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Final Progress Report and Addendum

(11-1-2007 to 10-31-2008; addendum 11-1-2008 to 10-31-2009)

DOD Grant W81XWH-06-1-0095 (27774): A mouse model to investigate postmenopausal biology as an etiology of ovarian cancer risk.

1. Abstract

We are completing this project to use a germ cell deficient *Wv* mouse model to test the hypothesis of a synergy between oncogenic mutations and postmenopausal biology in ovarian cancer development. We found that crossing of *Wv* mice into mutant *p53* or *pten* (+/-) background did not lead to a malignant tumor phenotype (**Aim 1**). Instead, the mutants rescue ovarian germ cells, a very interesting finding. The ovarian surface epithelia in *Wv/Wv:p27* (+/-) or *Wv/Wv:p27* (+/-) compound mutant mice develop unique lesions with peculiar morphology and formed large ovarian tumors in older mice (**Aim 2**). The analysis of tumor phenotypes (**Aim 3**) is ongoing and we hope to complete and report the findings in next several months.

Thus, in this project we have successfully developed *Wv/Wv:p27* (+/-) mice as suitable models of ovarian epithelial cancer. We conclude that the result support our hypothesis that the collaboration of reproductive factors and genetic mutations leads to the development of ovarian cancer. The study also provides us with future directions, and we plan to seek future support to use *flox-p53* mutant mice to create additional models. In sum, the project is completed as planned and is successful, and provides basis for further advance.

2. Introduction

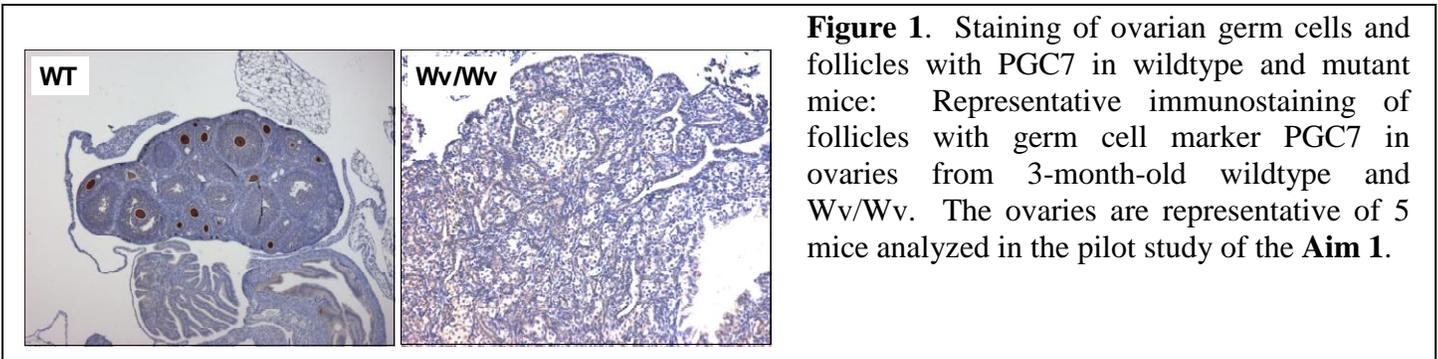
This project is to use a unique *Wv* mouse model to study the interaction of reproductive factors and genetic mutations in the development of ovarian cancer. Ovarian cancer often develops in women of perimenopausal age, when ovulation ceases but gonadotropin levels are increased. We found that the germ cell deficient *Wv* mice mimics postmenopausal biology and develop benign ovarian tumors. We plan to test the hypothesis that the synergy between oncogenic mutations and postmenopausal biology can be revealed by combining the germ-cell deficient phenotype of the *Wv/Wv* mice and genetic alterations such as *p53*, *pten*, or *p27kip1*, which are found in human ovarian cancer.

3. Body: Research Progress

In the first year of the project, we completed **Aim 1/Task 1**, to determine the optimal genetic changes that synergize with the Wv genotype for the development of malignant ovarian tumors in mice (Months 1-12). We carried out studies to use the Wv mouse model to study the interaction of reproductive factors and genetic mutations in the development of ovarian cancer. We tested the hypothesis that the synergy between oncogenic mutations and postmenopausal biology can be revealed by combining the germ-cell deficient phenotype of the Wv/Wv mice and genetic alterations such as p53, pten, or p27kip1, which are found in human ovarian cancer. We crossed and obtained several female Wv mice with additional p53 (-/-), p27 (-/-), or pten (+/-) mutation and analyzed the ovaries for tumor phenotype. The details of the results are described below for each combination of genotypes tested.

p53 deletion reverts ovarian tumor phenotype in Wv/Wv mice

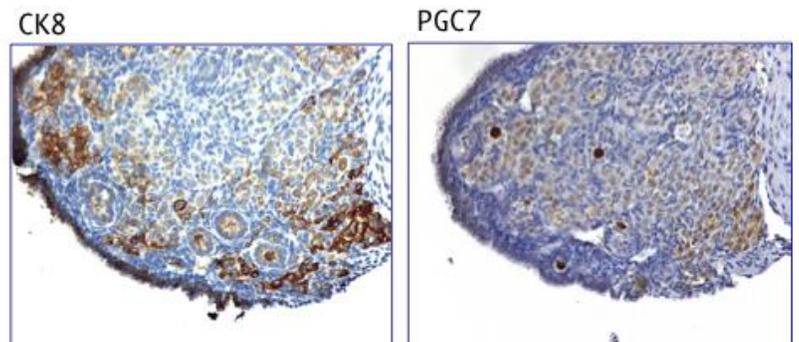
At 3 months of age, the wildtype ovary contains many germ cells and follicles that are indicated by positive staining with marker PGC7 (**Figure 1**). The Wv/Wv ovary shows infiltration with epithelial derived tumor cells and contains no germ cells or follicles (**Figure 1**).



We found that crossing Wv mice into mutant p53 did not lead to development of large tumors or a malignant tumor phenotype. Instead, the additional p53 mutation rescues ovarian germ cells, a very interesting finding. As shown in a representative figure (**Figure 2**), an ovary of Wv/Wv:p53 (-/-) mouse showed the absence of CK-8-positive epithelial tumor cells and the presence of PGC7-positive follicles (**Figure 2**). Thus, deletion of p53 in the Wv/Wv:p53 (-/-) ovary results in the absence of tumor and the presence of germ cells/follicles. This finding suggests p53 is highly important for the survival and lifespan of ovarian germ cells and follicles. Also, the finding suggests that the depletion of follicles is key causal factor for the ovarian tumor phenotype.

Figure 2. Ovarian morphology in Wv/Wv:p53 (-/-) ovaries.

An immunostaining with epithelial cell marker cytokeratin 8 (CK8) and germ cell marker PGC7 of a representative ovary of the 3-month-old Wv/Wv:p53 (-/-) mice. The staining shows that there is no tumor lesion in the ovary. The presence of several germ cells or follicles are indicated by the positive staining of PGC7 in an adjacent section of the same ovary. This ovary is representative of ovarian tissues harvested from 5 mice analyzed in the pilot study.



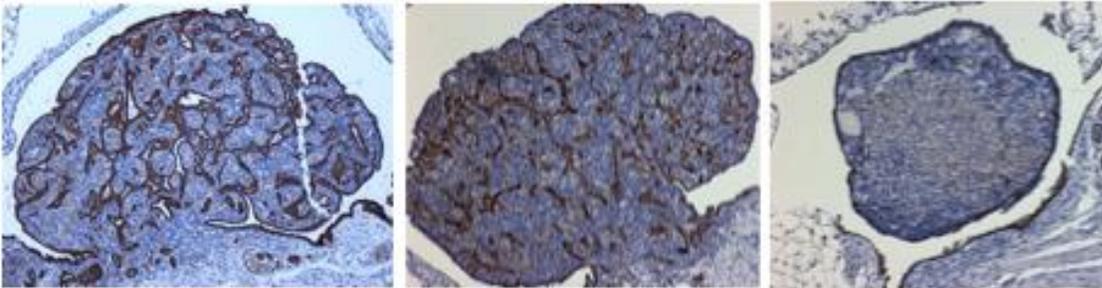
In future study, we plan to create a p53 mutation only in ovarian surface epithelial cells to avoid the rescuing/cell surviving activity in germ cells by a cre-lox conditional gene targeting approach. Currently, we are crossing Wv mice into flox-p53 mutant mice. We will introduce cre recombinase into ovarian surface epithelial cells in the mice by injecting adeno-cre into ovary to delete p53 only in surface epithelial cells but not in germ cells.

Reduction of pten gene dosage has subtle influence on Wv/Wv ovarian tumor phenotypes

The addition of pten mutation into Wv/Wv mice did not significantly alter the tumor phenotype (**Figure 3**), rather it reduced the presence of epithelial tumor lesions in about one third of the ovaries. Interestingly, follicle structure was observed in the ovaries with a reduced tumor phenotype, similar to the addition of p53 mutation. It is possible that a reduction of pten gene dosage leads to an increased survival of ovarian follicles and germ cells, and thus a reduced tumor phenotype. We conclude that we need to introduce the additional mutations only to the surface epithelial cells but not to the germ cells of the Wv ovaries.

Figure 3. Ovarian morphological features of Wv/Wv:pten (+/-) mutant mice: Representative cytokeratin staining (of epithelial cells) of three ovaries from 3-month-old Wv/Wv:pten (+/-) mice. One ovary shown (right panel) exhibits a reduced tumor lesion. These ovaries are representative of ovarian tissues harvested from 5 mice each analyzed in the pilot study of the **Aim 1**.

In future experiments, we should use Wv/Wv:pten (flox/flox) model to study pten deletion only in epithelial cells but not in follicles and germ cells.

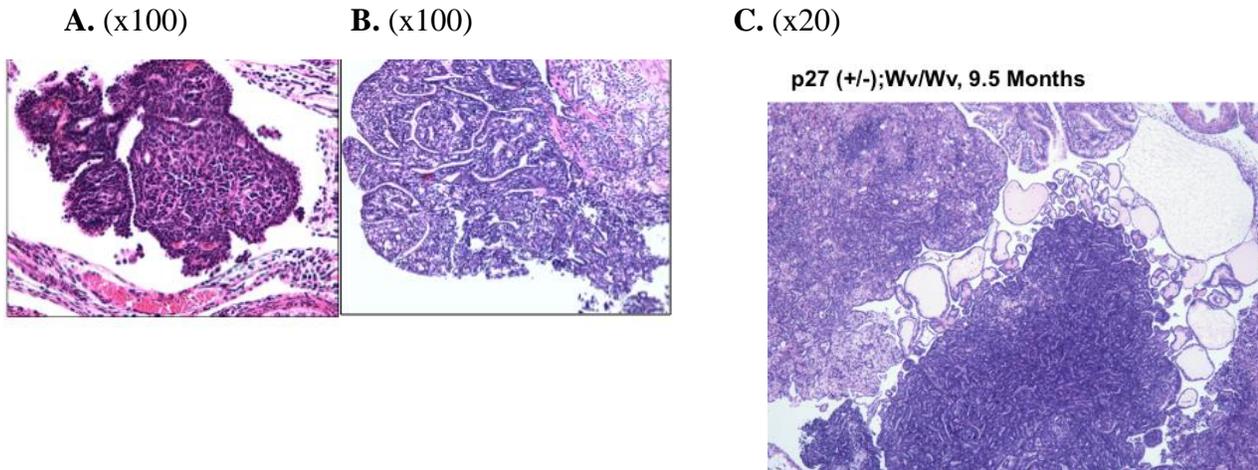


p27kip1 deletion enhances Wv/Wv ovarian tumor phenotypes

Reduction of p27 expression predicts ovarian prognosis in human ovarian cancer, and p27 loss is likely important for ovarian cancer development. The addition of p27 deletion into Wv/Wv mice enhanced the tumor phenotypes. The ovarian surface epithelia in these compound mutant mice, both Wv/Wv:p27 (-/-) and Wv/Wv:p27 (+/-), develop lesions with unique morphology (**Figure 4A,B**), resembling papillo endometrial and serous carcinoma morphology. The addition of p27 (-/-) background often led to lymphoma phenotype in 6-month or older mice. In aged Wv/Wv:p27 (+/-) mice, large ovarian tumors had developed, invaded and embedded in the entire region (**Figure 4C**), often causing lethality of the tumor-bearing mice.

Thus, the Wv/Wv:p27 (+/-) ovarian tumor mouse model appears highly promising and it is one of our successes in **Aim 1**. Thus, we used Wv/Wv:p27 (+/-) ovarian tumor model in the more detailed analysis in **Aim 2**. Currently, we are continuing the analysis of Wv/Wv:p27 (+/-) mouse ovarian epithelial tumors for proliferation and expression of proteases, etc. The results will be ready for preparation in report shortly.

Figure 4. Ovarian morphology in Wv/Wv:p27 (-/-) and Wv/Wv:p27 (+/-) ovaries. H&E images of a representative ovary of the 4-month-old Wv/Wv:p27 (-/-) (A) and Wv/Wv:p27 (+/-) (B) mice. H&E staining shows the unique morphology of the ovarian tumor. By 9 months of age, ovarian tumors from Wv/Wv:p27 (+/-) are much enlarged and embedded in the entire ovarian/uterine region, highly resembling human ovarian cancer (C).



We completed all the experiments in **Aim 1**, and we reached a conclusion that Wv/Wv:p53 (-/-) and Wv/Wv:pten (+/-) mice do not develop neoplastic ovarian tumors, due to the rescuing of ovarian germ cells and follicles in the compound mutant mice.

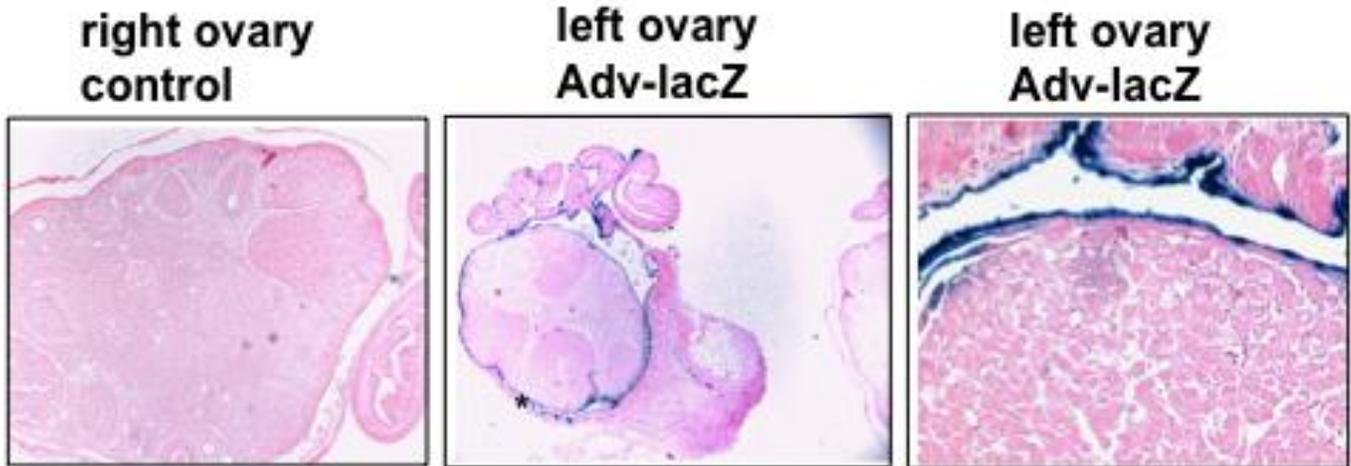
We found Wv/Wv:p27 (-/-) and Wv/Wv:p27 (+/-) mice develop more malignant and bigger ovarian tumors, and will be proper mouse models for continue studying. Currently, we are completing the analysis ovarian tumor phenotypes from of these p27 mutant mice (**Aim 3**). The results will be ready for report in a few months.

In additional future study, we created mutation only in ovarian surface epithelial cells to avoid the rescuing activity in germ cells by a cre-lox conditional gene targeting approach (**Aim 2**). We crossed Wv mice into flox-p53 mutant mice. Then, cre recombinase was introduced into ovarian surface epithelial cells in the mice by injecting adeno-cre into ovary to delete p53 only in surface epithelial cells but not in germ cells. This approach was considered in the original application as an alternative approach. Thus, *Task 2* (**Aim 2**) has been carried as planned. The experiments were carried out essentially as that were planned in the original application. The timeline and tasks in the Statement of Work have been well followed.

Adenoviral delivery of cre to ovarian surface epithelial cells

As part of **Aim 2**, we first established the approach for expressing cre in ovaries in the lab. When the mice are 2 and 6 months of age, the female Wv/+:p53 (flox/flox) mice will be injected into the ovarian bursa with Adv-cre to delete p53 and Adv-lacZ as controls in the ovarian surface epithelial cells. Currently, there is no suitable cre transgenic mouse line that can be used to specifically express in the ovarian surface epithelium. The protocol of adenoviral injection into ovarian bursa has been well established, and Adv-cre injection has been successfully used in several mouse models of ovarian tumors recently. Thus, we will use Adv-cre injection as a main approach in this aim. Adv-cre and Adv-lacZ were purchased commercially (Vector Lab, Iowa). Using Adv-lacZ as a reporter for transfection by Adenoviral injection, we observed expression of beta-galactosidase in cells of both the ovarian surface and the bursa inner layer of cells (**Figure 5**). We conclude that adenoviral delivery of cre into ovarian bursa is efficient and technically practical.

Figure 5. Delivery of Adenoviral lacZ to ovarian surface epithelial cells. Adv-lacZ was used as a reporter for adenoviral injection into ovarian bursa. The left ovary of a 2-month-old mouse was injected with 10^8 pfu of Adv-lacZ in 5 μ l, and the right ovary was injected with PBS as a control. Seven days after injection, ovaries were harvested, sectioned with a cryotome, stained for beta-galactosidase activity, and counterstained with Fast-Red. Area indicated by a "*" is shown in a higher magnification.



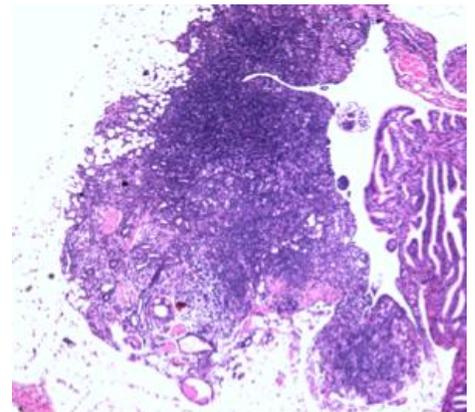
Ovarian tumor development in Wv/Wv:p53 (flox/flox)Adv-cre models

When the mice are 2 or 6 months of age, the Wv/Wv:p53 (flox/flox) female mice were injected with Adv-cre to delete p53 in the ovarian surface epithelial cells, and were sacrificed after 6-10 months to allow tumor development, until the mice are about 1 year of age.

By deleting p53 in ovarian surface epithelial cells through injection of adenovirus expression cre, we established another ovarian mouse model referred to as Wv/Wv:p53 (flox/flox):Adv-cre, mimicking both reproductive factors (postmenopause) and genetic mutation (p53) (**Figure 6**). These Wv/Wv:p53 (flox/flox):Adv-cre ovarian epithelial tumors appear malignant, though we will need more time and number of mice to reach a statistically significant conclusion..

We are currently characterizing in more details of these two mouse ovarian epithelial models. Thus, we have established two models of ovarian epithelial tumors, of Wv/Wv:p27 (-/-):Adv-cre and Wv/Wv:p53 (flox/flox):Adv-cre.

Figure 6. Ovarian tumor morphology in Wv/Wv:p53 (flox/flox);adv-cre mice. H&E images of a representative ovarian tumor of the 6-month-old Wv/Wv:p53 (flox/flox) mouse. The mouse was injected with cre delivering adenovirus (Adv-cre) into ovarian bursa at 3 months of age, and the ovarian tumor was analyzed at 6 months of age.



Studies of human cancer for relevance

Task 3 is “To analyze the alterations in signaling pathways in ovarian tumors and derived cell cultures from the mice (Months 30-36)”. The work is ongoing, to further analyze the Wv/Wv:p27 (-/-):Adv-cre and Wv/Wv:p53 (flox/flox):Adv-cre models.

To collaborate with the mouse model study, we have also examined human ovaries obtained from prophylactic oophorectomies for morphological changes as what we attempt to model using the Wv mice. We assembled a panel of archived ovarian tissues: 52 ovarian tissue blocks were from prophylactic oophorectomies of a high-risk (BRCA1/2 mutation or a family history of breast or ovarian cancer) population; 66 ovaries were from surgeries due to non-ovarian-related diseases, referred to as normal-risk group. The morphology of ovarian tissues was examined, and morphological changes including papillomatosis, invaginations, inclusion cysts, and epithelial stratification were assessed in a blinded fashion. We found that inclusion cysts and deep invaginations were found much more commonly in women age 45–54 of either high risk or normal risk groups. When age was categorized into two groups, 45-54 representing peri-menopausal status and the other group consisting of the remaining age groups (below 45 years and 55 years & above), a statistically significant difference was found between age group and frequency of occurrence of morphological features. The odds of occurrence of inclusion cyst were 5.43 times as high in women aged 45-54 relative to other women (p-value = 0.009). Likewise, the odds of occurrence of deep invagination were 6.42 times as high in women aged 45-54 relative to other women (p-value = 0.008), and the odds of occurrence of Pseudo-stratification were 3.77 times as high in this group of women as in other women (p-value = 0.039). This study suggests that the frequency of these histological features, especially inclusion cysts, may associate with age or menopausal status. We propose that ovulatory and perimenopausal gonadotropin stimulation produces ovarian morphological changes, and these histological features may promote the transformation of genetically compromised epithelial cells in the development of ovarian cancer. This finding provides additional support for relevance and rationale to study the Wv mice as models to investigate menopausal physiology on ovarian epithelial remodeling and cancer risk.

Therefore, we report that the project has been progressed well as planned in the last 3 years. We found that the additional mutations had more impact on the germ cells than the epithelial cells. We have also ayeared the basis for future studies of the models.

Use of the Wv ovarian tumor mouse models to test therapy

Because that our laboratory relocate from Fox Chase Cancer Center to the Sylvester Cancer Center in the University of Miami, the research project is delayed and we have requested an addendum/extension to complete the study in 11-1-2008 to 10-31-2009. In this period, besides ongoing project to complete the characterization of the models developed and preparing the results for publication, we performed study use the Wv mice.

We investigated the potential use of genetically engineered Vesicular Stomatitis Virus (VSV) to treat ovarian cancer using the Wv mouse models. We tested VSV oncolytic therapy using the immune competent Wv mice that develop ovarian tubular adenomas. We found that regardless of the inoculation route (intra bursal, IP, or IV), VSV specifically infected and replicated in the in situ ovarian tumors in the Wv mice without significant activity in any other organs and tissues (**Fig. 7**), and showed no detectable toxicity. The epithelial tumor lesions were greatly reduced in VSV-targeted ovarian tumors in the Wv mice (**Fig. 8**). The results demonstrated the explicit targeting of ovarian epithelial tumors by VSV in immune competent, ovarian tumor-bearing mouse models, and further support the utility of VSV as an effective and safe anti-cancer agent.

Figure 7. Targeting of VSV to ovarian tumors following IP and IV injection in Wv mice. Four-month-old Wv/Wv mice that bear ovarian tumors were injected IP or IV with 10^5 pfu of VSV-GFP into the control or Wv/Wv mice. Two mice per experimental group were injected. Ten days after injection, the mice were dissected and organs were examined under fluorescence microscopy for GFP signals. (A) The ovary of a representative tumor-bearing Wv mouse injected IV with VSV shows fluorescence indicating VSV proliferation (arrow). (B) The ovaries from wildtype control mice injected IP with VSV-GFP showed no GFP signal. (C) Other organs from VSV-GFP-injected mice by either IP or IV routes show minimal GFP signals.

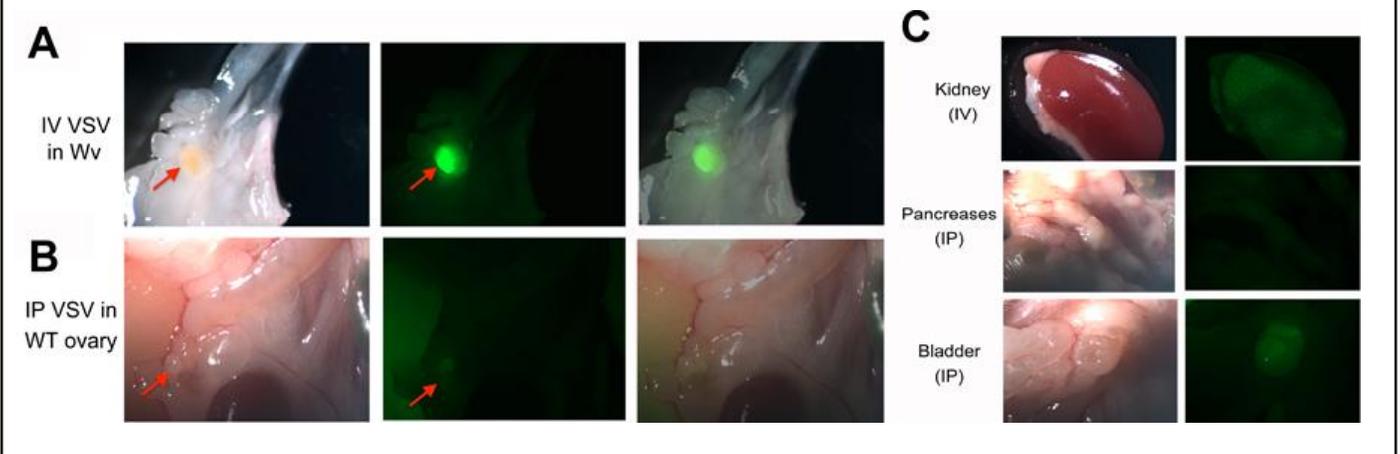
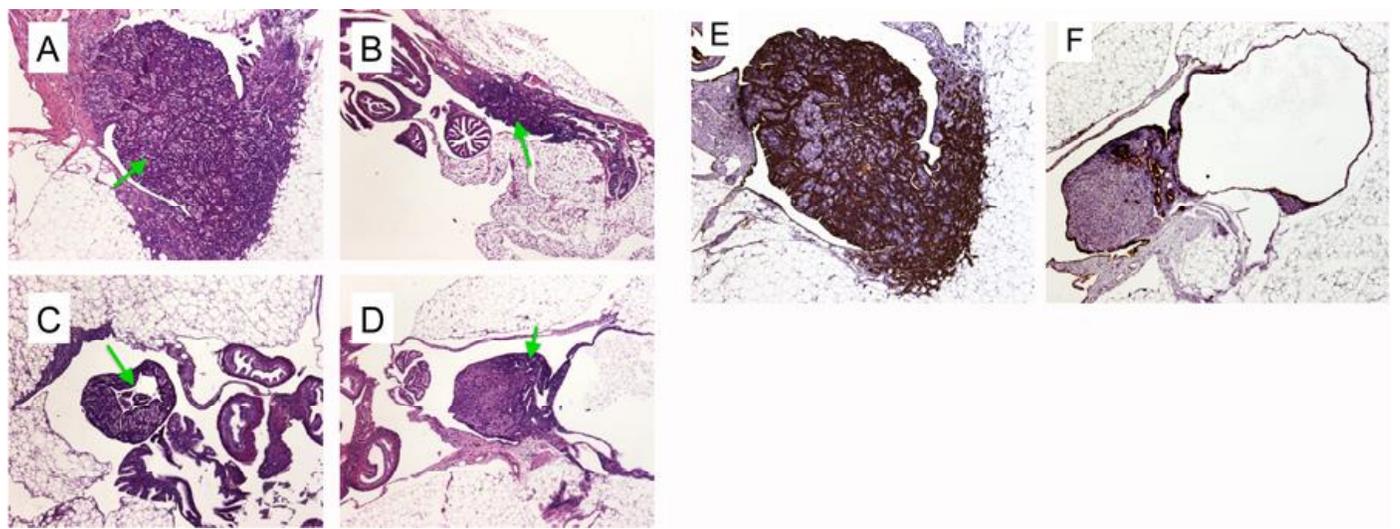


Figure 8. Oncolytic activity of VSV toward ovarian tumors in Wv mice. Female Wv mutant mice, two in each experimental group, were injected with VSV or inactivated VSV as control, through IB, IP, and IV routes. Ten days after a single viral injection, the ovarian tissues were harvested for histological analysis. H&E and cytokeratin staining of the sections of ovarian tumors at the widest cross section of the ovary are shown for comparison. Arrows indicate the ovarian tumors. (A) H&E staining of control, inactivated VSV injected IV; (B) H&E, active VSV injected IV; (C) H&E, active VSV injected IB; (D) H&E, active VSV injected IP; (E) cytokeratin-18 staining of control, inactivated VSV injected IV; (F) cytokeratin-18 staining, active VSV injected IP. The images were taken under a microscope with a 40 \times magnification and processed identically using Adobe Photoshop.



4. Key Research Accomplishments

- (1) Further verify the relevance of the Wv mouse model to human menopausal biology.
- (2) Verifying the influence of reproductive aging/menopause on human ovarian morphological changes. This finding provides additional support for relevance and rationale to study the Wv mice as models to investigate menopausal physiology on ovarian epithelial remodeling and cancer risk.
- (3) We made an unexpected but very interesting finding that the additional mutation of p53 in the Wv mice rescued ovarian tumor phenotype and preserved germ cells. This finding suggests p53 is very important for the survival and lifespan of ovarian germ cells and follicles. Also, the finding suggests that the depletion of follicles is key for the ovarian tumor phenotype.
- (4) We established a suitable ovarian cancer mouse model by combining reproductive factor (follicle depletion in Wv mice) and p27 suppression of p27 knockout background. The Wv/Wv:p27 (+/-) mice develop large ovarian tumors. Thus, we provide additional support for our hypothesis that the collaboration of reproductive factors and genetic mutations leads to the development of ovarian cancer.
- (5) Based on the new information learned from the ongoing study, we consider to make oncogenic mutation (such as deletion of p53 or pten) in only ovarian surface epithelial cells but in germ cells/follicles. We did preliminary study to establish the delivery of cre using adenoviral vector (Adv-cre) into ovarian surface epithelial cells. In a very limited number of mice tested, we are able to produce malignant ovarian tumors by conditionally deleting p53, in a Wv/Wv:p53 (flox/flox):Adv-Cre model. This result provides basis for a future project, and we will seek additional funding to support the experiments.
- (6) The Wv mouse ovarian tumor models are useful to test therapy: we successfully used the Wv ovarian tumor models to test oncolytic therapy of VSV.

5. Reportable Outcomes

- (1) Yang et al., Am. J. Pathology, 2007: We showed that the Wv mice are excellent models for human menopausal biology. This result is included in a paper (Yang et al, 2007).
- (2) Cai et al., Gyn. Oncology 2006: We found that the human ovaries show age-dependent morphological changes, suggesting follicle depletion and gonadotropin stimulation in perimenopausal period resulting in ovarian morphological changes. This observation has been published (Cai et al, 2006).
- (3) Cai, Yang, Smith et al., 2008 in preparation: We found that p53, pten, and p27kip1 genes have critical impacts on the survival of ovarian germ cells/follicle. We are doing additional experiments to characterize the mechanism further and also to obtain additional cases to determine the statistical significance. These results will be prepared for publication in 2-3 months.
- (4) Smith et al., 2008 in preparation: We have established the mouse models of ovarian cancer in Wv/Wv:p27 (+/-) and Wv/Wv:p53 (flox/flox):Adv-cre mice. Although it is clear that these

mouse models will be highly valuable to study ovarian cancer etiology and biology, we will need additional characterization and a larger number of mice to be analyzed in order to reach statistically significant conclusions. We may need to obtain additional funding support to complete these very interesting findings. We hope to publish these results in another 1-2 years.

- (5) Capo-chichi et al., Gyn. Oncology 2009 The Wv mouse ovarian tumor models are useful to test therapy: we successfully used the Wv ovarian tumor models to test oncolytic therapy of VSV. In the study, we showed that VSV explicitly target the ovarian tumors and show tumor-killing efficacy. This result is included in a paper (Capo-chichi et al, 2009).

6. Conclusions:

The experimental results are supportive of the hypothesis that. We need to delete p53 specifically in ovarian surface epithelial cells but not in germ cells in order to create a model of malignant ovarian tumor based on the Wv mice.

In the future study, we plan to create mutation only in ovarian surface epithelial cells to avoid the rescuing activity in germ cells. Currently, we are crossing Wv mice into flox-p53 mutant mice. We will introduce cre recombinase into ovarian surface epithelial cells in the mice by injecting adeno-cre into ovary to delete p53 only in surface epithelial cells but not in germ cells. This approach was considered in the original application as an alternative approach. Thus, the future experiments will be carried out essentially as that were planned in the original application.

In the study carried out last year (2009), we used the Wv mouse models to successfully test the tumor-killing efficacy of VSV. This study is an example showing the usefulness of the Wv ovarian tumor mouse models developed based on support from this award.

In sum, the project is progress well as planned. We have layered the basis in the first year of the project, and we hope to obtain conclusive results for the aims and questions proposed in the coming years.

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8. Appendices

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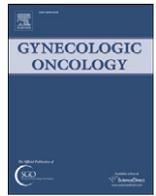
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Explicit targeting of transformed cells by VSV in ovarian epithelial tumor-bearing Wv mouse models

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Tumor mouse models

ABSTRACT

Objective. Current treatment options for epithelial ovarian cancer are limited and therapeutic development for recurrent and drug-resistant ovarian cancer is an urgent agenda. We investigated the potential use of genetically engineered Vesicular Stomatitis Virus (VSV) to treat ovarian cancer patients who fail to respond to available therapies. Specifically, we examined the toxicity to hosts and specificity of targeting ovarian tumors using a Wv ovarian tumor model.

Methods. We first tested recombinant VSV for oncolytic activity in a panel of human ovarian epithelial cancer, immortalized, and primary ovarian surface epithelial cells in culture. Then, we tested VSV oncolytic therapy using the immune competent Wv mice that develop tubular adenomas, benign tumor lesions derived from ovarian surface epithelial cells.

Results. The expression of GFP encoded by the recombinant VSV genome was detected in about 5% of primary ovarian surface epithelial cells (3 lines) up to 30 days without significantly altering the growth pattern of the cells, suggesting the lack of toxicity to the normal ovarian surface epithelial cells. However, VSV-GFP was detected in the majority (around 90%) of cells that are either “immortalized” by SV40 antigen expression or cancer lines. Some variation in killing time courses was observed, but all the transformed cell lines were killed within 3 days.

We found that regardless of the inoculation route (intra bursal, IP, or IV), VSV specifically infected and replicated in the in situ ovarian tumors in the Wv mice without significant activity in any other organs and tissues, and showed no detectable toxicity. The epithelial tumor lesions were greatly reduced in VSV-targeted ovarian tumors in the Wv mice.

Conclusions. VSV oncolytic activity depends on a cell autonomous property distinguishing primary and transformed cells. The efficient oncolytic activity of VSV for the “immortalized” non-tumorigenic ovarian surface epithelial cells suggests that the selective specificity extends from pre-neoplastic to overt cancer cells. The results demonstrated the explicit targeting of ovarian epithelial tumors by VSV in immune competent, ovarian tumor-bearing mouse models, and further support the utility of VSV as an effective and safe anti-cancer agent.

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Introduction

Epithelial ovarian cancer is a disease with poor prognosis, few early diagnostic markers, and limited treatment options [1–3]. Chemotherapeutic agents based on platinum derivatives have been widely used to treat a broad range of cancers including epithelial

ovarian cancer with some success. Currently, a platinum- and taxane-based combination regimen remains standard frontline chemotherapy for ovarian cancer [1–4]. Unfortunately, intrinsic and acquired resistance to cisplatin/taxane has greatly limited the efficacy of the therapy [4,5]. New agents, such as Gemcitabine, Doxorubicin, and Topotecan that convey anti-cancer activities via different mechanisms, are being evaluated in clinical trials, and some have been adopted for clinical application [4,5]. Nevertheless, current treatment options are still very limited, and development of resistance to the cytotoxic chemotherapy remains a key problem to be overcome, and most women ultimately die of the disease. Development of additional chemotherapeutic regimens, biological therapeutic agents, and other unique approaches for treatment of ovarian cancer is a high priority.

An idea is to use particular types of viruses as agents to selectively kill cancer cells [6]. These viruses, referred to as oncolytic viruses, are capable of replicating in cancer but not in normal cells [6]. The

Abbreviations: GFP, green fluorescence protein; HOSE cells, human ovarian surface epithelial cells; HIO cells, Human “immortalized” ovarian surface epithelial cells; IFN, interferon; IB, intra-bursal; IP, intra-peritoneal; IV, intra-venous; pfu, plaque forming unit; PI, propidium iodide; VSV, vesicular stomatitis virus; Wv mice, white spotting variant mutant mice.

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potential usefulness of Vesicular Stomatitis virus (VSV) as an anti-cancer therapeutic agent has been investigated [7,8]. VSV is a negative-stranded RNA virus that can infect a large range of cell types, through an as-yet-unidentified but likely ubiquitous cell surface receptor(s). VSV replicates in the cytoplasm of infected cells, though the 11-kilobase viral genome does not integrate into the host genome and has no transforming activity [9].

VSV can infect essentially all human cells in culture and undergo robust replication in certain (often cancerous) cells; however, VSV is relatively non-pathogenic for humans, likely due to the inability of the VSV to replicate and amplify in humans. While VSV is largely asymptomatic for humans, domestic and farm animals can become non-lethally infected, with symptoms such as lesions in the mucous membranes of the mouth and nose [7–9]. VSV also has been reported to be neuropathic in mice, following intranasal inoculation and subsequent infection and replication in the central nervous system. VSV infection can be cleared through activation of both the innate and adaptive immune responses [7–9]. The interferons are critically important in antiviral innate immunity and are a family of cytokines produced in response to VSV infection.

The selectivity of VSV to replicate and kill malignant, but not normal cells, has been well established in cultured cells and xenografts in mice of human cancer cells [10,11]. VSV has oncolytic activity in a large range of cancer types [12–20], including ovarian cancer cells [11]. Significant progress has been made to understand the mechanisms of VSV oncolytic activity and selectivity [7,21]. Neoplastic cells often have defective immune defense, probably involving the dis-regulation of the interferon system, which is an important factor for the ability of VSV to replicate in cancer cells [11,22]. A defective translational control in protein synthesis in cancer cells is another key factor for VSV oncolytic activity [23–26]. Additional mechanisms likely exist to account fully for the cell autonomous properties of sensitivity to VSV, and these are topics of active investigation.

While the efficacy of VSV for cancer therapy has been established in pre-clinical studies, further studying VSV oncolytic therapy in intact immune competent model organisms, rigorous evaluation of safety, and careful documentation of potential toxic side effects may move VSV oncolytic therapy to the next step, a clinical trial in human cancer patients. Here we report a study of VSV targeting of ovarian tumors in a Wv mouse model that is immune competent and spontaneously develop ovarian epithelial tumors. The Wv (white spotting variant) mice have a point mutation in the c-kit gene that reduces the tyrosine kinase activity and affects the development of germ cells [27,28]. The mutant mice live largely a normal lifespan with few developmental and physiological phenotypes, but the mutation results in a greatly reduced number of ovarian germ cells and follicles [27,28]. As a result of follicle depletion, ovarian morphological aging occurs, and ovarian tubular adenomas develop from ovarian surface epithelial cells in nearly 100% of Wv mice by 3–4 months of age [29,30]. These ovarian tumors are benign, but increasingly neoplastic features develop in older mice [29,30].

In this study, we examined the ability of VSV to target the ovarian tumors in Wv mice. The in situ ovarian tumor-bearing mice are anatomically correct and reflect more accurately the accessibility of VSV to tumor cells and the potential toxic side effects of the oncolytic therapy.

Materials and methods

VSV preparation and handling

Recombinant VSV of Indiana serotype was prepared in the laboratory as described previously [31]. The GFP transgene was inserted into the pVSV-XN2 plasmid that contains the entire VSV genome, and the expression of GFP can be used to monitor the

expression of VSV-GFP genome. Aliquots of VSV stock of 10^8 pfu/ μ l were stored at -80°C until use. As controls, aliquots of VSV-GFP were exposed to UV light for 30 min. The viruses were thawed slowly on ice and diluted in PBS (for animal injection) or RPMI medium (for cell culture experiments) to obtain a working stock of 10^5 pfu/ μ l. VSV was used according to bio-safety procedure in a P2-level safety facility/room, which was previously inspected by the Institutional Biosafety Committee (IBC). The use of VSV-GFP in this project was reviewed and approved by the IBC and IACUC (Institutional Animal Care and Usage Committee) committees at the University of Miami.

Cell culture and VSV oncolytic assay

Primary Human Ovarian Epithelial (HOSE) cells and Human Immortalized Ovarian epithelial (HIO) cells were provided by Dr. Andrew Godwin of Fox Chase Cancer Center, Philadelphia, PA [32]. All human ovarian cancer cells were purchased from ATCC. Recombinant VSV-GFP Indiana serotype was used to study viral cytolysis activity in normal human ovarian cells (HOSE60 and HOSE65), immortalized cells (HIO-80 and HIO-114), and ovarian cancer cell lines (OVCAR3, OVCAR5, OVCAR10, and ES2). Primary HOSE cells can commonly be maintained up to 10 passages in culture [33,34]. Transfection with the SV40 large T antigen (SV40Tag) expression vector to “immortalize” the primary cells can prolong the HIO cell lifespan in culture up to 20–30 passages [32–35]. Ovarian cancer cells were cultured and used as previously described [35].

For in vitro VSV infection assays, cells were seeded at a density of 10^5 cells/well in 6-well dishes 24 h prior to VSV-GFP infection. The cells were first rinsed twice with phenol red-free and FBS-free RPMI medium, and viruses were added in 1 ml of phenol red-free FBS-free RPMI medium containing VSV-GFP diluted to the appropriate viral dosage (10^5 , 10^6 , 10^7 , or 10^8 pfu/ml) and incubated for 2 h at 37°C . The “infection” medium was replaced by fresh phenol red-free RPMI medium containing 10% FBS for cancer cells, and 15% FBS for HOSE cells. VSV infection and proliferation were monitored by the expression of VSV-encoded GFP using a fluorescence microscope. Cell death was determined by staining with propidium iodide.

Additionally, VSV proliferation was measured by Western blot of the cell lysates for the expression of VSV viral proteins using either VSV antiserum (provided by Dr. Barber’s laboratory) or anti-VSV-G-protein (Santa Cruz Biotechnology, Santa Cruz, CA).

Wv ovarian tumor mouse models

The original founder Wv mice were purchased from Jackson Laboratory (Bar Harbor, ME) and a breeding colony was established at Fox Chase Cancer Center for 4 years [30] and at the University of Miami mouse facility for the last 2 years. The mice are maintained by mating between heterozygous mutant Wv mice and housed in micro-isolated cages with free access to autoclaved water and Purina 5001 standard mouse chow in barricaded viral pathogen-free rooms with husbandry provided by specialized animal technicians and monitored by veterinarians on duty.

Generally, the mice are genotyped by the coat color [30]. Wildtypes are evenly brown or black, heterozygous mice are mosaic with a dorsal or ventral white spot/patch on brown/black background, and homozygous mutant mice are all white coat with black eyes. All homozygous Wv mice were examined and confirmed to be non-albino (yellow coat with red eyes). If in the rare occasion albino mice were identified (through loss of eye pigment), the mice were removed from the colonies. Littermates of female Wv/Wv mutants (tumor bearing) and their heterozygous and wildtype littermates (controls, tumor free) were used for experiments. At 4 months of age, all female Wv homozygous mutant mice consistently develop bilateral ovarian tubular adenomas of similar size [30]. The heterozygous and wildtype littermates were used as controls for the specific tumor targeting by

VSV. The tumor-bearing Wv mice injected with UV-inactivated VSV were used as controls for auto-fluorescence.

Surgical procedure and viral delivery

Initially, various routes of VSV delivery, intraperitoneal (IP), intravenous (IV), and intrabursal (IB), were tested. Two female mice of around 4 months were used in each experimental group, and the experiments were repeated three times.

For IB injection, a surgical procedure was needed to access the ovary or ovarian tumor. Only one ovary (the left side) in each animal was injected. The mice were first anesthetized by intramuscular injection of a standard mix of 80–100 mg/kg Ketamine-HCl: Ketaset: Xylazine (5–10 mg/kg). The site of injection was shaved and washed with sterile saline and isopropanol. VSV was injected into the ovarian bursa using a syringe with 29-gauge beveled needle under a dissecting microscope. The ovary was first exposed via a small dorsal incision, then the needle was inserted, bevel up into the oviduct near the infundibulum, and moved toward the bursa. A volume of 10 μ l of

VSV-GFP (10^5 pfu/ μ l in PBS) was injected into the ovarian bursa. The incision was closed using Dissolvable-5.0, and skin was closed with 7-mm staples. Following surgery, the mice were kept warm with a heating pad until awake. The injected mice were kept separated individually and were closely observed on a daily basis for potential infection or discomfort.

The IV (standard tail vein injection) and IP injections were performed using a 27-gauge syringe without anesthesia.

The animals were euthanized 2 weeks later and infused with 10% buffered formalin. The tissues (ovary, uterine horns, bladder, gut, fat, bile duct, pancreas, liver, kidney, adrenal glands, spleen, heart, lungs, brain, eye, skeletal muscle, skin, fur, bedding, urine, and stool) were analyzed using a Zeiss (Stereo Discovery V8) stereo-fluorescence dissecting microscope equipped with a GFP 470 filter and digital camera (AxioCam MRC, with Axiovision 4.6 software). Representative images were recorded. The ovaries (or tumors) and selected tissues were collected in 10% buffered formalin and embedded in paraffin wax for further histology analysis. The epithelial lesions were examined following immunostaining for cytokeratin-18 [30].

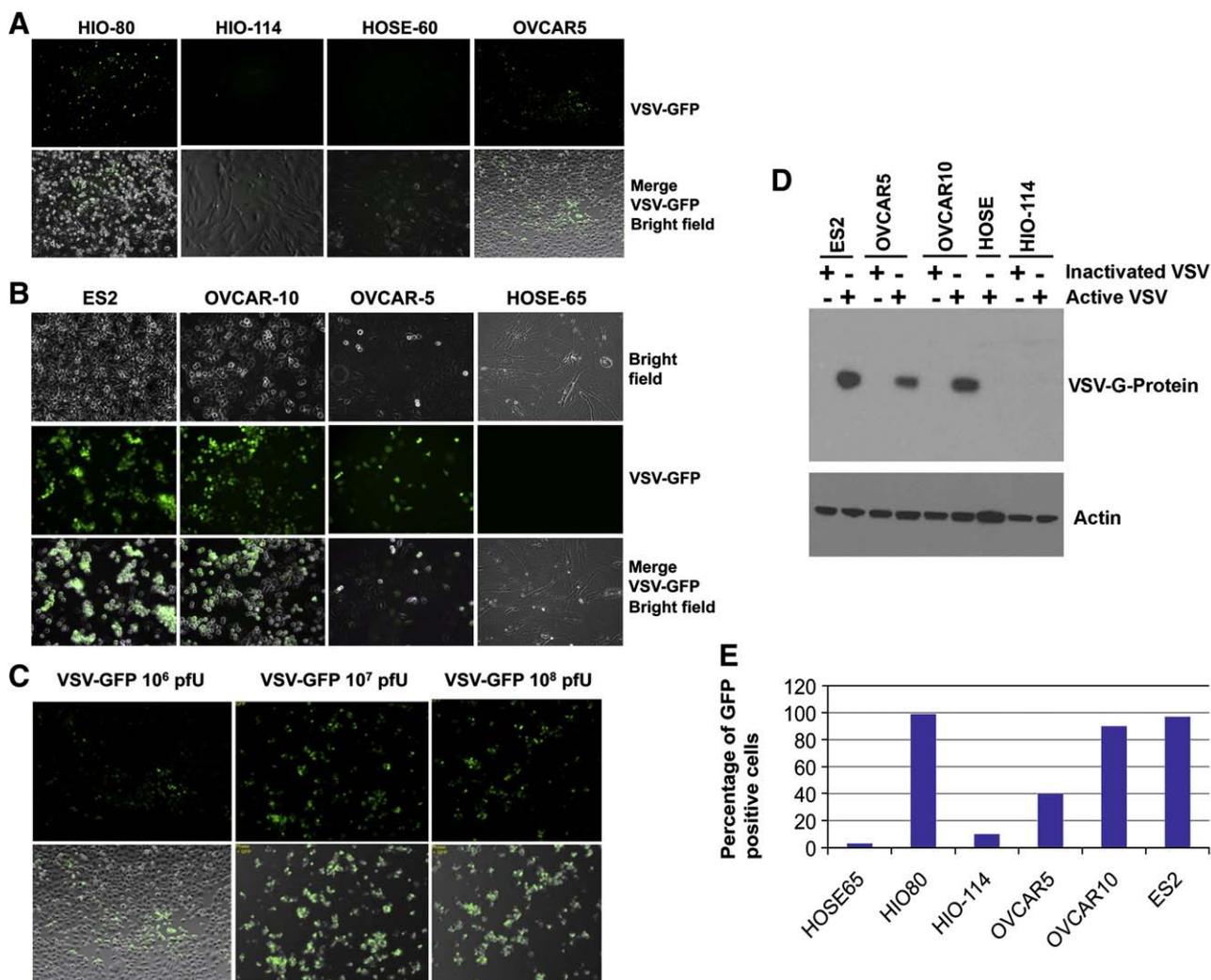


Fig. 1. Infection of VSV-GFP in cultured ovarian epithelial and tumor cells. (A) “Immortalized” ovarian surface epithelial cells HIO-80, HIO-114; primary ovarian surface epithelial cell preparation, HOSE-60; and ovarian cancer cells NIH:OVCAR5 in 6-well culture dishes were infected with 10^6 pfu of VSV-GFP. The cells were observed for GFP expression 24 h after infection. (B) Ovarian cancer cells ES2, NIH:OVCAR5, NIH:OVCAR10 [35], and primary ovarian surface epithelial HOSE-65 cells in 24-well dishes were infected with 10^7 pfu of VSV-GFP. The cells were observed for GFP expression 24 h after infection. (C) Ovarian cancer cells NIH:OVCAR5 in 24-well dishes were infected with increasing titers, 10^6 , 10^7 , and 10^8 pfu of VSV-GFP. The cells were observed for GFP expression 24 h after infection. (D) Ovarian cancer cells, ES2, NIH:OVCAR5, NIH:OVCAR10, the “immortalized” HIO-114 cells, and primary ovarian surface epithelial preparation HOSE-65 cells in 24-well dishes were infected with 10^7 pfu of VSV-GFP. The cell lysates were harvested 24 h after infection for Western blotting analysis of VSV-encoded G-protein, which indicates viral proliferation. (E) The percentage of VSV replicating cells (GFP positive) was quantified by cell counting 24 h following infection with 10^7 pfu of VSV-GFP. The value was calculated as an average of 5 images of GFP over-layering on bridge field, and the difference between HOSE cells and cancer cells is large and statistically significant as determined by Student’s *t*-test.

Results

Susceptibility to oncolytic activity of VSV of “immortalized” ovarian surface epithelial cells in cultures.

We further characterized the infectivity and replication of VSV in ovarian epithelial and cancer cells in culture using VSV-GFP. The VSV genome was modified by inserting the GFP transgene into the viral genome, and thus the presence of GFP in cells indicates VSV infection and replication [31]. Upon inoculation of VSV-GFP in cultured cells for 24 h, various degrees of infectivity as indicated by the expression of GFP were observed in a panel of cells (Fig. 1A, B): the majority of ovarian cancer cells NIH:OVCAR5, ES2, and NIH:OVCAR10 [35] were infected; only a small percentage of primary HOSE cells showed viral replication; all HIO-80 cells showed high degree of viral replication, while only a small percentage of HIO-114 showed viral infection. The infectivity depends on the dosage of VSV, as shown in an example using NIH:OVCAR5 cells (Fig. 1C). Only 10–20% of the cells expressed GFP 24 h after the addition of 10^6 pfu VSV-GFP; however, most NIH:OVCAR5 cells were infected when either 10^7 or 10^8 pfu of VSV was added. The selective replication of VSV-GFP in cancer cells but not in primary cells or HIO-114 non-tumorigenic “immortalized” cells was also confirmed by assaying VSV-encoded G-protein in cell lysates by Western blot (Fig. 1D). Some small variation in infectivity was observed of various cell lines in repeated experiments over time, and the percentage of infected cells was quantified for a representative experiment, as shown in Fig. 1E.

Following VSV infection, the cancer cells rapidly appeared in poor condition, rounded up, and underwent apoptosis. At 24 h after VSV infection of all cancer lines, a large percentage of cells showed viral infection and replication as indicated by robust signals from expressed GFP, and at 48 h, extensive cell detachment and death

were observed (Fig. 2A). Propidium iodide staining 2 days after VSV infection indicated that the majority of the cancer cells in culture had already died (Fig. 2A), and by day 3-post infection, essentially all ovarian cancer cells were detached and dead. UV-inactivated VSV as a control did not affect cell growth (not shown). In HOSE cells, faint and scattered expression of GFP persisted at day 3 and 7, but the GFP signal was essentially absent at day 14 (Fig. 2B), and viable cells were maintained up to 3 weeks. Thus, following exposure to VSV, the HOSE cells survived and showed no detectable phenotype, comparable to controls with the addition of inactivated VSV. Thus, the selectivity and oncolytic activity of VSV were demonstrated in cultured ovarian epithelial and cancer cells. The active viral replication in some lines of HIO cells suggests that oncolytic activity may depend on pre-neoplastic changes in the cells, since HIO cells are not tumorigenic.

Robust infectivity of VSV in ovarian tumors of Wv mice by ovarian inoculation

Next, we injected VSV-GFP into the ovarian bursa of 4-month-old wildtype (or Wv +/-) and Wv/Wv mutant mice to examine VSV infection and replication in the Wv ovarian tumors. The Wv ovarian tumor-bearing mice allow us to test VSV infection and treatment of in situ tumors in an immune competent mouse model. By 3 months old, nearly all female Wv/Wv mice develop ovarian tubular adenomas bilaterally. Initially, we delivered VSV-GFP directly into ovarian bursa (IB, intrabursal) by a simple surgical procedure. The virus was injected into the left ovary of each animal, and the right ovary was intended to be a control. Ten days after the virus was injected, ovaries and other organs were collected for analysis. The expression of GFP, as detected under a fluorescence stereomicroscope, indicates the degree of viral infection and replication. As shown in Fig. 3A, an intense GFP signal was detected in the injected left ovary, and surprisingly, GFP

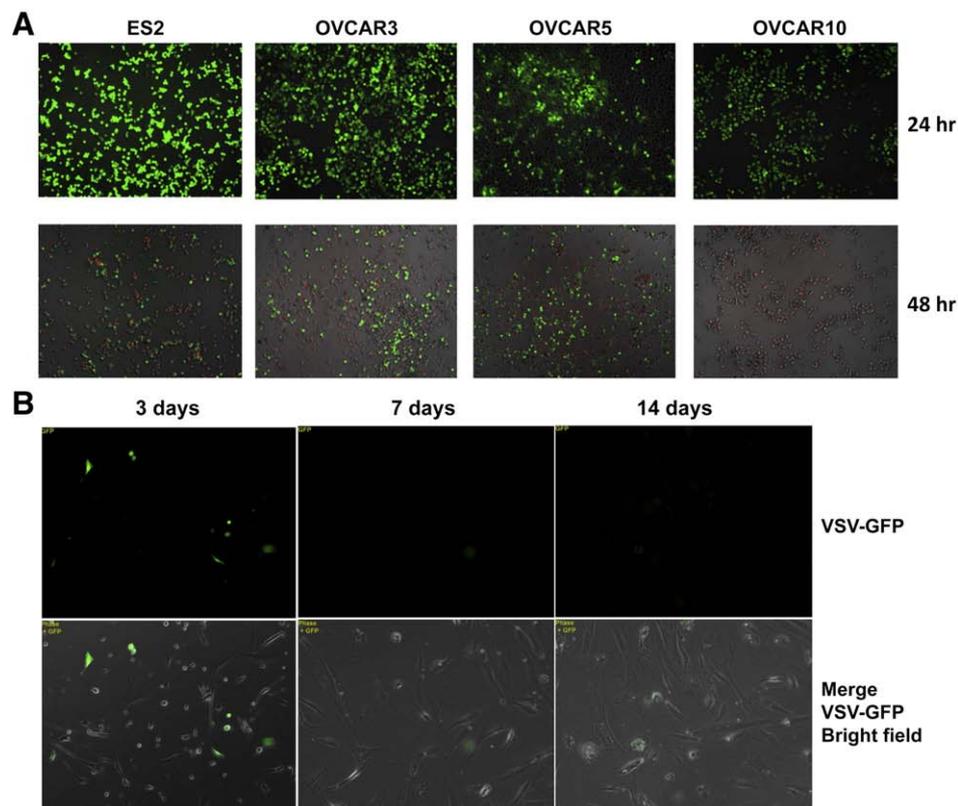


Fig. 2. Oncolytic activity of VSV-GFP in cultured ovarian epithelial and tumor cells. (A) Ovarian cancer cells ES2, NIH:OVCAR5, and A2780 in 24-well dishes were infected with 10^6 pfu of VSV-GFP. The cells were observed for GFP expression and for PI staining (red) to mark dead cells 2 days after infection. (B) Primary ovarian surface epithelial cell preparation HOSE-65 cells in 24-well dishes were infected with 10^8 pfu of VSV-GFP and monitored for GFP expression on days 3, 7, and 14 after infection. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

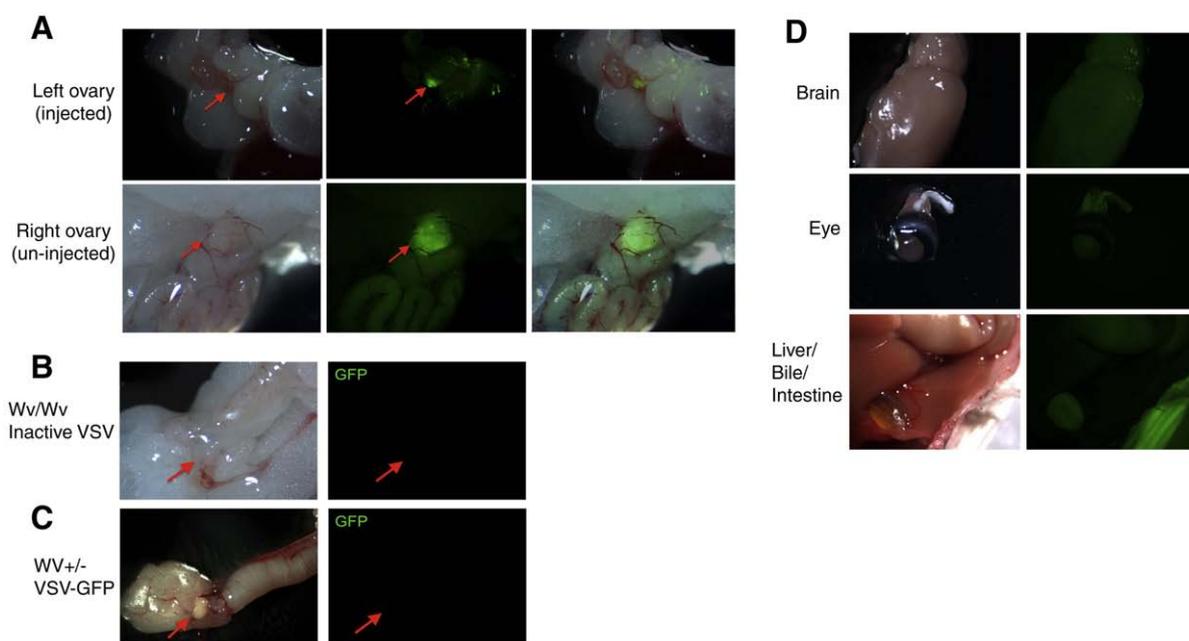


Fig. 3. Targeting of VSV following intra-ovarian bursa injection in Wv mice. Four-month-old Wv/Wv mice that bear ovarian tumors were injected with 10^5 pfu of VSV-GFP into the bursa of left ovary. Ten days after injection, the mice were dissected and organs were examined under fluorescence microscopy for GFP signals. (A) The injected left ovary of a representative tumor-bearing Wv mouse shows fluorescence (arrow). The non-injected right ovary from the same mouse also shows GFP fluorescence. As controls, no signals were detected in ovaries in Wv/Wv mice injected with inactivated VSV (B), or in Wv heterozygous (no ovarian tumor) mutant mice (C). (D) Other organs (brain, eye, and liver/bile/intestine are shown) from VSV-GFP-injected mice show no significant GFP signals.

expression was equally intense in the non-injected right ovary (Fig. 3A). Thus, the VSV injected in the left ovary apparently accessed the tumor of the right ovary. No GFP signals were detected in ovarian tumors from Wv mice injected with inactivated VSV-GFP (Fig. 3B). Active VSV-GFP also failed to infect and replicate in ovaries of wildtype mice (Fig. 3C). Essentially no significant GFP signal was detected in any other organ examined, including the brain, heart, liver, intestines, kidney, bladder, etc. (Fig. 3D).

Specific targeting of ovarian tumors in Wv mice by VSV through IP or IV routes

Because the VSV targeted both ovarian tumors when only one side was injected, we next examined the ability of VSV to target Wv ovarian tumors if delivered through intra-peritoneal (IP) or intravenous (IV) routes. After 10 days, the Wv mice injected with VSV-GFP or inactivated VSV as control were analyzed for GFP expression. Again, robust GFP signals were found only in both ovaries of the VSV-GFP-injected ovarian tumor-bearing animals (Fig. 4A). VSV-GFP injected

either by IP or IV routes did not proliferate in ovaries from wildtype mice (Fig. 4B). Other organs were dissected and carefully examined for GFP, and no significant sign of VSV replication was observed in any of the organs (Fig. 4C). Nevertheless, urine and solid wastes found on the bedding of the VSV-GFP-injected wildtype and Wv mice were found to be intensely fluorescent with GFP, suggesting VSV infection occurred and the viruses were cleared from the animals in tissues other than the ovarian tumors. As control, mock infection with UV-inactivated VSV was performed in parallel in all experiments to ensure the observed GFP signals specifically corresponded to VSV replication. Both IV and IP injection of VSV resulted in very similar targeting of ovarian tumors without significant replication in other tissues, suggesting that the injected VSV accessed cells throughout the entire circulation independent of the route of delivery.

Effective oncolytic activity of VSV toward ovarian tumors in Wv mice

We examined the impact of VSV infection on ovarian tumor histology 10 days after a single dosage of VSV injection. The ovarian

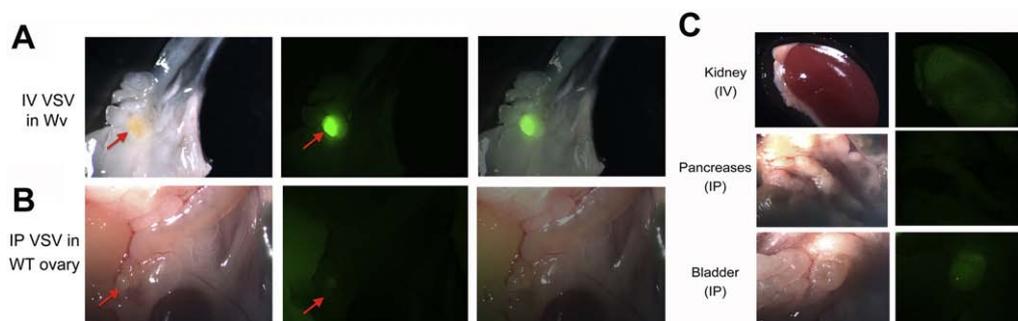


Fig. 4. Targeting of VSV to ovarian tumors following IP and IV injection in Wv mice. Four-month-old Wv/Wv mice that bear ovarian tumors were injected IP or IV with 10^5 pfu of VSV-GFP into the control or Wv/Wv mice. Two mice per experimental group were injected. Ten days after injection, the mice were dissected and organs were examined under fluorescence microscopy for GFP signals. (A) The ovary of a representative tumor-bearing Wv mouse injected IV with VSV shows fluorescence indicating VSV proliferation (arrow). (B) The ovaries from wildtype control mice injected IP with VSV-GFP showed no GFP signal. (C) Other organs from VSV-GFP-injected mice by either IP or IV routes show minimal GFP signals.

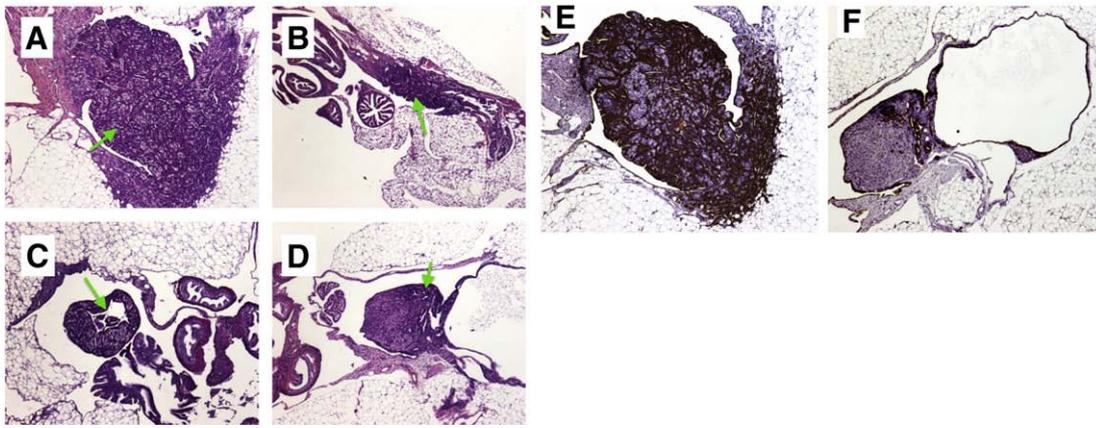


Fig. 5. Oncolytic activity of VSV toward ovarian tumors in Wv mice. Female Wv mutant mice, two in each experimental group, were injected with VSV or inactivated VSV as control, through IB, IP, and IV routes. Ten days after a single viral injection, the ovarian tissues were harvested for histological analysis. H&E and cytokeratin staining of the sections of ovarian tumors at the widest cross-section of the ovary are shown for comparison. Arrows indicate the ovarian tumors. (A) H&E staining of control, inactivated VSV injected IV; (B) H&E, active VSV injected IV; (C) H&E, active VSV injected IB; (D) H&E, active VSV injected IP; (E) cytokeratin-18 staining of control, inactivated VSV injected IP; (F) cytokeratin-18 staining, active VSV injected IP. The images were taken under a microscope with a 40 \times magnification and processed identically using Adobe Photoshop.

tumors were dissected and processed by formaldehyde fixing and paraffin embedding. The blocks were cut to obtain middle sections with the largest area of ovarian or tumor tissues for comparison. In all cases, we observed a substantial reduction in tumor sizes by VSV independent of the route of viral delivering (Fig. 5). As shown in representative examples in Fig. 5, compared to an average-size ovarian tumor in 4-month-old Wv mice treated with inactivated VSV (Fig. 5A), the ovarian tumor mass from the VSV-injected Wv mice was reduced approximately 5-fold (Figs. 5B–D). Based on the remaining expression of GFP shown in Figs. 3 and 4, the ovarian tumors still exhibited active VSV infection at the time of tissue harvest. Presumably, the VSV treatment might further reduce the tumor sizes in animals examined after a longer period after VSV treatment.

Additionally, compared to the ovarian tumors from control animals treated with inactivated VSV in which the cytokeratin-positive ovarian epithelial tumor permeated the entire ovary (Fig. 5E), the ovary from VSV treated mice often contains mainly tumor-free ovarian stromal area (Fig. 5F). These results suggest that VSV has robust activity in clearing epithelial tumor lesions.

Discussion

This current study compared the oncolytic activity of VSV in primary human ovarian surface epithelial (HOSE), non-tumorigenic “immortalized” human ovarian surface epithelial (HIO), and ovarian cancer cells in culture [32–35]. Confirming previous reports [10], the differential susceptibility of normal and cancer cells to VSV oncolytic activity is unequivocal. Several ovarian cancer cell lines tested were all sensitive to VSV and the cells died within 3 days following addition of VSV to the cultures. Active VSV replication in these transformed cells was revealed both by the expression of VSV-encoded GFP using immunofluorescence microscopy and by the expression of VSV encoded G-protein by Western blotting. Only a small number of primary ovarian surface epithelial cells were observed to express a low level of VSV-encoded GFP, and the cells maintained a normal growth pattern up to 3 weeks following exposure to VSV. We also observed that some lines of the HIO cells, which may resemble a pre-neoplastic state, are highly susceptible to oncolytic activity of VSV. Thus, it suggests that some subtle changes in cell properties without requirement of a fully neoplastic feature, are sufficient to render a cell type susceptible to killing by VSV. This element of VSV oncolytic selectivity may potentially be used to deal with pre-neoplastic lesions.

Previous reports have demonstrated the ability of VSV to target xenografts of human cancer cells in immune deficient mice [10,11].

The oncolytic activity and the efficacy of VSV as a cancer therapy are well demonstrated in mouse models [7,8], and the important remaining issues are the safety and selectivity. We tested VSV oncolytic therapy further in the immune competent Wv mouse model. The tumors that develop in the Wv mice are epithelial-derived and essentially the counterpart of epithelial proliferation and morphological changes in aging ovaries [30]. These ovarian epithelial tumors are known as tubular adenomas, and are benign, though some malignant features develop in older mice [29]. Using the Wv ovarian tumor models, the current study demonstrated the explicit targeting of VSV to ovarian tumors through various routes of delivery in immune competent mice, without significantly affecting any other organs or showing observable toxicity. The current Wv ovarian tumor model accurately represents the anatomical location of small ovarian tumor lesions and thus the results may be highly relevant to actual therapy in ovarian cancer patients.

The oncolytic virus that is commonly considered and developed is adenovirus, which have been actively investigated in the past decades [6,36,37]. Engineered conditional, replicative competent adenovirus can kill tumor cells but spare normal cells in pre-clinical studies, and the potential of oncolytic adenoviral therapy was regarded with much enthusiasm [36,37]. However, clinical studies of oncolytic adenovirus encountered several difficult barriers including the lack of tumor-specific viral targeting/infection and clearance of the virus by the immune system [36,37]. In humans, the weak tumor cell targeting by adenovirus is a serious shortcoming [36,37]. Although adenovirus can efficiently infect most mammalian cells through specific receptors, the infectivity seems reduced in neoplastic cells, and the majority of adenovirus delivered is sequestered in the liver [36,37]. While oncolytic adenovirus still has good potential and promise, strategies to modify the basic viral structure will be needed to overcome the existing shortcomings.

In comparison, VSV with its specificity for transformed cells and the ability to target tumors without accumulating in other organs may be superior and have great potential as a useful oncolytic agent to treat drug-resistant ovarian cancer. Additional studies of the biology of VSV oncolytic activity such as identification of VSV receptor(s) and its regulation in benign and malignant cells, and rigorous testing in additional ovarian cancer mouse and rat models for safety and efficacy, may bring VSV oncolytic therapy closer for treatment of drug-resistant ovarian cancer patients.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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