

ORIGINAL ARTICLE

## Fusogenic vesicular stomatitis virus for the treatment of head and neck squamous carcinomas

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**Objectives:** This study investigates the efficacy of recombinant fusogenic VSV [rVSV-NDV/F(L289A) or rVSV-F] in the treatment of head and neck squamous cell carcinoma (HNSCC).

**Study and Design Setting:** The *in vitro* replication and cytotoxicity of rVSV-F were studied in two human SCC cell lines, in one murine SCC cell line, and in human keratinocytes. The effects on tumor size and animal survival were investigated following *in vivo* rVSV-F treatment of floor-of-mouth tumor model C3H/HeJ mice.

**Results:** Recombinant VSV-F preferentially induced rapid syncytia formation, and replicated in ( $p < 0.04$ ) and killed ( $P < 1 \times 10^{-5}$ ) all three SCC lines tested. The virus had no observable effect on human keratinocytes. Tumor size was smaller ( $P < 0.03$ ) and overall survival was better ( $P < 0.001$ ) for treated animals than for control animals.

**Conclusion / Significance:** Recombinant VSV-F confers a modest survival benefit for HNSCC in this orthotopic murine model. This oncolytic virus holds promise as a novel cancer treatment for recurrent HNSCC.

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Head and neck cancer has an estimated yearly incidence of 38,530, with 11,060 associated mortalities. As many as 33% of treated patients develop recurrent disease.

The treatment of these patients is a challenge because of their poor prognosis, despite the options of salvage surgery and / or re-irradiation (with or without chemotherapy). One prospective study has shown that surgical salvage of resectable disease gives a median disease-free survival interval of 17.9 months, with an overall two-year disease free survival rate of 44%. Unfortunately, most patients present with unresectable disease, in which case the chances of survival are even more dismal. Systemic chemotherapy, is an option for carefully selected patients but gives only slightly better results. Given these unsatisfactory results, new treatments are urgently required for patients with recurrent head and neck cancer. A novel approach is the use of oncolytic viruses.

Vesicular stomatitis virus (VSV) has demonstrated potency as an oncolytic virus in preclinical tumor models of glioma, hepatocellular carcinoma, breast carcinoma, and melanoma. By taking advantage of the inherent attenuated antiviral responses of tumor cells, viruses, such as VSV, can be development of more virulent oncolytic viruses with shorter replication cycles is required.

Ebert et al (2004) have generated a fusogenic VSV with enhanced oncolytic potential for the treatment of hepatocellular carcinoma. In this modification, VSV is genetically engineered to incorporate the sequence that codes for a mutated Newcastle disease virus (NDV) protein, NDV - F(L289A), to form rVSV - NDV/F (L289A), which is capable

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of inducing syncytia. Syncytia formation enhances the cytopathic effects of VSV. The combination of natural life cycle transmission and cell-cell fusion allows for more efficient replication and transmission of the viral vector, and the oncolytic ability of this virus has been shown to be superior to that of nonfusogenic VSV. Other fusogenic oncolytic viruses have shown improved local control and

survival in several tumor models.<sup>7-13</sup>

Given that fusogenic viruses have been shown to be more effective than the corresponding wild-type viruses in other tumor models, we investigated the efficacy of rVSV-NDV/ F(L289A) (abbreviated as rVSV-F) for the treatment of human and murine head and neck squamous cell carcinoma (HNSCC) cell lines in vitro, as well as in a murine orthotopic floor-of-mouth (FOM) squamous cell carcinoma model in vivo.

## MATERIALS AND METHODS

### Cell Lines

The BHK-21 cell line (used for virus generation) was obtained from the American Type Culture Collection (Manassas, VA) and maintained in 10% fetal bovine serum-supplemented DMEM (Mediatech, Herndon, VA). The human SCC cell lines, SCC 09 and SCC 38, were kindly gifted by Dr Dianne Duffey, and the murine SCC cell line SCC VII was a kind gift from Drs Richard Wong and Bert W. O'Malley, Jr. These cells were maintained in media that contained 100 U/mL penicillin-streptomycin (Mediatech). Human keratinocytes (HuK) were obtained from Gibco, Invitrogen Corporation (Carlsbad, CA) and maintained in serum-free keratinocyte medium (Defined Keratinocyte-SFM; Gibco, Invitrogen Corporation).

### Generation of rVSV-F

The recombinant virus was generated by the methods described previously. Briefly, the plasmid was constructed by PCR amplification of NDV/F, followed by PCR site-directed mutagenesis, to generate the mutated plasmid NDV/ F(L289A). The mutated plasmid was then cloned and incorporated into the VSV by reverse genetics, to form rVSV-NDV/F(L289A).

To generate recombinant VSV vector that expresses the mutant (L289A) F protein, the pVSV-NDV/F(wt) plasmid was modified by PCR site-directed mutagenesis. The sequence that encodes the NDV/F protein was assembled from two overlapping PCR fragments. The first PCR fragment was generated using the forward primer 5'-CGG-CCGCTCGAGATGGGCTCCAGACCTTCTAC-3' and the reverse primer 5'-GTT000GACTGAAGGTGCAGTTACTGTATACC-3'. The residues introduced into the primer to change the amino acid sequence at position 289 from leucine to alanine are underlined. The second PCR fragment was generated using the forward primer 5'-GGTATACAGGTA-ACTGCACCTTCAGTCGGGAAC-3' and reverse primer 5'-AAGCTGGCTAGCTCACA111111GTAAGTGGCTC-3'. The two overlapping PCR fragments were joined together in a this PCR, and the resulting fragment, which contained the mutant (L289A) F protein sequence, was cloned into pVSV-XN2 generate pVSV-NDV/F(L289A). Sequencing of the plasmids was conducted at the DNA Sequencing Core Facility of Mount Sinai School of Medicine.

### Assessment of In Vitro Viral Infection

To investigate the ability of rVSV-F to form syncytia in SCC cells in vitro, the human SCC cell lines SCC 09 and SCC 38, the murine SCC cell line SCC VII, and HuK were infected with this virus at a multiplicity of infection (MOI) of 0.1. At 18 hours postinfection, all the cell lines were examined under light microscopy for large multinucleated syncytia. To document the ability of rVSV-F to form syncytia and to cause cell death, 1 X 10<sup>6</sup> SCC VII cells were infected at an MOI of 0.01. Photographs were taken under bright-field microscopy at 24, 36, and 48 hours postinfection.

For the quantitative evaluation of viral replication, 1 x 10<sup>6</sup> cells were grown in six-well plates. After 24 hours, the cells were infected with the recombinant virus at an MOI of 0.01. The cells were washed twice with phosphate-buffered saline (PBS) after one hour, and fresh medium was added. Supernatants were harvested at 4, 16, 24, 36, and 48 hours postinfection. Each time point was tested in duplicate. Total RNA samples from the culture supernatants were prepared. These RNA samples were then analyzed for the presence and concentrations of genomic VSV RNA by real-time RT-PCR using the specific sense primer 5'-TTGGCAAGTATGCTA AGTCAG-3'; the antisense primer 5'-AGGACTTGAGAT-ACTCACGAA-3'; and the probe 5'-FAMACAAATGAC'-CCTATAATTCTCAGA-3'. The experiments were performed on two separate occasions.

For measurements of viral cytotoxicity, 5 X 10<sup>4</sup> cells were grown in six-well plates, and after 24 hours, the cells were infected with the recombinant virus at an MOI of 0.01. The cytotoxic effects on the cells were quantified by the MTT assay and expressed as a fraction of the mock-infected cells at each time point. Each sample was tested in duplicate, and the experiments were repeated in triplicate to ensure consistency.

### Assessment of In Vivo Intratumoral Infection of Murine SCC VII FOM Tumors

All the procedures that involved animals were approved by and performed according to the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the Mount Sinai School of Medicine. For the in vivo studies, we studied the FOM tumor model in C3H/HeJ mice. This orthotopic model was chosen as it has been characterized as being aggressive and reflective of the human counterpart. On day 0, syngeneic SCC VII cells (5 X 10<sup>5</sup>) were transcutaneously implanted into the FOM at the level of the mylohyoid. By day five, tumors had grown to an average diameter of five to six mm. Daily injections were administered directly into the tumors for five consecutive days (days five-nine). Each tumor was injected with rVSV-NDV

(L289A) at  $1 \times 10^7$  plaque-forming units (pfu) in 15  $\mu$ L PBS. Control animals were injected with PBS, and treated animals were injected with rVSV-NDV/F(L289A). The endpoint examined was the progression of tumor size and animal survival. The results shown are the combination of two separately performed experiments.

### Statistical Analysis

For comparisons of individual time points, the Student t test is applied to determine statistical significance. The animal survival curves were plotted according to the Kaplan-Meier method, and statistical significance in different treatment groups was compared using the log-rank test.

## RESULTS

### In Vitro Phenotypes of Cells Infected with rVSV-F

Recombinant VSV-F induced syncytia formation in the murine and human tumor cell lines but not in the normal human keratinocytes (Fig 1A). At an MOI of 0.01, infected HuK showed no significant change in cell phenotype or population size, as compared to the PBS control-infected group. In contrast, recombinant VSV-F infection of tumor cells resulted in the formation of multinucleated giant cells, with the greatest effect seen for the murine SCC VII tumor cell line. Cell death occurred by 48 hours postinfection (Fig 1B).

### In Vitro Replication

Recombinant VSV-F replicated preferentially in the human and murine SCC cell lines (Fig 2). In all tumor cell lines, replication reached a peak of  $1 \times 10^8$  copies of viral RNA within 48 hours of infection, and the number of viral RNA copies was 10,000-fold higher than that obtained for viral replication in HuK ( $P < 0.04$ ).

### In Vitro Cytotoxicity

Recombinant VSV-F preferentially and efficiently killed the human and murine SCC cells (Fig 3). Cell viability decreased significantly over time in the infected tumor cell lines. The HuK population was essentially unaffected by infection with rVSV-F.

### In Vivo Tumor Size

Treatment with VSV-F reduced tumor volume (Fig 4). The tumor area, as determined by the maximal tumor height and width, was found to be smaller in treated animals ( $n = 19$ ) than in the control PBS group ( $n = 19$ ) (comparisons on days 15, 19, and 21,  $P < 0.03$ ). By day 27, the last control group animals were sacrificed in compliance with the

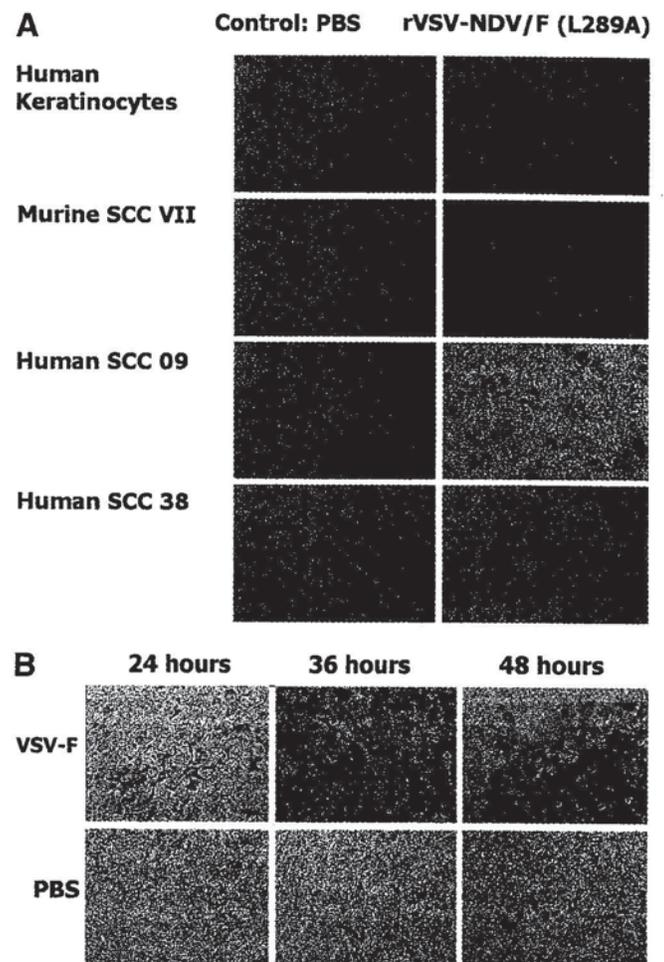
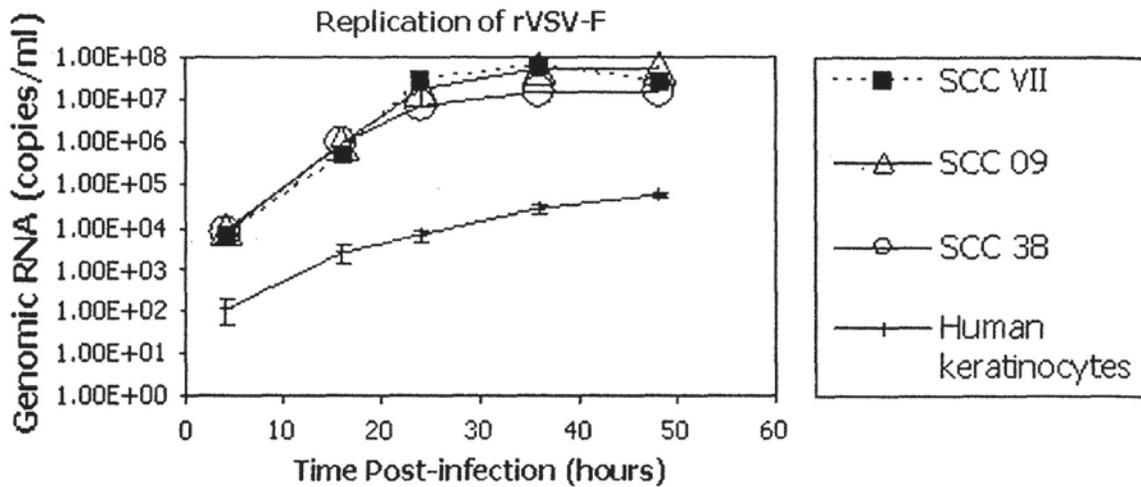


Figure 1 (A) In vitro phenotypic changes. Under bright-field microscopy at 18 hours postinfection, the PBS control group shows no syncytia formation and no cell population size differences between the different cell lines. In the rVSV-F group (MOI of 0.01), the HuK show no significant changes in cell phenotype or population size, as compared to the control group. The rVSV-F-infected tumor cell lines form multinucleated giant cells, with the greatest effect seen for SCC VII cells. Magnification X20. (B) In vitro cytotoxicity. Both the infected and control group SCC VII cells were observed over 48 hours under bright-field microscopy. In the rVSV-F-infected group (MOI of 0.01), early syncytia form with significant cell death at 36 hours postinfection, and almost all the cells are dead at 48 hours postinfection. Magnification X20.

IACUC criteria set (tumor size, ruffled fur, and weight loss), to prevent undue suffering.

### In Vivo Survival

Kaplan-Meier survival curves demonstrated that treated animals ( $n = 19$ ) had better survival rates than control animals ( $n = 19$ ) (Fig 5). During the first five days of viral administration, the only side effect was weight loss. This weight was regained by about day 10. On day 10, one of the treated animals developed hind limb paralysis and was sacrificed. By day 27, all of the control PBS mice were sacrificed; in contrast, the oldest treated animal survived more than twice.



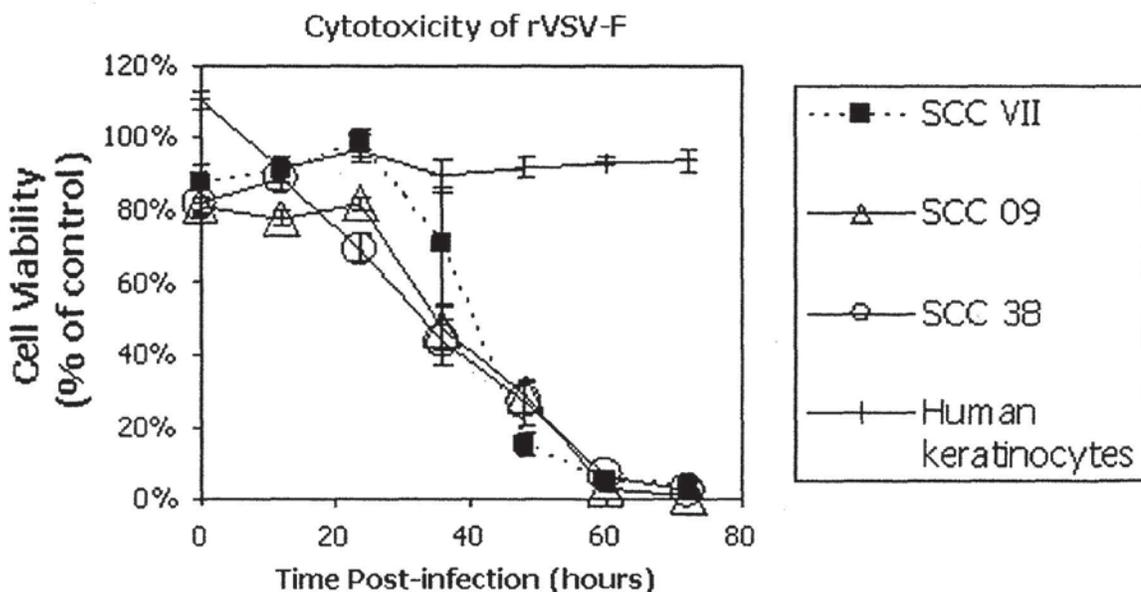
**Figure 2** Viral replication expressed as the number of RNA copies on a log scale. In all the tumor cell lines, replication reached a level of  $1 \times 10^8$  within 48 hours of infection, which represents a 10,000-fold higher viral replication rate than that seen in HuK ( $P < 0.04$ ). Error bars represent the SEM.

as long (59 days). This animal was free of tumors at the last VSV measurement; the cause of death is unknown. The difference in the survival curves was significant ( $P < 0.001$ ).

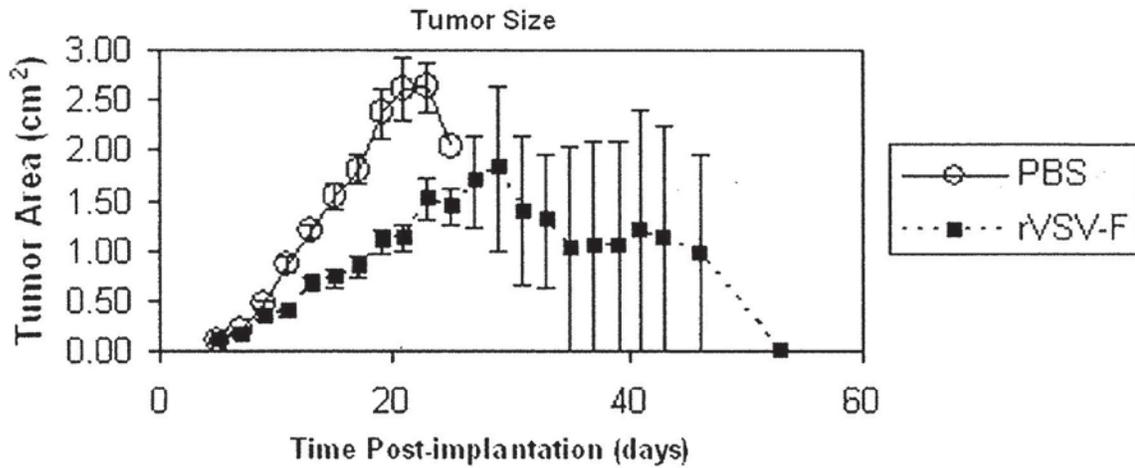
## DISCUSSION

Since the 1990s, oncolytic virotherapy has become a rapidly developing field. Preceding VSV, other oncolytic viruses had been studied and clinical trials initiated for three DNA viruses (herpes simplex virus [HSV], adenovirus, and Vaccinia) and two RNA viruses (NDV and reovirus).<sup>15</sup> However, all of these agents have restrictions in terms of their use as effective therapeutic agents.

VSV, which is a member of Rhabdoviridae family, is a promising candidate for oncolytic therapy. This negative stranded RNA virus has inherent specificity for replication in many tumor cell types, owing to their attenuated antiviral responses. The wide range of susceptible tumor types suggests that different target membranes may have a common receptor (possibly phosphatidylserine-related) for the VSV/G membrane glycoprotein. VSV naturally has an efficient replication mechanism that functions via budding and occurs within one to two hours (Fig 6). Mediated by the VSV/G glycoprotein this mechanism results in the immediate inhibition of host cellular RNA and protein synthesis such that cytotoxicity becomes apparent within hours of infection. Another attractive feature of VSV is the limited host response.



**Figure 3** Cell viability expressed as a percentage of the control. Cell viability decreases significantly over time in the tumor cell lines (at 60 hours postinfection,  $P < 1 \times 10^{-13}$ ). Error bars represent the SEM.

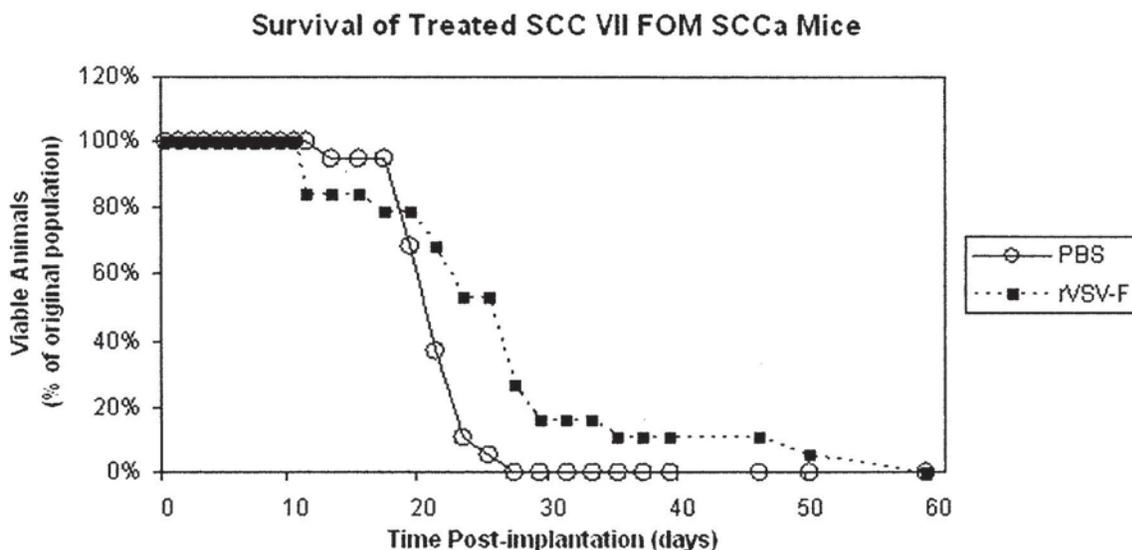


**Figure 4** FOX SCR VII tumor area (maximum tumor height X width) after treatment with five daily intratumoral injections of rVSV-F ( $1 \times 10^7$  pfu) or PBS starting on day five post implantation of tumor cells (in both groups, n = 19). The tumor size is smaller in the treated animals than in the control PBS group (on days 15, 19, and 21,  $P < 0.03$ ). By day 27, all of the control group animals had died. Error bars represent the SEM. Of the 19 treated mice, survival is extended to 59 days ( $P < 0.001$ ). The error bars begin to enlarge after day 19 because fine treated animal was free of disease, which contributes an area of  $0 \text{ cm}^2$  to the calculations.

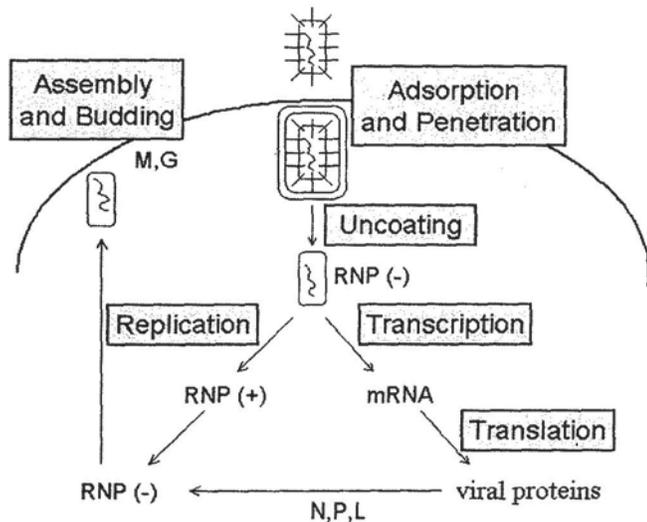
Alike the ONYX-015 adenovirus and HSV-1, VSV is of endemic to the human population. As a result, there no significant pre-existing innate or adaptive immunity drat would limit its initial use as an oncolytic virus delivered locally or systemically.

Exert et al (2004) have generated a novel VSO vector plat expresses the mutant F(L289A) protein of NDV. This virus replicates through the formation of multinucleated giant cells, called syncytia. In this method, the infected cell fuses with adjacent tumor cells, thereby spreading the infection by direct extension. In addition to skipping certain steps of the viral life cycle (budding and endocytosis), the

direct cell-to-cell fusion allows for more efficient viral spreading. In addition, these viral particles remain intracel-lular, so they avoid humoral immune mechanisms that could neutralize the viral particles. The modified VSV effectively induces syncytia formation in hepatocellular carcinoma cells.6 In vitro, fusogenic VSV killed almost 100% of the tumor cells within 36 hours, whereas nonfusogenic VSV killed only 25% to 50% of the tumor cells. In addition, fusogenic VSV exhibits effective oncolysis in the rat model in vivo (single hepatic arterial injection). Multiple admin-istrations of rVSV-F conferred a significant improvement in tumor-free, long-term survival when compared to a single



**Figure 5** Animal survival rates. Kaplan-Meier survival curves of mice with FOX SCC VII tumors after treatment with five daily intratumoral injections of rVSV-F ( $1 \times 10^7$  pfu) or PBS starting on day five post implantation of tumor cells (control mice, n = 19; treated mice, n = 19). By day 27, all the control PBS mice have been sacrificed. Of the 19 treated mice, survival is extended to 59 days ( $P < 0.001$ ). One animal died disease-free on day 59.



**Figure 6** VSV infection and replication. The virus is internalized by the host cell. Endocytosis releases nucleocapsids into the cytoplasm, where replication, transcription, and translation occur. The virions are then assembled and bud from the host cell. These new viruses infect the neighboring host cells.

administration (18% vs 0%). Those animals treated with a single administration of rVSV-F had a median survival time of 17 days (total up to 25 days), whereas those animals treated with multiple administrations had a median survival time of 27 days (total up to 100 days).<sup>16</sup>

These promising results have stimulated further evaluation of this oncolytic virus (rVSV-F) in other types of cancers. Recurrent HNSCC is a particularly appropriate target for rVSV, as these tumors are accessible to direct injection. Local delivery of the virus may limit the VSV induced toxicity seen with systemic delivery. Finally, VSV with its broad tissue tropism, readily infects HNSCC. This is the first study to evaluate the oncolytic effectiveness of VSV in head and neck cancer.

The present study demonstrates that rVSV-F is highly effective in the three SCC cell lines tested. All three cell lines were sensitive to rVSV-F in vitro at an MOI of 0.01. As seen in Figure 1, the recombinant virus induced syncytia formation in SCC cell lines but not in normal HuK, and few tumor cells were observed by 48 hours postinfection. Quantitative results confirm these qualitative observations. Replication was preferential and rapid, approaching  $1 \times 10^8$  copies of viral RNA within 48 hours (Fig 2). Cytotoxicity was also rapid and specific, with nearly complete eradication of tumor cell lines within 72 hours. Animal survival was also improved with virus treatment. The established FOM tumors in syngeneic mice all responded to injections of rVSV-F. In the treated mice, there was significant slowing of tumor growth and improved survival (median survival increased by six days,  $P < 0.001$ ). Control animals survived for only 27 days (median survival 21 days), whereas treated animals survived for up to 59 days (median survival 27 days). The inclusion of cured animals (about 10% of the animals) in the tumor size analysis accounts for

the wide variance in tumor size; all of the tumors diameters of five to six mm at the start of treatment. Further analyses of tumors undergoing regression and cure (about 10%) may reveal differences in viral propagation or immune response when compared to nonregressing tumors, and may suggest future avenues of investigation to improve this oncolytic virus. The high oncolytic activity in vitro incongruous with the more modest oncolytic activity in vivo. This discrepancy suggests that the SCC VII tumor is highly aggressive and undergoes rapid growth in vivo. In addition, in preliminary studies, injections of lower concentrations of rVSV-F did not have as large an effect as  $1 \times 10^8$  pfu of the virus (data not shown). Injections of higher viral concentrations were necessary to affect tumor size and survival in established and rapidly growing tumors in the FOM of mice.

Toxicity is always a concern with viral oncolytic therapy. Weight loss of 5% to 10% was associated with viral administration however, the weight returned to normal by about 10 days postinjection. In addition, neurotoxicity was observed in one animal as hind limb paralysis on day 10. Paralysis was presumed to be secondary to viral spread (although this was not confirmed immunohistologically and was our first observation of toxicity at this dose of VSV).

The current experiments used oncolytic rVSV-F as a single agent treatment for SCC. Recombinant VSV-F may be particularly useful for treating recurrent head and neck cancers, for which treatment options are limited. The preoperative administration of these viruses may reduce tumor volume, making otherwise inoperable tumors surgically resectable. Alternatively, oncolytic VSV can be used in the surgical bed after resection to treat residual disease. As the mechanisms of action of oncolytic viruses differ from those of standard therapies such as chemotherapy and radiation, strategies that combining oncolytic VSV therapy with either radiation or chemotherapy may significantly enhance the oncolytic effect. Similar enhancement of the oncolytic effect has been documented with other oncolytic viruses, such as HSV.<sup>17</sup>

The potential neurotoxicity of VSV has prompted investigations to improve its safety. In our test group, one animal did show signs of neurotoxicity (hind limb paralysis). In humans, VSV usually produces infections without any symptoms, or with mild, flu-like illnesses.<sup>8</sup> Despite the low risk and rarity of occurrence of viral encephalitis, experiments investigating the neurotropism of VSV and the host central nervous system immune response are ongoing. TYPO I interferons and IL-12 have been shown to attenuate VSV infection and/or to improve recovery from VSV infection. Further studies that combine oncolytic VSV with these types of immune system modifiers may improve the safety of VSV and increase its therapeutic index.

## CONCLUSION

This study demonstrates that rVSV-F is a potentially effective oncolytic agent in the treatment of HNSCC. Recombi-

nant VSV-F replicates rapidly and efficiently kills both human and murine SCCs in vitro. Multiple direct intratumoral injections result in tumor oncolysis, as evidenced by reduced tumor size and prolonged survival. Further modifications of rVSV-F that improve its efficacy may result in a novel form of cancer treatment for HNSCC.

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