi), rMV-EGFP-SLAMblind had efficiently infected HEK293/canine Nectin-4 cells and induced syncytia but had infected neither HEK293/canineSLAM cells nor the parental HEK293 cells (Figure 1b,c). To confirm that the canine SLAM expressed on HEK293/canineSLAM cells was functional, these cells were inoculated with rMV-EGFP, the parental strain of rMV-EGFP-SLAMblind.<sup>32</sup> rMV-EGFP efficiently infected both HEK293/canineSLAM cells and HEK293/canineNectin-4 cells and induced syncytia (Figure 1b,c). To further ascertain that rMV-SLAMblind infected HEK293/canine-Nectin-4 cells via canine Nectin-4, we performed a blocking assay of rMV-SLAMblind infection by anti-Nectin-4 antibody. The number of infected cells and syncytia-formed areas were dramatically reduced by treatment with the anti-Nectin-4 antibody (Figure 1d,e). These results suggest that rMV-SLAMblind uses canine Nectin-4 but not canine SLAM as a receptor, whereas wild-type MV potentially uses both canine SLAM and canine Nectin-4 as receptors.

#### Nectin-4 expression on CMC cells

To examine whether canine Nectin-4 is expressed on CMC cells, nine CMC cell lines were analyzed with flow cytometry (Table 1). CHMp and CHMm, CTBp and CTBm, and CIPp and CIPm cells were derived from three different dogs. CHMp, CTBp, and CIPp were established from primary lesions, and CHMm, CTBm, and CIPm cells were established from metastatic lesions.<sup>33</sup> Four of the nine CMC cell lines (CF33, CHMm, CTBp, and CTBm) expressed canine Nectin-4 (Figure 2a,b). Two of these CMC cell lines (CHMm and CTBm) from metastatic lesions expressed Nectin-4 (Figure 2b). Interestingly, although CHMm derived from metastatic lesions expressed Nectin-4, CHMp derived from primary lesions of the same dog did not. These results suggest that canine Nectin-4 is expressed not only in primary lesions but also in metastatic lesions.

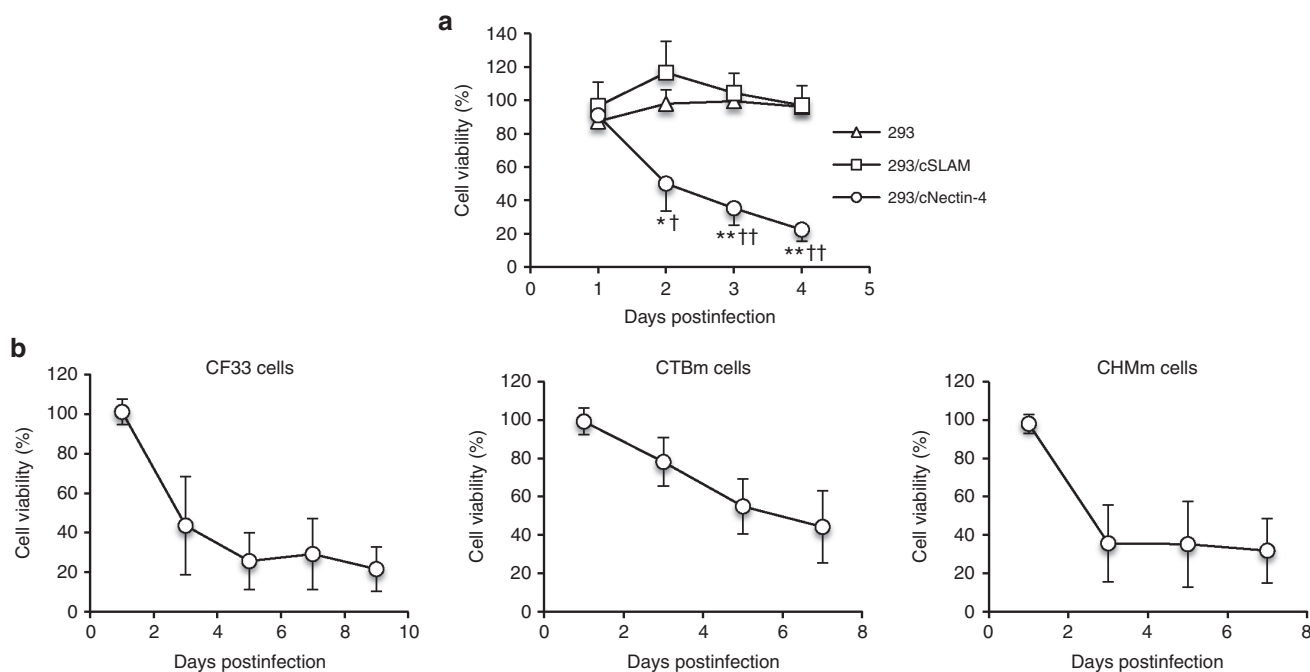
#### Infectivity and replication of rMV-SLAMblind in CMC cells

To examine the infectivity of rMV-SLAMblind in CMC cells, CMC cells were inoculated with rMV-EGFP-SLAMblind at an MOI of 2. Although CBrC, AZACB, CHMp, CIPp, and CIPm cells, which do not express canine Nectin-4, were negligibly infected, CF33, CHMm, CTBp, and CTBm cells, which express canine Nectin-4, were efficiently infected and developed syncytia (Figure 3a). To determine whether rMV-EGFP-SLAMblind replicates in canine cells, CF33 cells were inoculated with rMV-EGFP-SLAMblind at an MOI of 0.01 or 0.1. The virus grew well until 5 dpi, and both the cell-free and cell-associated viral titers peaked at 5 dpi (MOI of 0.01) or at 2–3 dpi (MOI of 0.1), respectively (Figure 3b,c). When we compared the virus growth in CF33 to that in a human breast cancer cell line (MCF7), the peak titers were similar, whereas the growth speed of the virus in CF33 was slightly slower than in MCF7 when the inoculation titer was lower at an MOI of 0.01 but was similar at an MOI of 0.1. MCF7 is the most susceptible cell lines derived from human breast cancers to rMV-SLAMblind in our hands,<sup>25</sup> and there are many other ones which are less susceptible but well killed by the virus (data not shown). Thus, these results demonstrate that, as well as within human breast cancer cells, rMV-EGFP-SLAMblind efficiently infects and replicates within CMC cells.

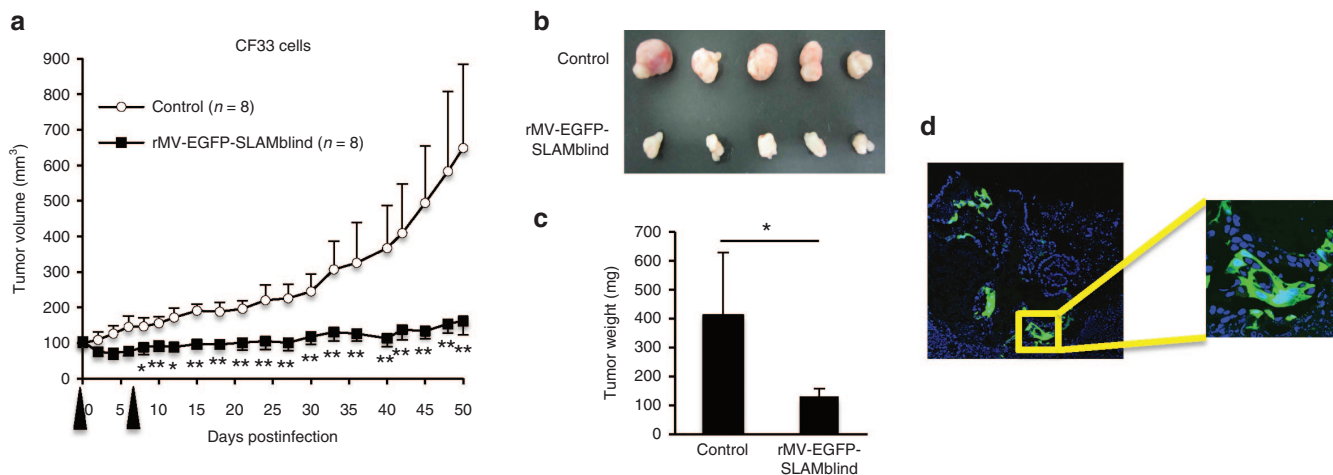
#### *In vitro* cytotoxicity of rMV-SLAMblind depends on Nectin-4 expression

To examine whether rMV-SLAMblind is specifically cytotoxic to Nectin-4-expressing cells, we tested the viability of HEK293/canine Nectin-4 cells after infection with rMV-EGFP-SLAMblind and compared it with the viability of HEK293 and HEK293/canineSLAM cells. The viability of HEK293/canineNectin-4 cells was dramatically reduced at 2 dpi, although the viability of HEK293 cells and HEK293/canineSLAM cells was maintained until 4 dpi (Figure 4a).

Three CMC cell lines expressing Nectin-4 were also infected with rMV-EGFP-SLAMblind to examine its cytotoxicity. rMV-EGFP-SLAMblind killed 71% of CF33 cells, 56% of CTBm cells,



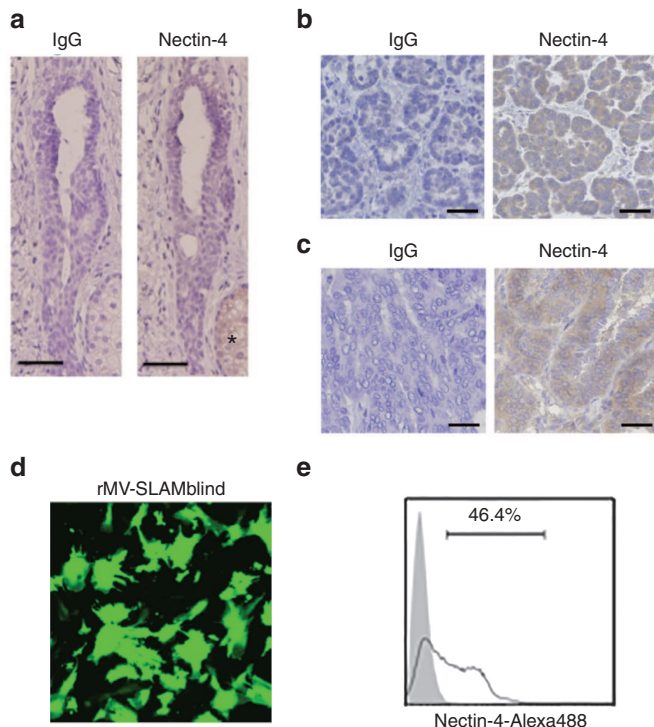
**Figure 4** *In vitro* cytotoxicity of rMV-SLAMblind. (a) HEK293, 293/cSLAM, and 293/cNectin-4 cells were infected with rMV-SLAMblind at MOIs of 0.1. Cell viability was measured every 24 hours with a WST-1 assay. The viability of 293/cNectin-4 cells was dramatically reduced at 2 dpi, although that of HEK293 cells and 293/cSLAM cells was maintained until 4 dpi. Error bar indicates the SD of three independent experiments. \* $P < 0.05$ ,  $^{\dagger}P < 0.05$  and \*\* $P < 0.01$ ,  $^{\dagger\dagger}P < 0.01$  on the Tukey test compared with the viability of HEK293 and 293/cSLAM cells. (b) Three canine cancer cell lines, CF33, CTBm, and CHMm, were infected with rMV-SLAMblind at MOIs of 2. Cell viability was measured every 48 hours with a WST-1 assay. Error bar indicates the SD of three independent experiments. MOI, multiplicity of infection.



**Figure 5** Antitumor activity of rMV-EGFP-SLAMblind in xenografts. (a) CF33 tumors were established in SCID mice. Mice were inoculated intratumorally with either rMV-EGFP-SLAMblind at a dose of  $10^6$  TCID<sub>50</sub> or HBSS as the control. The virus was inoculated on days 0 and 7 (arrowheads). The virus-inoculated group (black square) suppressed tumor growth compared to the control group (white circle). Welch's *t*-test was used to compare the two groups. Error bar represents SD. \* $P < 0.05$  was considered statistically significant. \* $P < 0.05$ , \*\* $P < 0.01$ . (b) The tumor samples were collected at 50 days after the first inoculation. The tumors of the control group (upper samples) were larger than those of the virus-inoculated group (lower samples). (c) The weights of the tumors collected were measured at day 50. The weights of the tumors in the virus-inoculated group were significantly less than that of the tumors in the control group. Error bar represents SD. \* $P < 0.05$  was considered statistically significant on Welch's *t*-test. (d) At 4 dpi, frozen sections were prepared and stained with Hoechst 33342. EGFP fluorescence and cell nuclei were observed under a confocal microscope. EGFP-positive syncytia caused by virus growth could be observed. Magnification was  $\times 100$  in the left panel and  $\times 600$  in the right panel. HBSS, Hank's balanced salt solution; SCID, severe combined immune deficiency; TCID<sub>50</sub>, 50% tissue culture infective dose.

and 68% of CHMm cells by 7 dpi (Figure 4b). These results suggest that rMV-EGFP-SLAMblind exerts antitumor activity *in vitro* in a Nectin-4-dependent manner.

Oncolytic activity of rMV-SLAMblind in a CF33 xenograft model  
To assess the oncolytic ability of rMV-SLAMblind *in vivo*, CF33 cells were transplanted into severe combined immune deficiency



**Figure 6** Expression of Nectin-4 in normal mammary tissues and clinical CMT tissues. **(a)** Paraffin sections (3  $\mu\text{m}$ ) of normal mammary tissues were incubated with control IgG (left panel) or anti-Nectin-4 antibody (right panel) as the primary antibody. ImmPRESS Reagent, Anti-Goat Ig and 3,3'-diaminobenzidine were used to visualize the samples. The sections were counterstained with hematoxylin. Nectin-4 was not detected in normal mammary tissues except in sebaceous glands (\*). Bar: 100  $\mu\text{m}$ . Magnification,  $\times 100$ . **(b)** Strong Nectin-4 expression was detected in invasive adenocarcinoma in dog No. 1 stained with control IgG (left panel) and anti-Nectin-4 antibody (right panel) in a paraffin section. Bar = 50  $\mu\text{m}$ . Magnification,  $\times 200$ . **(c)** Nectin-4 was detected in simple tubular adenocarcinoma from dog No. 2 stained with control IgG (left panel) or anti-Nectin-4 antibody (right panel) in a frozen tissue section. Bar = 50  $\mu\text{m}$ . Magnification,  $\times 200$ . **(d)** Primary tumor cells from dog No. 2 in Table 2 were disaggregated to single cells and inoculated with rMV-EGFP-SLAMblind at an MOI of 0.01. At 2 dpi, EGFP-positive cells caused by the virus infection could be observed. Magnification,  $\times 100$ . **(e)** Flow-cytometric analysis of canine Nectin-4 in primary CMT tissue. Tumor from dog No. 2 in Table 2 was digested and stained with control IgG (gray histogram) or anti-Nectin-4 antibody (white histogram). The main cell population was selected with FSC and SSC based on 4',6-diamidino-2-phenylindole incorporation. The histogram indicates the expression levels of Nectin-4 in the primary tumor cells. Around 46% of tumor cells were positive for Nectin-4. FSC, forward scatter; SSC, side scatter; MOI, multiplicity of infection.

(SCID) mice. The xenograft mice were inoculated twice intratumorally with  $10^6$  TCID<sub>50</sub> (50% tissue culture infective dose) of rMV-EGFP-SLAMblind on days 0 and 7. The tumors in the control group grew larger, but the virus-inoculated group displayed significantly suppressed tumor growth (Figure 5a). The mice were euthanized and autopsies performed at 50 days after the first inoculation. The tumors in the mice inoculated with rMV-EGFP-SLAMblind were clearly smaller than those in the control mice (Figure 5b,c). To observe the viral infection in the tumors, some of the mice inoculated with rMV-EGFP-SLAMblind were euthanized at 4 dpi. EGFP fluorescence was observed in many tumor cells, and syncytia were observed at higher magnification (Figure 5d). These results suggest

**Table 2** Expression of canine Nectin-4 in clinical tissue samples

Case no.	Pathological diagnosis	Malignant or benign	Expression of Nectin-4
1	Invasive AC	Malignant	+
2	Simple tubular AC	Malignant	+
3	Simple AC	Malignant	+
4	Complex AC	Malignant	-
5	Anaplastic carcinoma	Malignant	-
6	Complex AC	Malignant	-
7	Complex adenoma	Benign	+
8	Complex adenoma	Benign	+
9	Multiple complex adenoma	Benign	+
10	Complex adenoma	Benign	-
11	Complex adenoma	Benign	-
12	Complex adenoma	Benign	-
13	Adenoma	Benign	-
14	Adenoma	Benign	-
15	Complex adenoma	Benign	-
16	ND	ND	+

AC, adenocarcinoma; ND, no data.

that rMV-SLAMblind also replicates well *in vivo* and displays antitumor activity with cell-cell fusion.

#### Nectin-4 expression in clinically isolated CMT tissues and infection with rMV-SLAMblind *ex vivo*

We have demonstrated that Nectin-4 is often expressed in CMC cell lines and that rMV-SLAMblind kills CMC cells in a Nectin-4-dependent manner. Thus, it is important to determine Nectin-4 expression in clinical CMTs to predict the applicability of virotherapy with rMV-SLAMblind. We analyzed the expression of canine Nectin-4 in clinical tumor tissues using immunohistochemistry or fluorescent antibodies, compared with that in normal mammary tissues to determine the basal level of Nectin-4 expression. In normal mammary tissues, canine Nectin-4 was not detected in mammary glands, epithelial cells, or myoepithelial cells (Figure 6a). Positive Nectin-4 signals were only observed in sebaceous glands. In tumor tissues, canine Nectin-4 was detected in three malignant tumors, three benign tumors, and one unclassified tumor tissue among the 16 tested (Nos. 1-16) (Figure 6b,c and Table 2). To analyze the infectivity of rMV-EGFP-SLAMblind in clinical tumors, primary cells derived from mammary cancer in dog No. 2 were inoculated with rMV-EGFP-SLAMblind (Table 2). Approximately half of the cells were efficiently infected and killed (Figure 6d). To confirm the proportion of Nectin-4-expressing cells among these primary cells, the Nectin-4 expression in the cells was also analyzed with flow cytometry. Canine Nectin-4 was detected in 46% of viable cells in the main population with forward scatter and side scatter (Figure 6e). The proportion of Nectin-4-expressing cells in the population was similar to that in the

infected cells, suggesting that rMV-SLAMblind specifically infects Nectin-4-positive cells. These results suggest that canine Nectin-4 was expressed in nearly half of the clinical CMT samples and that rMV-SLAMblind was able to kill the tumor cells *ex vivo*.

## DISCUSSION

In this study, we have shown that rMV-SLAMblind effectively infected CMC cells and exerted an antitumor effect on cells expressing canine Nectin-4 *in vitro* and in xenografts. We also demonstrated that canine Nectin-4 was expressed in clinical CMT tissues but was not detected in normal mammary tissues. These data indicate that rMV-SLAMblind is a novel candidate oncolytic virus for the treatment of CMC that express Nectin-4.

In the veterinary field, treatments for metastatic CMCs are rarely given because therapeutic drugs for human breast cancers are mostly ineffective in dogs.<sup>6,7</sup> Therefore, an effective method for treating metastatic cancer in dogs must be developed. In this study, we have shown that canine Nectin-4 is expressed in CHMm and CTBm cells derived from metastatic lesions and that rMV-SLAMblind is cytotoxic to these cells. Notably, CHMm cells expressed canine Nectin-4, whereas the CHMp cells derived from a primary lesion did not. In addition, immunohistochemical analysis of clinical tissues revealed that canine Nectin-4 was expressed in 50% of the malignant cancers and in only 33% of the benign tumor samples. Although the number of samples examined in this study was not large, the expression of canine Nectin-4 seemed to correlate with the malignancy of CMT. Consistent with this, increasing Nectin-4 expression rates are reportedly significantly correlated with the size and malignancy of human breast cancers.<sup>34</sup> Although CMTs comprise a heterogeneous population of Nectin-4-positive and -negative cells as shown by the flow cytometric analysis of clinically isolated CMT cells (Figure 6e), rMV-SLAMblind is expected to preferentially kill Nectin-4-positive cells in dogs with Nectin-4-positive tumors, which are considered to have poor prognoses. Therefore, rMV-SLAMblind is a promising novel candidate for the treatment of CMTs, particularly malignant tumors that are unresponsive to anticancer drugs. Even if the tumor contains Nectin-4-negative tumor cells, the combined use of rMV-SLAMblind with other conventional drugs should effectively induce regression of the tumor.

While CHMm and CTBm expressed Nectin-4 at similar levels, infectivity with rMV-SLAMblind differed between these two cell lines. In our previous study using human lung cancer cell lines, the cytotoxicity with rMV-SLAMblind tended to correlate with the Nectin-4 expression level, but there were some exceptional cell lines.<sup>35</sup> Therefore, the expression level of Nectin-4 is one factor responsible for defining virus infection, but other factors also influence virus growth in each cell line. The molecular mechanism underlying cytotoxic activity of rMV-SLAMblind remains to be studied.

rMV-SLAMblind should be administered intravenously in the treatment of metastatic CMCs. A major impediment to the systemic application of oncolytic viruses is the presence of preexisting neutralizing antibodies.<sup>12,23</sup> Most dogs are vaccinated against CDV, which is related to MV. In one study, however, no MV-specific neutralizing antibodies to CDV were detectable in sera.<sup>36</sup> Therefore, the influence of CDV vaccination on intravenous administration of rMV-SLAMblind is expected to be small. Once MV infects the tumor, the immune reaction will support the antitumor effects of the virus<sup>37</sup> because MV infection activates the T-lymphocyte-mediated immune response, followed by the expansion of MV-specific CD8 T

cells.<sup>38-41</sup> Additionally, treatment of human ovarian cancer with the MV-vaccine strain reportedly triggers cellular immunity against the patients' tumors.<sup>37</sup> Therefore, treatment with rMV-SLAMblind may also activate tumor-antigen-specific T cells, which would enhance its therapeutic effect. Future studies are needed to investigate the ability of rMV-SLAMblind to reach the tumor cells, spread within the tumor, and trigger an immune response against the tumor in dogs with cancer.

Cells become infected with wild-type MV via the receptors canine Nectin-4 and canine SLAM. This raises the possibility that MV infects dogs. However, there are no reports of measles in dogs; thus, MV is not expected to cause pathogenicity in dogs, even a wild-type strain. We selected rMV-SLAMblind as an oncolytic agent to better ensure the safety of treated dogs and the people in contact with them. To confirm the safety of rMV-SLAMblind in dogs, we administered rMV-SLAMblind intravenously to six normal dogs. None of the treated dogs showed any clinical symptoms, and no viral RNA was detected in their lungs, urine, saliva, or other tissues 28 days after administration (data not shown). These results suggest that rMV-SLAMblind is not only safe but also not discharged into the environment. To date, there are two reports suggesting that Nectin-4 is expressed in several tissues in dogs, although there are some discrepancies in the expression patterns.<sup>42,43</sup> Since virus replication was not observed in the organs when we administered the virus to healthy dogs as described above, it is needed to study expression of Nectin-4 in each organ and capability of MV growth in those organs carefully. Such information will be important for further consideration of the safety of rMV-SLAMblind therapy for dogs.

Clinical trials of rMV-SLAMblind therapy for CMCs are required in the near future. The implementation of rMV-SLAMblind therapy in dogs will provide important findings on the efficacy, safety, and mechanism underlying this therapy, including the associated immune reaction. These findings will also have important implications for oncolytic MV therapy in human patients with cancer.

## MATERIALS AND METHODS

### Cells

MCF7 cells were purchased from the American Type Culture Collection (Manassas, VA). Six CMC cell lines, CHMp, CHMm, CTBp, CTBm, CIPp, and CIPm, were kindly provided by T. Nakagawa (The University of Tokyo, Japan) and were grown in RPMI 1640 medium (Life Technologies, Carlsbad, CA) containing 10% fetal bovine serum (FBS; SAFC Biosciences, Lenexa, KS), streptomycin (100 mg/ml), and penicillin (100 U/ml). CF33 cells were kindly provided by R. Asano (University of Nihon, Japan).<sup>44</sup> The CBrC cell line was established from an inflammatory adenocarcinoma (Y. Naya, Y. Okamoto, K. Hata, H. Ochiai, and K. Ogihara, unpublished data). CF33, CBrC, and HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) containing 10% FBS and antibiotics. AZACB cells were purchased from COSMO BIO (Hokkaido, Japan) and cultured in minimum essential medium (Sigma, St Louis, MO) containing 10% FBS and 0.295% tryptose phosphate broth (Sigma). All cells were cultured at 37 °C with 5% CO<sub>2</sub>.

### Plasmids

The plasmids expressing canine SLAM or Nectin-4 were generated as follows. The coding region of *SLAM* was amplified with Phusion High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA), using primers specific for canine *SLAM* (5'-GAAGATCTGAATGGATTCCAGGGGCTTCCT-3' and 5'-GCGTCGACTCAGCTCTCTGGGAACGTCA-3'). The PCR product was cloned between the *Bgl*II and *Sal*I sites of the pCAGGSneo vector, which is a pCAGGS vector containing a neomycin-resistance gene. The coding region of *Nectin-4* was amplified with Phusion High-Fidelity DNA Polymerase, using primers specific for canine *Nectin-4* (5'-AAGCTTGCCACCATGCCTCTATCCCTGGGAGC-3' and 5'-GAATTCTCAGCCCATCACAGAGCAGC-3'). The PCR product was cloned into the *Hind*III and *Eco*RI sites of the pcDNA3.1 vector (Invitrogen, Carlsbad, CA).

### Transfected cells

HEK293 cells were transfected with the plasmid pCAGGSneo-canineSLAM or pcDNA3.1-canineNectin-4 using Lipofectamine LTX (Life Technologies), according to the manufacturer's instructions. To select cells expressing SLAM or Nectin-4, the transfected cells were cultured in medium containing 0.5–1.0 mg/ml G418 for 3 weeks. The expression of canine SLAM and canine Nectin-4 was confirmed with reverse transcription PCR, followed by cell cloning. These transfectant cells were designated HEK293/canineSLAM and HEK293/canineNectin-4, respectively.

### Viruses

rMV-EGFP and rMV-EGFP-SLAMblind were propagated in MCF7 cells, as described previously.<sup>25</sup> Each viral titer was determined as the TCID<sub>50</sub> with the Reed–Muench method<sup>45</sup> in CF33 cells.

### Reverse transcription PCR

Total RNA was extracted from the cells with ISOGEN (Nippon Gene, Tokyo, Japan), according to manufacturer's instructions. Reverse transcription was performed with PrimeScript Reverse Transcriptase (Takara, Otsu, Japan). PCR amplification was performed with AmpliTaq Gold (Life Technologies). The canine *SLAM*, canine *Nectin-4*, or hypoxanthine guanine phosphoribosyl transferase (*HPRT*) gene was amplified with the following primers: canine *SLAM* forward 5'-TCATGACCCCTGGAGGAGAAC-3' and reverse 5'-GGTCAATCCCCAGTTTCTCA-3'; canine *Nectin-4* forward 5'-GTCACTTCGGAGTTCCACCT-3' and reverse 5'-TGAGTGATGCGCCTTCTC TG-3'; and *HPRT* forward 5'-TGCTCGAGATGTGATGAAGG-3' and reverse 5'-TCCCCTGTTGACTGGTCATT-3'.

### Infection assay in transfected cells

HEK293, HEK293/canineSLAM, and HEK293/canineNectin-4 cells (5 × 10<sup>5</sup> cells each) were seeded in 12-well plates. After incubation at 37 °C for 1 hour, the cells were infected with rMV-EGFP or rMV-SLAMblind at a MOI of 0.01. Viral infection was detected as enhanced green fluorescent protein (EGFP) under a confocal laser scanning biological microscope (FV-1500; Olympus Optical, Tokyo, Japan). Image J 1.48v (National Institutes of Health, Bethesda, MD) was used to calculate the percentages of infected cells.

### Inhibition of infection

HEK293/canineNectin-4 cells were seeded in a 96-well plate and pretreated for 1 hour at 37 °C with medium containing 10 µg/ml affinity-purified goat antihuman Nectin-4 polyclonal antibody (anti-Nectin-4 antibody; R&D Systems, Minneapolis, MN) or goat control IgG (control IgG; R&D Systems). The cells were infected with rMV-EGFP-SLAMblind at an MOI of 0.01. The cells were observed under a confocal microscope 48 hours after infection.

### Growth kinetics

CF33 cells were infected with rMV-EGFP-SLAMblind at an MOI of 0.01 or 0.1 and incubated in DMEM supplemented with 0.5% FBS. The cell-free virus was obtained from the culture supernatants every 24 hours, and the cell-associated virus was harvested with three freeze–thaw cycles. The viral titers were determined with CF33 cells. The growth kinetics analysis in MCF7 cells was conducted similarly to CF33, except that the cells were incubated in RPMI1640 supplemented with 2% FBS after the virus inoculation.

### Cell viability

HEK293, HEK293/canineSLAM, and HEK293/canineNectin-4 cells were infected with rMV-SLAMblind at an MOI of 0.1. CF33, CTBm, and CHMm cells were infected with rMV-EGFP-SLAMblind at an MOI of 2. Cell viability was determined every 48 hours with the Premix WST-1 Cell Proliferation Assay System (Takara) and by the absorbance at 450 nm on a Microplate Reader model 450 (Bio-Rad, Hercules, CA). The viability of the cells infected with virus was calculated as the mean absorbance value divided by the mean absorbance value for uninfected cells and was expressed as a percentage.

### Assessment of oncolytic activity *in vivo*

The animal experiments were approved by the Experimental Animal Committee of the University of Tokyo.

Six-week-old female SCID mice (C.B-17/lcr-scld/scldJc1) were purchased from Clea Japan (Tokyo, Japan). CF33 cells (5 × 10<sup>6</sup>) were suspended in 50 µl of Hank's balanced salt solution (HBSS; Life Technologies) containing 2% FBS, and the suspension was mixed with 50 µl of Matrigel (BD Biosciences, San Diego, CA). The mixture was injected into the flanks of the mice. Nine days after implantation, the mice were intratumorally

administered 10<sup>6</sup> TCID<sub>50</sub> of rMV-EGFP-SLAMblind (*n* = 8) or Opti-MEM (Life Technologies) (*n* = 8). Viral inoculation was repeated 7 days after the first inoculation. The tumor diameters were measured with calipers every 2–3 days for 50 days after the first inoculation. The tumor volumes were calculated based on the formula (width × width × length)/2. All the mice were euthanized 50 days after the first viral inoculation, and tumor samples were collected. Some mice were euthanized 4 days after the first inoculation, and their tumor samples collected.

### Collection of clinical samples

The collection of clinical samples was approved by the Experimental Animal Committee of the Tokyo University of Agriculture and Technology. Informed consent was obtained from the owners of the diagnosed dogs. Spontaneous canine mammary tumor tissues were collected from 16 female dogs that had undergone surgical resection at animal hospitals.

### Pathological analysis

The samples collected from the xenografts were fixed in 4% paraformaldehyde in phosphate buffer solution (Wako, Osaka, Japan). After dehydration in sucrose solution, the samples were embedded in OCT compound (Sakura Finetek, Tokyo, Japan). The frozen tissues were sectioned to a thickness of 5 µm in a cryostat (Leica CM1900; Leica Microsystems, Wetzlar, Germany). The cell nuclei were stained with 2 µg/ml Hoechst 33342 (Cambrex, NJ). EGFP fluorescence, indicating viral infection, and Hoechst 33342 were observed with a confocal microscope.

Frozen sections (5 µm) or paraffin sections (3 µm) were analyzed immunohistochemically. The frozen sections were fixed in acetone for 5 minutes at –20 °C. The paraffin sections were deparaffinized in xylene, rehydrated in graded ethanol, and washed in phosphate-buffered saline (PBS). Antigen retrieval was performed with autoclaving in 0.01 mol/l citrate buffer (pH 6.0) for 15 minutes. The frozen and paraffin sections were incubated in 3% H<sub>2</sub>O<sub>2</sub> in PBS for 10 minutes to quench any endogenous peroxidase activity. After the slides were washed in PBS, they were incubated in 2.5% normal horse serum for 20 minutes to block nonspecific reactions and then stained at 4 °C overnight with 2 µg/ml anti-Nectin-4 antibody or 2 µg/ml control IgG as the primary antibody. They were then washed in PBS for 15 minutes and incubated with ImmPRESS Reagent, Anti-Goat Ig (Vector Laboratories, Burlingame, CA) for 30 minutes at room temperature. After the slides were washed in PBS for 15 minutes, they were visualized with 3,3'-diaminobenzidine and hematoxylin as the counterstain.

### Flow cytometry

Cells (1 × 10<sup>6</sup>) were stained with 0.2 µg of primary antibody in 100 µl of sample buffer (PBS containing 2% FBS and 0.02% Na<sub>2</sub>S<sub>2</sub>O<sub>8</sub>) on ice for 45 minutes. The primary antibodies used were anti-Nectin-4 antibody and control IgG. The cells were washed once and stained with 0.1 µg of Alexa-Fluor-488-conjugated rabbit anti-goat IgG antibody (Molecular Probes, Eugene, OR) in 100 µl of sample buffer on ice for 45 minutes. After the cells were washed, they were resuspended in PBS containing 7-aminoactinomycin D (Beckman Coulter Immunotech, Massielle, France). The flow-cytometric analysis was performed with a BD FACSCalibur or BD FACSVers flow cytometer (BD Biosciences). The data were analyzed with the FlowJo software ver. 9.7.5 (TreeStar, San Carlos, CA).

### Primary mammary tumor cell culture

The tumor samples were obtained during clinical surgery at an animal hospital. The solid tumor samples were digested with HBSS supplemented with 1 mg/ml collagenase (Wako) and 100 µg/ml DNase I (Worthington, Lakewood, NJ) at 37 °C for 2 hours. After the cells were washed twice with HBSS containing 2% FBS, they were analyzed with flow cytometry, followed by culture in DMEM containing 10% FBS in a six-well plate. The primary tumor cells were infected with rMV-EGFP-SLAMblind at an MOI of 0.01.

### Statistical analysis

The statistical analysis of the *in vitro* experiments was performed with Student's *t*-test or one-way analysis of variance with the Tukey test, and the statistical analysis of the *in vivo* experiments was performed with Welch's *t*-test. *P* < 0.05 was considered statistically significant.

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