ORIGINAL ARTICLE

Vaccinia virus preferentially infects and controls human and murine ovarian tumors in mice

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Vaccinia virus has been shown to efficiently infect tumor cells. Therefore, vaccinia virus represents a potentially safe and effective antitumor agent against ovarian cancer. Here, we assessed the ability of vaccinia virus to preferentially infect and control both human and murine ovarian tumors in vivo. We used the non-invasive luminescence imaging system to monitor the infection and suppression of ovarian tumors by vaccinia in live mice. Our data indicated that vaccinia was able to effectively infect and kill both human

and murine ovarian tumors. Vaccinia virus administered to mice intraperitoneally was specifically targeted to the murine or human ovarian tumors and led to antitumor responses. These findings suggest that vaccinia virus is capable of selectively targeting and controlling ovarian tumors. Thus, intraperitoneal injection with vaccinia virus may provide a potentially effective strategy for treating advanced-stage ovarian cancers.

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Introduction

Metastatic ovarian cancer is an almost incurable disease and bears the highest mortality rate among patients with gynecologic malignancies. Current efforts to reduce this mortality rate, including improvement of early detection and treatment, have been relatively unsuccessful. Existing therapies for advanced disease, such as chemotherapy and radiation therapy, rarely result in long-term benefits in patients with locally advanced and metastatic disease.^{1–3} Thus, there is an urgent need to develop an innovative therapeutic approach to control advanced-stage ovarian cancer.

Cancer therapy using oncolytic viruses represents a promising new approach for controlling tumors. Oncolytic viruses such as measles, vaccinia and sindbis viruses have been shown to infect and kill cancer cells.^{4–6} These biological agents are able to replicate within the target cell and lyse the cells directly. The viruses are also capable of spreading to adjacent tumor cells, thereby eradicating the target cells without harming the normal tissues. In addition, as the cellular pathways by which these viruses lyse the cells are highly

complex, the emergence of virus-resistant tumor cells is unlikely. Therefore, the selective targeting and replication of these viruses offer a potentially safe and effective alternative for combating cancers.

Several studies have shown that oncolytic viruses can control ovarian cancer in preclinical models. When administered intraperitoneally, these viruses may directly target ovarian tumors in the peritoneal cavity. For instance, Tseng *et al.*⁶ demonstrated that intraperitoneal injection using Sindbis viral vectors selectively infected and controlled human ovarian tumors in C.B-17-SCID (SCID) mice. In another study, intraperitoneal injection of live attenuated Edmonston B vaccine strain of measles virus (MV-Edm) specifically induced infection and regression of established human ovarian tumors in athymic mice. This recombinant measles virus-infected human ovarian tumors with minimal cytopathic effects on normal tissues.⁴ Nakamori *et al.*⁷ demonstrated that intraperitoneal delivery of herpes simplex virus-controlled human ovarian cancer cells in athymic mice. Similarly, Yaba-like disease virus has also been shown to selectively infect established murine ovarian tumors in mice after intraperitoneal delivery.⁸ The vaccinia virus, popular for its role in the eradication of smallpox, has also been used as an oncolytic virus because of its ability to preferentially infect tumors over normal tissues.^{9–12} A substantial amount of clinical trials have demonstrated the ability of vaccinia virus to treat various types of tumors.^{13,14} The virus has been safely injected into patients through subcutaneous,¹⁵ intramuscular,¹⁶ intra-

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tumoral¹⁷ and intravesicle¹⁸ routes of administration. The oncolytic ability of vaccinia, as well as the safety record of this virus, makes it a potentially ideal choice for cancer treatment.^{5,19,20}

To demonstrate *in vivo* infection of cells by vaccinia virus, it is crucial to use a non-invasive imaging technique that can trace the replication of vaccinia in live organisms. The luminescence imaging system has previously been shown to detect *in vivo* infection of various cell types in several studies using live animals. For instance, Luker *et al.*²¹ used luminescence imaging to monitor the effects of interferons on the progression of vaccinia infection in mice. The green fluorescent protein has also been used to detect the tumor-specific replication of vaccinia virus.²² Thus, the employment of imaging systems for the detection of vaccinia infection in live animals has become increasingly popular.

In the current study, we assess the feasibility of using vaccinia to preferentially infect and control human and murine ovarian tumors. To monitor the infection and suppression of ovarian tumors by vaccinia in live mice, we use the luminescence imaging system. Our findings indicate that vaccinia virus is capable of preferentially infecting and controlling both human and murine

ovarian tumors in tumor-challenged mice. Thus, oncolytic virus therapy using vaccinia represents a promising new approach for the treatment of ovarian cancer. The clinical implication of this approach is discussed.

Results

Vaccinia virus is capable of infecting human and murine ovarian cancer cells in vitro

To determine whether vaccinia virus can infect human and murine ovarian cancer cells *in vitro*, we used a human ovarian cancer cell line (ES2) as well as a murine ovarian cancer cell line (Defb29 Vegf). The ES2 and the Defb29 Vegf cells were infected with increasing titers of a thymidine kinase-deleted (tk⁻) version of vaccinia that encodes luciferase (Vac-luciferase). Luciferase expression was characterized by luminescence imaging. As shown in Figure 1, luciferase activity was detected in both cell lines infected with Vac-luciferase. In addition, increasing luciferase expression was observed in cells infected with increasing titers of Vac-luciferase. Overall, the infected Defb29 Vegf cell line showed greater luciferase expression compared to the infected ES2 cell line. These data

Figure 1 Characterization of Vac-luciferase expression in infected human and murine ovarian cancer cell lines *in vitro*. Human and murine ovarian cancer cell lines were either infected with increasing titers of Vac-luciferase (0.005, 0.05 or 0.5 MOI/well) for 24 h or non-infected. A measure of 15 μ g/ml luciferin was added and incubated for 5 min. An integration time of 10 s was used for luminescence image acquisition. Luminescence images of (a) human ovarian cancer cell line (ES2) and (b) murine ovarian cancer cell line (Defb29 Vegf). Bar graph depicting the total photon counts for each well (mean $\pm$ s.d.) of the (c) Vac-luciferase-infected ES2 cell line and (d) Vac-luciferase-infected Defb29 Vegf cell line.

indicate that vaccinia virus can effectively infect both human and ovarian cancer cell lines *in vitro*.

Vaccinia virus can lyse human and murine ovarian cancer cells in vitro

As we have established that vaccinia can effectively infect ovarian cancer cells, we next determined whether infection with vaccinia virus could lead to the killing of human or murine ovarian cancer cells. In order to follow the lysis of ovarian cancer cells, we transduced ES2 and Defb29 cell lines with a retrovirus encoding luciferase (ES2-luciferase and Defb29 Vegf-luciferase, respectively). The transduced cell lines were subsequently infected with increasing titers of wild-type vaccinia (Vac-WT). Luciferase expression of the infected cells was characterized by luminescence imaging acquisition. As shown in Figure 2, although the cell lines that were not infected with Vac-WT continued to show luciferase activity, luciferase activity decreased in cell lines infected with increasing titers of vaccinia. Reduced luciferase expression indicates the lysis of the ovarian cancer cell lines. Thus, these data indicate that infection with vaccinia virus can cause lysis of both murine and human luciferase-expressing ovarian cancer cell lines *in vitro*.

Vaccinia virus is capable of infecting and controlling murine ovarian cancer cells in vivo

Given the ability of vaccinia virus to infect and kill murine ovarian cancer cell lines *in vitro*, we next determined whether vaccinia virus could also effectively infect and lyse murine ovarian cancer cells in C57BL/6 mice. To demonstrate preferential infection of murine ovarian cancer cells by vaccinia virus, C57BL/6 mice were either challenged with Defb29 Vegf cell line or were not challenged. The mice were subsequently infected with Vac-luciferase (tk⁻). Luminescence images of the murine ovarian tumor-bearing mice showed high luciferase activity on days 1 and 3 after Vac-luciferase infection (Figure 3). In comparison, mice without tumor challenge showed significantly lower or no luciferase expression after infection with Vac-luciferase (Figure 3). These data suggest that vaccinia virus may preferentially infect murine ovarian cancer cells in tumor-challenged mice. Interestingly, little or no luciferase expression was observed in both the murine ovarian tumor-challenged and non-challenged mice on day 5 after Vac-luciferase infection (Figure 3a). This observation suggests the clearance of the luciferase-expressing vaccinia in mice within 5 days of infection.

Figure 2 Characterization of the lysis of luciferase-expressing human and murine ovarian cancer cell lines after infection with wild-type vaccinia *in vitro*. Luciferase-expressing human and murine ovarian cancer cell lines were either infected with increasing titers (0.005, 0.05 or 0.5 MOI/well) of wild-type vaccinia (Vac-WT) for 48 h or were non-infected. Luciferin (15 μ g/ml) was added and incubated for 5 min. An integration time of 10 s was used for luminescence image acquisition. Luminescence images of the (a) luciferase-expressing human ovarian cancer cell line (ES2-luciferase) and (b) luciferase-expressing murine ovarian cancer cell line (Defb29 Vegf-luciferase). Bar graph depicting the total photon counts for each well (mean $\pm$ s.d.) of the (c) Vac-WT-infected, ES2-luciferase cell line and (d) Vac-WT-infected, Defb29 Vegf-luciferase cell line.

Figure 3 Characterization of luciferase expression in murine ovarian tumor-bearing mice infected with Vac-luciferase. C57BL/6 mice (five mice were per group) were either challenged with Defb29 Vegf cell line (2×10^5 /mice) or without tumor challenge. One day after tumor challenge, the mice were infected with 1×10^7 PFU/mouse of Vac-luciferase. Mice challenged with PBS were used as a negative control (data not shown). Luciferase expression in mice was characterized by luminescence imaging on days 1, 3 and 5 after vaccinia challenge. An integration time of 2 min was used for the acquisition of luminescence images. (a) Luminescence images of the Defb29 Vegf tumor-bearing mice. (b) Luminescence images of the naïve mice. (c) Line graph depicting the kinetic expression of luciferase (mean $\pm$ s.d.) in Vac-luciferase-infected mice.

We have also attempted to treat tumor-challenged mice with phosphate-buffered saline (PBS). The PBS-treated mice showed a background level of photon counts (1000–2000 counts) that were indistinguishable from that of non-tumor-challenged mice infected with Vac-luciferase at day 5 (Figure 3b).

We then investigated the ability of vaccinia virus to control murine ovarian tumors in C57BL/6 mice. The mice were intraperitoneally challenged with Defb29 Vegf-luciferase, and then subsequently infected with Vac-WT or PBS. The antitumor effects of vaccinia infection were determined by characterization of luciferase expression using luminescence imaging. As shown in Figure 4, the Defb29 Vegf-luciferase-challenged mice that were treated with Vac-WT demonstrated significantly lower overall expression of luciferase compared to mice treated with PBS. In addition, Defb29 Vegf-luciferase-challenged mice that did not receive treatment with Vac-WT continued to increase their luciferase activity over time. These data suggest that infection with vaccinia can lead to the killing of tumor cells in animals. Vaccinia

infection has been shown to lead to tumor necrosis in vaccinia-treated animals.¹² We have further confirmed the killing of tumor cells mediated by vaccinia. We dissected mice challenged with luciferase-expressing tumors and found that all of the Vac-WT-treated mice with little or no luciferase activity had no grossly appreciable tumors. In contrast, we observed grossly appreciable tumor in all of the mice showing high luciferase activity (data not shown). These results indicate that vaccinia is capable of killing tumor cells *in vivo*, and that luciferase activity correlates with tumor load.

Taken together, our findings indicate that vaccinia virus is able to control murine ovarian tumors in C57BL/6 mice. We also examined survival rates in tumor-challenged mice receiving either Vac-WT or PBS treatment. The Kaplan–Meier survival curve demonstrated that 100% of the Defb29 Vegf-luciferase-challenged mice treated with Vac-WT were able to survive 63 days after the treatment (Figure 4d). In contrast, all of the Defb29 Vegf-luciferase-challenged mice that did not receive

Figure 4 Characterization of antitumor effects against luciferase-expressing, murine ovarian tumors in mice infected with vaccinia. C57BL/6 mice (five per group) were intraperitoneally challenged with luciferase-expressing, murine ovarian tumors (Debf29 Vegf-luciferase) at a dose of 2×10^5 tumor cells/mouse. One day after tumor challenge, the mice were treated with either 1×10^7 PFU/mouse of Vac-WT (WR strain) or an equal volume of PBS. The antitumor effects were determined by the characterization of luciferase expression using luminescence imaging taken on days 1, 5, 10 and 17. An integration time of 2 min was used for luminescence imaging acquisition. (a) Luminescence images of the murine ovarian tumor-challenged mice treated with PBS over time. (b) Luminescence images of the murine ovarian tumor-challenged mice treated with Vac-WT (WR strain). (c) Line graph depicting the kinetic expression of luciferase (mean $\pm$ s.d.). (d) Kaplan-Meier curve to show the survival of murine ovarian tumor-challenged mice after treatment with Vac-WT or PBS.

Vac-WT treatment died within 42 days after treatment with PBS.

Vaccinia virus is capable of infecting and controlling human ovarian cancer cells in vivo

We also assessed the ability of vaccinia virus to infect and control human ovarian tumors in nude mice that are immunocompromised. We initially determined whether vaccinia virus could infect human ovarian tumors in nude mice. The mice were either challenged with the ES2 cell line or received no tumor challenge. The mice were subsequently infected with Vac-luciferase (tk⁻). As shown in Figure 5, the ES2 tumor-challenged mice infected with Vac-luciferase showed significantly higher luciferase activity compared to the Vac-luciferase-infected mice that did not receive tumor challenge. These results suggest that vaccinia virus is also capable of preferentially infecting human ovarian tumors over normal tissues in nude mice.

The variability in photon counts in the ES2 tumor-bearing mice on days 12 and 15 after Vac-luciferase injection (Figure 5c) may be contributed by variability in detection signal at these low levels as well as variability between animals. The detection of low-level luciferase activity on day 15 after Vac-luciferase injection implies persistent vaccinia infection in athymic mice. Gnant *et al.*¹⁰ has also reported that vaccinia infection in immunocompromised mice can lead to prolonged infection compared to immunocompetent mice. It is interesting to note that the clearance of luciferase-expressing vaccinia in C57BL/6 mice (Figure 3) is more rapid compared to immunocompetent nude mice (Figure 5).

We next investigated whether vaccinia virus could control human ovarian tumor growth in nude mice. We intraperitoneally challenged nude mice with ES2 cell lines, and subsequently treated them with either Vac-luciferase or PBS. The increase of abdominal girth following tumor challenge was indicative of ascites and therefore tumor burden. Growth of tumors was followed

Figure 5 Characterization of luciferase expression in human ovarian tumor-bearing mice infected with Vac-luciferase. Nude mice (five per group) were either challenged with human ovarian tumors (ES2) at a dose of 1×10^6 tumor cells/mouse or were without tumor challenge. One day after tumor challenge, the mice were infected with 1×10^7 PFU/mouse of Vac-luciferase. Mice treated with PBS were used as a negative control (data not shown). Luminescence images were taken on days 1, 3 and 15 after Vac-luciferase or PBS treatment to characterize luciferase expression. (a) Luminescence images of human ovarian tumor-bearing mice after Vac-luciferase treatment. (b) Luminescence images of mice without tumor challenge after treatment with Vac-luciferase. (c) Line graph depicting the kinetic expression of luciferase (mean $\pm$ s.d.) after Vac-luciferase infection.

by inspection. As shown in Figure 6, the ES2-tumor-challenged mice that did not receive Vac-luciferase treatment continued to exhibit tumor growth, as demonstrated by the increase of abdominal girth. In contrast, the ES2-challenged mice treated with Vac-luciferase exhibited a significant reduction in tumor growth (Figure 6b). We have also evaluated the survival rates of the tumor-challenged mice treated with Vac-luciferase or PBS. As shown in Figure 6c, 100% of the ES2-challenged mice injected with vaccinia were able to survive 70 days after the treatment. In contrast, all of the ES2-challenged mice that did not receive vaccinia treatment died within 28 days after PBS treatment. Taken together, our data suggest that vaccinia virus is capable of controlling human ovarian tumors in nude mice.

Discussion

In the current study, we have demonstrated the ability of vaccinia virus to control murine and human ovarian tumors in preclinical models. Our data indicate that vaccinia virus can effectively infect and kill both human and murine ovarian cancer cells *in vitro*. Furthermore, vaccinia virus (tk⁻) administered to mice intraperitoneally preferentially targeted human ovarian tumor cells and led to antitumor responses. The tested virus was derived from the vaccine strain (Lister) used in the international program on smallpox eradication. Thus, our data provided a strong rationale for the clinical translation of vaccinia as an oncolytic viral vector for the control of ovarian cancer.

Figure 6 Characterization of anti-tumor effects against human ovarian tumors in mice treated with vaccinia. Nude mice (five per group) were intraperitoneally challenged with human ovarian tumors (ES2) at a dose of 1×10^6 tumor cells/mouse. One day after tumor challenge, the mice were treated with either 1×10^7 PFU/mouse of Vac-luciferase or an equal volume of PBS. Tumor growth was followed by inspection and palpation. (a) Representative images of ES2 tumor-challenged mice treated with PBS at 20 days. (b) Representative images of ES2 tumor-challenged mice treated with Vac-luciferase. (c) Kaplan-Meier curve depicting the survival of mice after Vac-luciferase or PBS treatment.

Our findings are consistent with previous studies that demonstrate preferential infection of tumors over normal tissues by vaccinia virus. For example, Yu *et al.*²² demonstrated that subcutaneous C6 glioma tumors were preferentially infected by vaccinia virus in nude mice. In another study, Puhlmann *et al.*⁹ showed that vaccinia administered intraperitoneally preferentially infected intraperitoneal tumors compared with normal vital organs, including lung, liver, heart and kidney. Minimal vaccinia infection was detected in some of the intra-abdominal tissues, such as peritoneal lining and spleen compared to tumor cells within the peritoneal cavity.⁹ Furthermore, intravenous administration of the luciferase-expressing recombinant vaccinia virus led to significantly higher luciferase activity in different tumor models compared to normal tissues assayed.^{9,23} All these studies indicate that vaccinia virus is a potentially useful oncolytic viral vector that can preferentially infect tumors over normal tissues, although differences in results may reflect sensitivity of the assays, tumor models used, or strain of vaccinia employed in these studies.

Normal ovaries have also been shown by others to be infected by vaccinia.^{9,12,24,25} Immunohistochemistry revealed viral infection in ovarian follicles and in adjacent

ovarian stromal tissues.⁹ Although the ovary was found to have one of the highest luciferase activity among all normal organs after infection with vaccinia encoding luciferase, Puhlmann *et al.*⁹ demonstrated that luciferase activity was much higher in tumors than ovary. For clinical translation in patients with ovarian cancer, infection of normal ovarian cells by vaccinia should not be a significant concern, as bilateral ovaries are usually resected during surgery for the management of ovarian cancer patients.

In the current study, we used two different strains of vaccinia (Vac-luciferase and Vac-WT) to demonstrate its ability to infect and kill murine and human ovarian tumors. The Vac-luciferase (Lister strain, rVV4) is a hyper-attenuated recombinant derivative of vaccinia virus. It contains two reporter genes (luc and lacZ) inserted into the thymidine kinase region of vaccinia virus (tk-) as described.²⁶ The Vac-WT is based on the WR strain that is not attenuated.²⁷ We used the Lister strain for all of our *in vivo* studies, except for the murine ovarian tumor killing experiment, in which murine ovarian tumor-challenged C57BL/6 mice were treated with Vac-WT. Vac-WT cannot be used to treat tumors in nude mice, as Vac-WT would likely kill the immuno-compromised mice.

Our data indicated that nude mice showed significantly decreased luciferase activity 9 days after Vaccinia infection (Figure 5). One possible explanation is that vaccinia virus infection may have induced natural killer (NK) cell activation, proliferation and accumulation at the infection site. Although athymic mice do not have T cells, they do have sufficient NK cells. It is known that vaccinia virus-infected cells demonstrate increased susceptibility to NK cell lysis, and NK cells play a role in the protective response against poxvirus.²⁸ However, the mechanisms by which NK cells recognize cells infected with vaccinia are not fully understood.²⁸

The oncolytic effects of vaccinia viruses can be enhanced by insertion of genes encoding anticancer therapeutic agents into the viral genome. Indeed, intratumoral and intravesical therapy of experimental glioma and bladder tumors, respectively, by recombinant vaccinia virus expressing p53 was more effective than identical therapy using Vac-WT.^{29,30}

Although the employment of vaccinia represents an appealing strategy to control ovarian cancer, some concerns associated with its usage as a biotherapeutic agent include the presence of antivaccinia antibodies and the generation of immune responses in previously vaccinated patients. For instance, in the current study, significant reduction in luciferase activity was observed in mice 5 days after vaccinia infection (Figure 3a). This rapid clearance of the luciferase-expressing virus may be related to the high level of attenuation of vaccinia virus and effective innate immune response of infected mice. Furthermore, as the vaccinia virus has been used for the eradication of smallpox, many individuals have been previously vaccinated with vaccinia. These individuals may have a pre-existing immunity against the vaccinia virus and may not be suitable candidates for the treatment with the same kind of vaccinia vector. One strategy to resolve such limitations is to use different poxviruses for the control of ovarian cancer. For example, Yaba-like disease virus may be used as an alternative replicating poxvirus vector for the treatment of cancers. Hu *et al.*⁸ have demonstrated that the anti-vaccinia antibodies does not crossreact with the Yaba-like disease virus. Therefore, such viruses could be suitable for the further treatment of ovarian cancer in patients who have existing anti-vaccinia antibodies.

The route of administration for the vaccinia vector is also an important factor for effective treatment. In this study, we demonstrated that the intraperitoneal administration of vaccinia resulted in the selective infection and lysis of the human and murine ovarian tumors in live mice. For ovarian cancer treatment, intraperitoneal injection is likely the ideal route of administration. Intraperitoneal delivery allows the vaccinia virus to be directly targeted to the ovarian tumors, enhancing its ability to selectively infect and lyse ovarian cancer cells. We have also attempted to introduce the vaccinia to mice by tail vein injection. However, we have not observed any significant antitumor effects against tumors located in the peritoneal cavity. Instead, vaccinia infection was mainly observed in the lung cavity (data not shown).

Although our data indicated that intravenous injection with vaccinia could not control intraperitoneal tumors, other studies using different strains of vaccinia or tumor models suggest that intravenous injection may be feasible. For example, intravenous administration of

vaccinia virus has been shown to lead to significant antitumor effects in several tumor models.⁹ In addition, recombinant vaccinia virus (WR strain) has been shown to treat murine liver metastases through several routes of administration, including portal venous, intraperitoneal or intravenous injection.¹¹ Thus, differences in vaccinia strains, tumor models and tumor locations may affect the optimal route of administration of vaccinia in controlling tumors.

Although treatment with the virus is believed to be optimal during the early stage of ovarian cancer, most ovarian tumors are already in the advanced stage by the time they are diagnosed. Therefore, the best time to treat ovarian tumors with the virus is probably after the majority of the tumor has been removed (tumor debulking). At this stage, only minimal residual tumor remains. This makes oncolytic viral therapy more plausible for the control of ovarian cancer.

In summary, our findings suggest that the intraperitoneal administration of vaccinia virus may provide effective therapeutic effects against ovarian cancer. The selective infection and lysis of the ovarian tumors by vaccinia represent an appealing approach to control advanced-stage ovarian cancers. In addition, the selection of an optimal vaccinia viral vector and appropriate route of administration are important factors to consider for an effective control of ovarian cancer.

Materials and methods

Mice

The C57BL/6 and nude (BALB/c *nu/nu*) mice were acquired from the National Cancer Institute. All animals were maintained under specific pathogen-free conditions, and all procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals.

Vaccinia

The Vac-luciferase (Lister strain, rVV4) was generated using a previously described protocol. The rVV4 is a hyper-attenuated recombinant derivative of the vaccine strain Lister of vaccinia virus. It contains two reporter genes (*luc* and *lacZ*) inserted into the thymidine kinase region of VV (*tk*–) as described.²⁶ The Vac-WT (WR strain) was prepared as described previously.²⁷

Cell lines

The ES2 cell line was purchased from the American Type Culture Collection (Manassas, VA, USA). The Defb29 Vegf cell line was prepared as described previously.³¹ The ES2-luciferase and Defb29 Vegf-luciferase cell lines were generated by transducing ES2 and Defb29 Vegf cells with a retrovirus containing luciferase, respectively. In order to generate a retrovirus containing luciferase, a pLuci-thy1.1 construct expressing both luciferase and thy1.1 was made. Firefly luciferase was amplified by polymerase chain reaction from pGL3-basic (Promega, Madison, WI, USA) using the 5' primer CGGAGATC-TATGGAAGACGCCAAAAAC and the 3' primer CGG GTTAACTTACACGGCGATCTTCC. The amplified luciferase cDNA was inserted into the *Bgl*III and *Hpa*I sites of the bicistronic vector pMIG-thy1.1. Both luciferase and thy1.1 cDNA are under the control of a single

promoter element and separated by an internal ribosomal entry site. The pLuci-thy1.1 was transfected into Phoenix packaging cell line and the virion-containing supernatant was collected 48 h after transfection. The supernatant was immediately treated using a 0.45-mm cellulose acetate syringe filter (Nalgene, Rochester, NY, USA) and used to infect ES2 and Defb29 Vegf cells in the presence of 8 mg/ml Polybrene (Sigma, St Louis, MO, USA). ES2-luciferase and Defb29 Vegf-luciferase cells were isolated using preparative flow cytometry of stained cells with Thy1.1 antibody (BD, Franklin Lakes, NJ, USA). The growth rates of ES2-luciferase and Defb29 Vegf-luciferase cell lines were comparable to those of ES2 and Defb29 Vegf cell lines (data not shown).

In vitro infection of tumor cells by vaccinia

For tumor infection studies, 2×10^5 ES2 or Defb29 Vegf cells were plated in 24-well plates and grown overnight. The cells were infected with Vac-luciferase at various titers (multiplicity of infection (MOI)) of the virus for 24 h. Luciferin (15 μ g/ml) was added and incubated for 5 min. An integration time of 10 s was used for luminescence image acquisition by the IVIS 200 system (Xenogen Corp., Alameda, CA, USA).

In vitro lysis of tumor cells by vaccinia

For tumor killing studies, 2×10^5 ES2-luciferase or Defb29 Vegf-luciferase cells were plated in 24-well plates and grown overnight. The cells were infected with increasing titers of Vac-WT (MOI) for 48 h. Luciferin (15 μ g/ml) was added and incubated for 5 min. An integration time of 10 s was used for luminescence image acquisition.

Characterization of tumor infection by vaccinia in mice

To demonstrate that vaccinia virus can infect murine ovarian tumors, C57BL/6 mice (five per group) were injected with 2×10^5 Defb29 Vegf cells/mouse intraperitoneally. One day after the tumor challenge, the mice were injected with 1×10^7 pore-forming unit (PFU)/mouse of either Vac-luciferase or PBS intraperitoneally. Luminescence images were recorded from day 1 after the Vac-luciferase injection. An integration time of 2 min was used for luminescence image acquisition.

To determine whether vaccinia can infect human ovarian tumors, the nude mice (five per group) were injected with 1×10^6 ES2 cells/mouse intraperitoneally. One day after the tumor challenge, the mice were treated with either 1×10^7 PFU of Vac-luciferase or PBS. Luminescence images were taken from day 1 after the Vac-luciferase injection. The mice were injected with 0.2 ml of 15 mg/ml beetle luciferin (potassium salt; Promega). After 10 min, the mice were imaged using the IVIS 200 system (Xenogen Corp., Alameda, CA, USA). An integration time of 30 s were used for luminescence image acquisition.

Characterization of antitumor effects by vaccinia in mice

To demonstrate that vaccinia virus is capable of controlling murine ovarian tumors, C57BL/6 mice (five per group) were intraperitoneally challenged with Defb29 Vegf-luciferase at a dose of 2×10^5 tumor cells/mouse. One day later, the mice were either injected with

1×10^7 PFU/mouse of Vac-WT or treated with PBS intraperitoneally. Luminescence images were recorded from day 1 after vaccinia injection. An integration time of 2 min was used for luminescence image acquisition. The survival of mice was recorded.

To demonstrate that vaccinia virus is capable of controlling human ovarian tumors, nude mice (five per group) were challenged with ES2 at a dose of 1×10^6 tumor cells/mouse. One day after the tumor challenge, the mice were injected with 1×10^7 PFU/mouse of Vac-luciferase or equal volume of PBS. Tumor growth was followed by inspection and palpation. The gross pictures of mice were taken 20 days after tumor challenge and the survival of mice was recorded.

Statistical analysis

All data expressed as means $\pm$ s.d. are representative of at least two different experiments. For analysis of the tumor protection experiment, we used Kaplan–Meier analysis.

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