

# Directed evolution as a tool for the selection of oncolytic RNA viruses with desired phenotypes

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**Abstract:** Viruses have some characteristics in common with cell-based life. They can evolve and adapt to environmental conditions. Directed evolution can be used by researchers to produce viral strains with desirable phenotypes. Through bioselection, improved strains of oncolytic viruses can be obtained that have better safety profiles, increased specificity for malignant cells, and more efficient spread among tumor cells. It is also possible to select strains capable of killing a broader spectrum of cancer cell variants, so as to achieve a higher frequency of therapeutic responses. This review describes and analyses virus adaptation studies performed with members of four RNA virus families that are used for viral oncolysis: reoviruses, paramyxoviruses, enteroviruses, and rhabdoviruses.

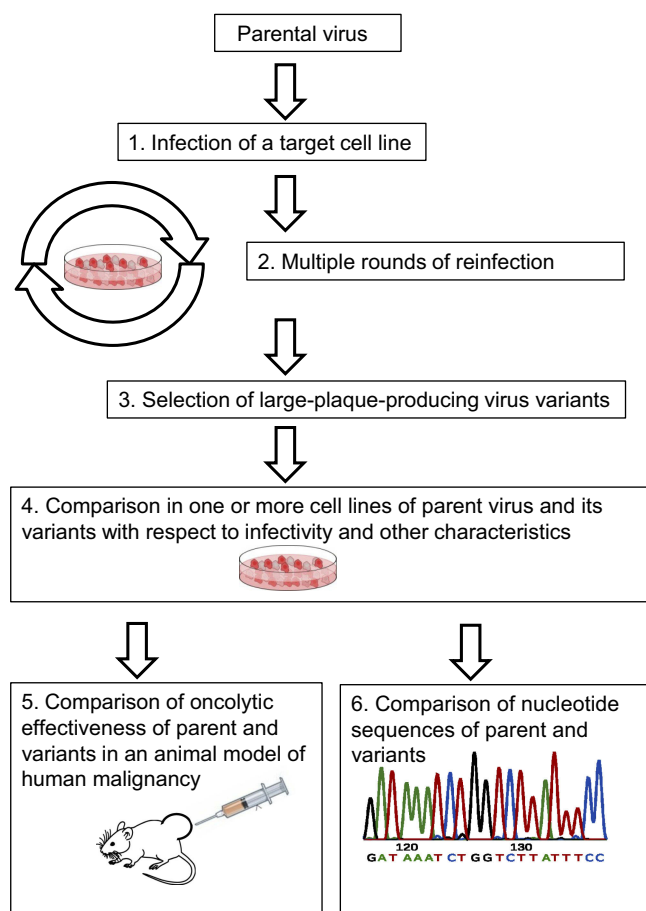
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## Introduction

Tumor regression after naturally acquired viral infections or vaccination was observed more than 100 years ago.<sup>1-3</sup> Following these earlier random observations, the number of studies in which a virus was used as an anticancer therapeutic increased dramatically during the 20th century.<sup>3,4</sup> As a result, the term oncolytic virus (OV) was introduced. OV has tumor-selective replication abilities and can kill tumor cells directly through infection and lysis of cancer cells, as well as indirectly through initiation of systemic antitumor immune responses.<sup>3-5</sup> The viruses that have been investigated as oncolytic agents belong to different families and represent a diverse group of wild-type and genetically engineered viral strains. OV research has intensified in the 1990s leading in 2015 to the first US Food and Drug Administration-approved OV strain (Talimogene laherparepvec (T-VEC or Imlygic)) for the treatment of advanced melanoma.<sup>6</sup> This first approved oncolytic viral agent is based on genetically engineered herpes simplex virus (HSV-1). While T-VEC showed encouraging durability of responses, its overall efficacy remains limited.<sup>7</sup> The field of oncolytic viral therapy faces multiple challenges, including optimizing viral delivery and viral spread inside tumors, as well as overcoming tumor resistance and antiviral immunity. So far no known biomarkers can reliably identify cancer patients, who are likely to respond to viral therapy. However, extensive preclinical and clinical studies are gradually addressing these challenges.

This review describes and analyzes studies that employed directed evolution via multiple rounds of virus selection for improving, testing, or studying OV properties. In most selection experiments, an OV is passaged in a target cell line for achieving genetic changes that produce desirable characteristics (Figure 1). Alternative routes of viral selection include selective passaging in embryonated chicken eggs or tumor

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**Figure 1** Selection process for broadening virus infectivity. Steps 5 or/and 6 might be omitted in some experiments.

tissues. Various selection and testing schemes that have been applied to representatives of four RNA virus families—*Reoviridae*, *Paramyxoviridae*, *Picornaviridae*, and *Rhabdoviridae* are presented in this review.

## Reoviruses

*Reoviridae* is a family of non-enveloped double-stranded RNA viruses with a very wide host range that includes animals, plants, and fungi.<sup>8</sup> The genome length of reoviruses ranges from 18.2 to 30.5 kb.<sup>8,9</sup> The name “reovirus” stands for Respiratory Enteric Orphan virus.<sup>10</sup> The abbreviation resulted from the observation that the virus usually could be isolated from the mammalian respiratory and enteric tracts, but it causes very few, if any clinical symptoms (hence “orphan”). Antibodies to reoviruses have been observed in almost all human subjects.<sup>8,11</sup> The family includes *Orthoreovirus* among its genera. *Orthoreovirus* infection is common in humans, but in most cases, it causes very mild symptoms. For simplicity, *Orthoreovirus* representatives are referred to as reoviruses in the text that follows.

Prompted by observations that reoviruses have oncolytic potential, viral modifications that may improve reovirus oncolytic potency and/or specificity were studied and described.<sup>12,13</sup> Four mammalian orthoreovirus serotypes, T1, T2, T3, and T4, are known. For each serotype, representative strains have been isolated: for type 1, Lang (T1L); for type 2, Jones (T2J); for type 3, Abney (T3A) and Dearing (T3D)<sup>9</sup> and for type 4, Ndelle (T4N). Among reovirus strains, T3D has been the most studied. One of its derivative strains was given the commercial name Pelareorep (REOLYSIN®) by Oncolytics Biotech, the company that is developing anticancer therapeutic agents using this reovirus.<sup>10,14</sup>

Reoviruses can enter cells using Junctional Adhesion Molecule-A (JAM-A) as the cell’s entry receptor.<sup>9,15</sup> JAM-A protein in humans is encoded by the F11R gene. Reoviruses are capable of using glycans as co-receptors; for example, some serotype T3 viruses use sialic acid as a co-receptor.<sup>15</sup>

Extensive preclinical cell-based and animal studies showed that T3D reovirus and related strains are capable, as many other OV, of killing a broad range of cancer cells both directly and also indirectly through the activation of the immune system.<sup>16,17</sup> Subsequent clinical trials have shown promise for REOLYSIN efficacy. Intra-lesion REOLYSIN injections generated one complete response, two partial responses and four stable diseases among 19 patients with variable solid tumors. Remaining patients from 19 were non-responders.<sup>18</sup> In clinical trials, multiple intravenous REOLYSIN injections were combined with simultaneous administration of chemotherapeutic agents. Such treatment schemes failed to show any efficacy for recurrent ovarian, tubal, peritoneal<sup>19</sup> or pancreatic<sup>20</sup> adenocarcinomas. However, overall survival time of patients with metastatic breast cancer who were treated with a combination of REOLYSIN and chemotherapy was seven months longer than that of patients treated by chemotherapy alone.<sup>21</sup> Side effects of the combined REOLYSIN-chemotherapy treatment were comparatively mild (grade 3 or less) and included pyrexia, chills, myalgia, pain, fatigue, and nausea.<sup>21</sup> Rare severe adverse events (grade 4) were also registered. They included neutropenia and severe respiratory problems.<sup>19</sup>

## Adaptation of reoviruses

Adaptation studies have been directed toward enhancing and broadening reovirus infectivity as well as increasing

its safety (Table 1). Theoretically, these two goals could be mutually exclusive because increased infectivity could compromise safety. Therefore, virus strains that have been adapted for higher infectivity should receive extra scrutiny for safety.

## Infectivity enhancement

Shmulevitz's team managed to enhance reovirus infectivity in a few steps.<sup>22</sup> First, three strains of reovirus (T1L, T2J, and T3D) were used in combination to infect murine L929 cells. (L929 cells are highly permissive to reovirus infection and are used for its propagation.) Second, four rounds of virus selection in L929 cells were performed; the largest plaques were selected in each round. Third, after all the selection rounds, two variants were isolated that consistently formed plaques larger than those formed by all parental virus strains. These reovirus variants produced larger plaques on several human and mouse cell lines with moderate susceptibility to reovirus, including human colorectal carcinoma cells, pancreatic ductal carcinoma cells, and murine ovarian cancer cells. Mutations in  $\lambda 2$  vertex and  $\sigma 1$  cell attachment proteins were identified in these strains. Authors of the study concluded that these mutations were responsible for an increase in the proportion of infectious progeny virus particles produced by the selected strains. Later they showed that reduction of the reovirus virion-associated  $\sigma 1$  protein results in earlier depletion of  $\sigma 1$  during viral uncoating and promotes the establishment of productive virus infection in malignant cells.<sup>23</sup>

Finally, the oncolytic efficacy of these two reovirus variants was tested in a syngeneic murine model of melanoma. Animals treated with both selected viral variants survived significantly longer than animals treated with parental virus strains.<sup>22</sup> Thus, the study demonstrated that it is possible to select reovirus variants with better oncolytic infectivity and therapeutic potential without compromising safety. It would be interesting to learn if these preclinical observations can be translated into beneficial effects in clinical studies.

## Infectivity broadening

In the Vero cell line, infectivity of wild-type T3D reovirus is not as good as in the L929 cell line. However, through infection persistence establishment, a virus variant was selected that was capable of infecting Vero cells efficiently.<sup>24</sup> This variant had amino acid substitutions in two reovirus proteins,  $\sigma 1$  and  $\mu 1$ .<sup>24,25</sup> The first protein,  $\sigma 1$ , participates

in virus attachment to the cell surface; the other protein,  $\mu 1$ , participates in virus disassembly (uncoating) from its outer capsid. As a result of the amino acid changes in the  $\sigma 1$  and  $\mu 1$  proteins, three phenotypic differences between the selected reovirus variant and the parental strain were observed.<sup>24,25</sup> First, the virus binding to the Vero cell surface became five times stronger. Moreover, binding became sensitive to neuraminidase treatment, indicating the involvement of sialic acid residues attached to the cell outer membrane in the binding process. Second, the rate of disassembly of the virions inside infected cells increased. Third, the virus became very sensitive to interferon (IFN).<sup>26</sup> Later this sensitivity was attributed to a unique mutation in the S1 gene, overlapping the  $\sigma 1$  and  $\sigma 1$  s reading frames.<sup>26</sup> The adapted virus's oncolytic efficiency and its safety profile remain to be tested in animal models.

Wild-type T3 reovirus (along with T1 reovirus) uses JAM-A as a receptor.<sup>15,27</sup> Human gliomas, melanomas, ovarian, prostate, and some other cancers express little or no JAM-A.<sup>28–30</sup> Low or no JAM-A expression makes many cancer cells resistant to reovirus T3. Virus variants that enter cancer cells in a JAM-A-independent manner could overcome this resistance. An adaptation study was performed in pursuit of this goal.<sup>31,32</sup> It included establishment of persistent reovirus infection of a murine erythroleukemia (MEL) cell line which does not express JAM-A. Analysis of reassorted viruses created from various combinations of parental and MEL adapted strains demonstrated that the  $\sigma 1$  protein is responsible for the phenotypic change. Viral variants with a point mutation in the  $\sigma 1$  protein have an increased ability to bind sialic acids located on the cell surface. Their binding to human cholangiocarcinoma cells was improved 100-fold<sup>31–33</sup> compared with the T3 parent. In addition, selected viral variants were capable of causing much higher levels of apoptosis of HeLa cells than the parental virus strain.<sup>31,32</sup> However, the safety profile of the parental virus was better than that of the sialic acid binding mutants.<sup>33</sup>

A similar goal of adapting reovirus to infect a broader range of cancer cells was pursued by another research team.<sup>34</sup> This team performed selection rounds of T3D virus variants in glioblastoma cells, which do not express JAM-A. Three viral variants were isolated that acquired the ability to infect the previously reovirus resistant U118 glioblastoma cell line.<sup>34</sup> It was demonstrated that all three selected virus variants rely on sialic acids for cell entry. Not surprisingly, this viral phenotypic change was associated with mutations located close to the sialic acid

**Table 1** Adaptation studies of oncolytic viruses

Virus family	Virus	Goal of selection	Method	Cell culture	Animal model	Phenotype	Genotype	Ref.
REOVIRIDAE	Reovirus	↑infectivity	Four rounds of selection in cell culture	L929	Syngeneic murine model of melanoma	↑animal survival	Mutations in $\lambda 2$ and $\sigma I$ proteins	22
		Infectivity broadening	Multiple selection rounds in cell culture	Vero	None	↑infection of Vero and HeLa cells through sialic acids	Mutations in $\sigma I$ and $\mu I$ proteins	25
				MEL		↑binding to sialic acids, ↑100-fold binding to human cholangiocarcinoma cells, ↑HeLa cells apoptosis	Mutation in $\sigma I$ protein	31–33
				Glioblastoma		↑infection of U118 glioblastoma, ↑infection of many other malignant cell lines	Mutations in $\sigma I$ protein	34
		↑safety		HT1080	Human xenografts of HT1080 cells	↑suppression of HT1080 xenografts, ↓infection of L929 and HCT116 cells, ↓toxicity	Premature stop codon in $\sigma I$ protein	40

(Continued)

Table 1 (Continued).

Virus family	Virus	Goal of selection	Method	Cell culture	Animal model	Phenotype	Genotype	Ref.
PARAME	Newcastle Disease Virus (NDV)	↑intratumoral spreading	Two rounds of selection in human HT1080 fibrosarcoma	HT29		↑Killing of cells in HCT116 colon carcinoma spheroids, ↑regression of fibrosarcoma in xenografts		59
	Sendai Virus (SeV)	↑titer in cell culture	Multiple selection rounds in cell culture	4,647 and 293	None	↑titer in 4,647 and 293 cells, ↓killing of malignant Me18 and U87MG cells	Several non-synonymous mutations in F and HN proteins	77
PICORNAVIRIDAE	Coxsackievirus B2 Ohio-1 (CVB2/O)	↑infectivity		Rhabdomyosarcoma cells		↑ability to use DAF as a cell entry receptor, ↑infection of rhabdomyosarcoma	Nucleotide changes in the virus capsid region	90
	Six different serotypes of Coxsackievirus B						Single amino acid change in the virus capsid	91
	Coxsackievirus non-lytic B6 (CV-B6-Schmitt)			Human pancreatic duct epithelial cells		↑binding to DAF as a cell entry receptor, ↑infection of pancreatic duct epithelial cells		93
IRIDAE	Coxsackievirus B6 (CV-B6)			A431, A549, RD and MCF7	Human xenografts	↑infection of previously non-permissive malignant cells, ↑oncolytic properties in xenografts		94
	Poliovirus construct (PVSR1PO)	Maintaining non-pathogenic phenotype in gliomas	One round of propagation in a few human glioma xenografts	Human glioma cells		Absence of neurovirulence	Absence of genetic determinants of neurovirulence	110

(Continued)

Table 1 (Continued).

Virus family	Virus	Goal of selection	Method	Cell culture	Animal model	Phenotype	Genotype	Ref.
R H A B D O V I R I D A E	VSV	<div>↑infectivity in mammary gland cancer cells</div> <div>↑infectivity in glioblastoma</div> <div>↑infectivity of p53 negative cells</div>	Multiple selection rounds in cell culture	<div>D2F2/E2</div> <div>U-87 MG</div> <div>Primary mouse embryonic fibroblasts derived from p53 –/– C57BL6 mice</div>	<div></div> <div>Human xenografts</div> <div>Murine syngeneic models for breast cancer</div>	<div>↑infectivity in target malignant cells</div> <div>↑killing of transplanted human glioblastoma cells in murine model</div> <div>↓growth of p53 negative transplanted cancer cells</div>	<div>Two point mutations in artificially inserted single-chain antibody domain</div> <div></div> <div></div>	<div>114</div> <div>115</div> <div>116</div>

**Notes:** Description of cell lines (in order mentioned in Table 1). L929, murine fibroblasts; Vero, African green monkey kidney cells; Hela, human cervical cancer cells; MEL, murine erythroleukemia cells, which do not express JAM-A; HT1080, human fibrosarcoma cells; HT29, human colon carcinoma cells; 4.647, African green monkey kidney cells; 293, human embryonic kidney cells; Mel8, human melanoma cells; U87MG, human glioma cells; A43 I, human epidermoid carcinoma cells; A549, human lung carcinoma cells; MCF7, human breast adenocarcinoma cells; D2F2/E2, mouse HER-2 positive mammary gland carcinoma cells; U-87 MG, human glioblastoma cells.

binding motif of  $\sigma 1$  cell attachment protein. One of the virus mutants gained the ability to infect a wide range of new cell lines that were not permissive to T3D reovirus infection. These cell lines included chicken hepatocellular carcinoma LMH, murine endothelioma, human bone osteosarcoma U2OS, and Ewing sarcoma STA-ET2.<sup>34</sup> As such, the goal of broadening the range of cancer cells that could be infected by reovirus was achieved. However, virus dissemination specificity and safety profiles were not reported.<sup>34</sup> The possibility that broadening the reovirus infectivity compromised its safety, by analogy with the observations reported earlier,<sup>33</sup> could not be ruled out.

### Safety improvement IFN sensitivity increase

IFN pathways are frequently defective in malignant cells, making them more sensitive than normal cells to OV. However, viruses, in general, can inhibit some components of the IFN defensive system of their host cells, whether malignant or not. Consequently, the virus ability to inhibit IFN defenses in normal cells could promote infection of these cells and decrease OV safety.

So, for the best discrimination between malignant and normal cells, OVs should be sensitive to IFN. Such reovirus variants were obtained by chemical mutagenesis and tested in normal parental and Ras-transformed mouse NIH-3T3 cells. As expected, a correlation was found between increased virus IFN sensitivity and decreased virus ability to infect normal cells. An IFN-hypersensitive reovirus variant was highly dependent on Ras activation, and it discriminated between normal and transformed cells much better than the parental strain.<sup>36</sup> A single amino acid substitution in one region of  $\lambda 2$  methyltransferase was found to be the major determinant of IFN sensitivity in this variant.<sup>37</sup> The results of the study suggest that optimized reoviruses with improved safety could be selected by modification of virus IFN sensitivity.

### Attenuation of JAM-A receptor binding

In a separate study, Kim and coauthors created attenuated versions of reovirus by establishing persistent infection of wild-type T3D variant in human HT1080 fibrosarcoma cells.<sup>38–40</sup> Attenuated reovirus kept its oncolytic efficiency but demonstrated reduced pathogenicity in the animal model.<sup>40</sup> It was able to infect three tested lymphoma cell lines, HBL-2, Granta, and Z138C, and was able to suppress the growth of HCT116 colon carcinoma cells. This reovirus variant maintained its ability to kill parental



HT1080 cells *in vitro* but developed reduced ability to infect L929 and HCT116 cells. In murine xenografts, attenuated reovirus suppressed HT1080 cell growth as efficiently as its parental T3D strain. However, in contrast to the parental strain, mice injected with the mutant virus showed no visible toxicity after 1 month post-treatment and only began to develop black tail syndrome after 3–7 months. It was shown that the attenuated virus has a premature stop codon in the  $\sigma 1$  cell surface-attachment protein, resulting in a truncated translational product. The stop codon prevents translation of the  $\sigma 1$  head, thereby preventing binding to JAM-A.<sup>40</sup>

## Genomic changes that affect infectivity and safety

Almost all studies cited above demonstrated that critical genotype changes were responsible for increase or broadening of reovirus infectivity. The changes typically occurred in viral gene for cell attachment protein  $\sigma 1$ . Moreover, these studies also indicated that genotype changes, which were responsible for the improvement of the safety profile of reoviruses, could also be mapped to the same protein. Collectively, these data suggest that small changes in reovirus genes cause gain or loss of  $\sigma 1$  ability to bind sialic acids or JAM-A located on the host cell surface. Gaining the ability to bind new cell entry receptors increases and broadens reovirus infectivity, while losing it most likely increases virus safety. Achievement of the optimal interplay between this gain or loss as well as the creation of relevant optimal reovirus variants is challenging and critical tasks for future research.

## Paramyxoviruses

### Newcastle disease virus (NDV)

NDV is a negative-sense, single-stranded RNA virus that belongs to the genus *Orthoavulavirus* in the family *Paramyxoviridae*.<sup>41</sup> Its genome size is approximately 15 kb. NDV causes highly contagious and serious disease in birds<sup>42</sup> but only mild conjunctivitis in humans.<sup>43</sup> The virus' oncolytic properties are well studied.<sup>44–49</sup> It infects a broad spectrum of cancer cell lines, including fibrosarcoma, osteosarcoma, cervical and bladder carcinomas, neuroblastoma, and Wilm's tumor.<sup>50</sup> Among all viral proteins, one (F protein) is of special interest for approaches that aim to improve viral oncolysis. This glycoprotein is responsible for viral fusion with the cell membrane and viral spread from cell to cell through syncytia

formation. F protein is synthesized as an inactive precursor (F0) and is activated through proteolytic cleavage by cellular proteases. The amino acid sequence of the cleavage site determines NDV virulence. F0 proteins of low pathogenicity viruses have monobasic cleavage sites that are susceptible to trypsin-like proteases only, resulting in restricted infection. In contrast, F0 proteins of the most pathogenic virus strains have polybasic cleavage sites that are susceptible to a larger spectrum of proteases, resulting in systemic infection. Genetic modification of the F0 cleavage site could increase virus fusogenic potential and improve its spread among malignant cells, thereby increasing its oncolytic potential.<sup>45</sup>

In animal models, NDV promotes both direct and immuno-modulated cancer cell death.<sup>44,46,48</sup> When injected intratumorally or peritumorally in syngeneic animals with transplantable colon carcinomas, the virus caused significant slowing of tumor growth, which prolonged animal survival. Intravenous NDV injection in the same animal model did not retard the growth of the tumors but did promote significant animal weight loss.<sup>51</sup> Systemic virus application was also shown to be much less effective than local in treatment of murine metastatic melanoma as well as in treatment of colon and renal carcinomas.<sup>52</sup>

NDV-based oncolytic therapy has been reported to be beneficial in several clinical trials that included treatment of melanomas, glioblastomas, head, and neck squamous cell carcinomas and other malignant diseases. Descriptions of these trials are compiled in summary tables in recently published reviews.<sup>46,49</sup> Two larger trials deserve special mentioning. One was a Phase III trial that involved approximately 50 colorectal cancer patients.<sup>53</sup> The trial tested efficacy of injections of patients' with their autologous tumor cells infected with NDV, following resection of patients' liver metastases. In the subgroup of colon cancer patients, statistically significant improvement in 10-year overall survival was observed.<sup>53</sup> Another trial was an NDV dose escalation study performed with approximately 100 patients with different advanced malignancies. In this trial variable dosages of NDV were injected intravenously. A few responses to the therapy were observed at the highest dose levels.<sup>54–57</sup>

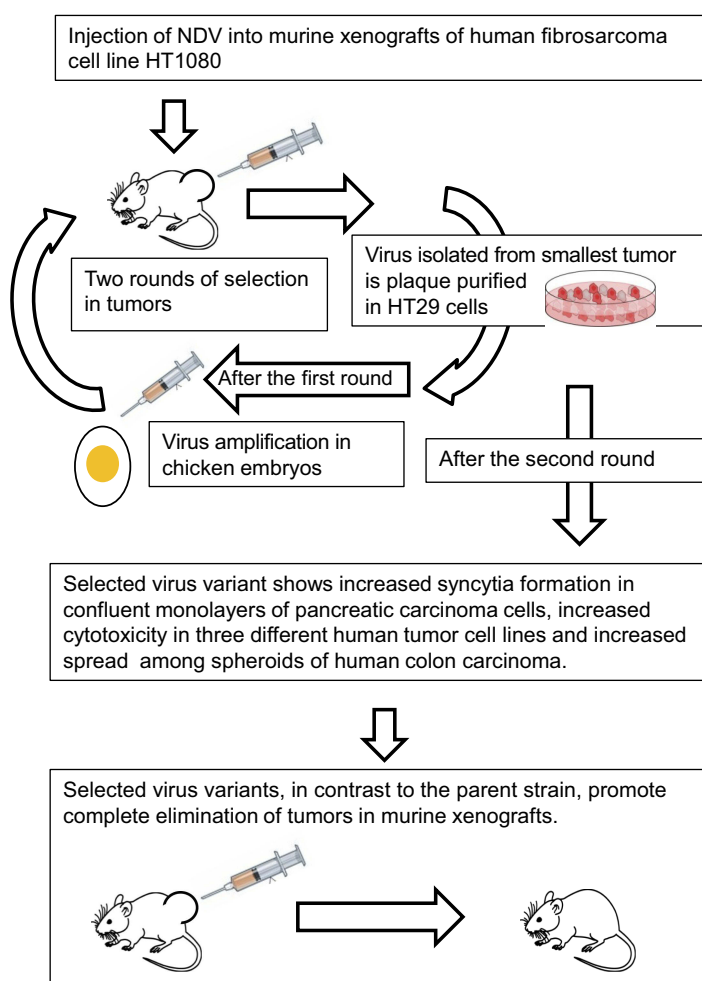
Because NDV can cause epidemic disease in poultry, its further development as oncolytic therapy must address concerns about potential environmental contamination, which could cause viral outbreaks in domestic birds. Hopefully, genetic modifications such as those introduced into an NDV strain by Cheng and coauthors<sup>58</sup> will address this issue by attenuating the virus sufficiently to make it nonpathogenic to birds.

## Improving intratumoral spreading ability

Inside tumors, viral infection spread could be limited due to tissue barriers that do not exist in a cell culture. For example, the human fibrosarcoma HT1080 cell line is highly sensitive to NDV infection, but NDV spread within the tumor formed by HT1080 cells is restricted.<sup>59</sup> Because of this limitation, elimination of HT1080 tumors by virotherapy is usually incomplete.<sup>59</sup> Beier and coauthors<sup>59</sup> hypothesized that virus selection could improve NDV intratumoral spreading ability. To confirm their hypothesis, the authors performed two rounds of virus selection in tumor xenografts of HT1080 fibrosarcoma (Figure 2). In the first round, they injected NDV into a number of HT1080 fibrosarcoma xenograft carrying animals, and identified an animal with a tumor, which was most affected by the virus treatment. They excised this tumor from the euthanized animal, dissected it into fragments and incubated them on top of HCT116 carcinoma cells' monolayer. NDV variants were plaque purified from this monolayer and further amplified in the allantoic fluid of embryonated chicken eggs.

In the second round of selection, the virus harvested from the eggs was used for reinfection of the tumors of a new set of animals with HT1080 fibrosarcoma xenografts. The virus variants from the animal with the smallest tumor were again plaque purified in a HCT116 colon carcinoma cell culture and further characterized.

The characterization included viral infection of various malignant cell types' monolayers and of colon carcinoma's spheroids. The latter represents a more clinically relevant three-dimensional cancer model than monolayers of malignant cells. The authors<sup>59</sup> concluded that through the selection steps phenotypically distinct virus clones were produced. Their fusogenicity, viral spread, and growth rate distinguished them from the parental strain. Some of the selected virus variants also exhibited stronger cytotoxicity in a variety of cancer cell monolayers. Besides, a few of the selected clones were able to kill spheroids from HCT116 carcinoma cells completely, while their parental strain was capable of only partial killing.<sup>59</sup>



**Figure 2** Selection process for increasing Newcastle Disease Virus (NDV) intratumoral spreading ability.



However, the most clinically relevant test was done by comparing the virus variants' antitumor effects in HT1080 fibrosarcoma murine-human xenografts. Tumor growth inhibition was stronger in animals that were injected with the virus variants than in animals that were injected with a parental strain. Moreover, the selected NDV variants not only slowed tumors growth but also led to their complete elimination in a few animals, an effect which had not been observed with the parental virus strain.<sup>59</sup> The animals that were treated with the selected virus variants did not lose weight or demonstrate any other signs of viral toxicity. The authors concluded that NDV intratumoral spreading ability could be improved by rounds of viral adaptation without compromising viral safety.<sup>59</sup>

The study described above shows that multiple viral progenies of the same viral strain are diverse with respect to their spreading capability, and rapid selection of clones with increased spreading capability is possible. However, this study did not connect viral phenotypic changes with any genomic changes. It left unanswered the question if the selected virus strains had changes in the gene region that corresponds to the proteolytic cleavage site of the F0. Therefore, the mechanism of improved intra-tumor spreading remains a mystery. The study also raises many other questions. How stable is the acquired phenotype? How universal is the spreading ability of selected clones for different tumor types? If the clones were selected using xenografts, tumors of which were formed by one type of malignant cells, could the selected viruses equally efficiently spread in tumors formed by other types of malignant cells? Hopefully, future research will provide answers to these pressing questions.

## Sendai virus (SeV)

SeV is a negative-sense single-stranded RNA virus with a genome of 15.3 kb. It belongs to the genus *Respirovirus*, family *Paramyxoviridae*.<sup>8</sup> SeV causes respiratory infections in mice, hamsters, guinea pigs, rats, and other rodents.<sup>60</sup> It spreads through aerosols or direct animal contact. The virus is a very common murine pathogen; it can be isolated from mice colonies worldwide<sup>60</sup> including the US.<sup>61</sup> SeV and human parainfluenza virus type 1 (HPIV-1), which causes human disease, induce production of cross-reactive antibodies. Thus, in the US, SeV has been used in clinical trials of immunization of both adults and children against HPIV-1. SeV administration was well tolerated and it triggered the production of neutralizing antibodies towards HPIV-1.<sup>62,63</sup> These studies represent essential proof of SeV safety for humans.

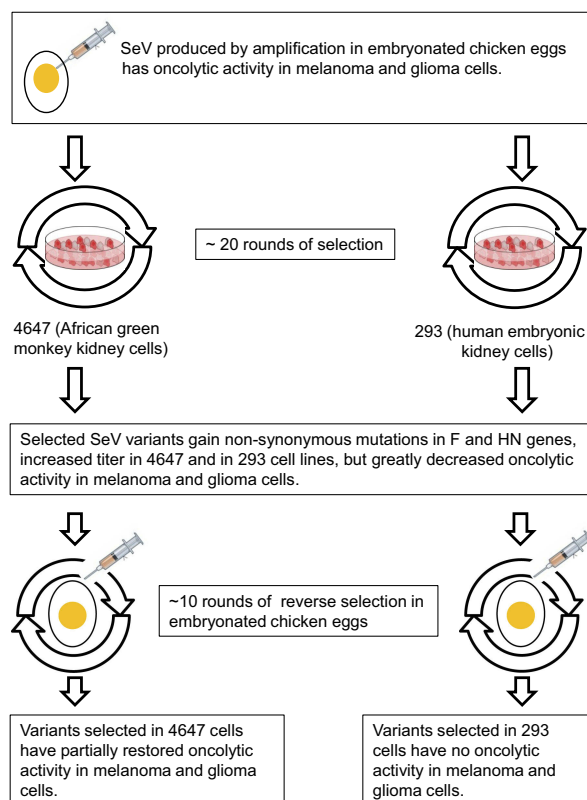
SeV causes severe murine disease, so a genetically modified strain was created to be nonpathogenic for experimental mice to study this virus' oncolytic properties.<sup>64-67</sup> This recombinant strain suppressed or eradicated tumor growth in a variety of human xenograft tumors, including sarcoma, melanoma, neuroblastoma, pancreas, colon, hepatocellular, and prostate carcinomas.<sup>64-67</sup> Complete eradication of established brain tumors was also observed in a few animals as a result of treatment with another recombinant variant of SeV.<sup>68</sup>

Wild-type SeV is infectious and is immuno-suppressive for rodents. However, UV-inactivated SeV virions have immune-stimulating properties: they are capable of promoting immuno-modulated tumor regression of colon,<sup>69,70</sup> bladder,<sup>71</sup> and kidney<sup>72</sup> cancers in syngeneic mice. UV-inactivated SeV virions also promote human prostate cancer eradication in murine xenografts.<sup>73</sup>

In the 1990s, the Moscow strain of SeV was tested as an anticancer agent in a few dozen patients affected by various malignancies with metastatic growth.<sup>74</sup> In the majority of patients, SeV was ineffective or caused transient improvement. However, a few patients achieved long-term remission even when the virus was used as a monotherapy. In these cases, complete disappearance of metastatic disease was observed without signs of its recurrence for 5–10 years or more after viral treatment. Short descriptions of these cases are presented in the patent.<sup>74</sup>

## Adaptation of Sendai virus to grow in cell culture

High titers of SeV can be produced in the allantoic fluid of embryonated chicken eggs.<sup>75,76</sup> However, such virus growth depends on a supply of specific pathogen-free eggs, which are expensive and not readily available in all countries. SeV propagation in cell culture might be a cheaper alternative to virus growth in eggs. In a study by Zainutdinov and colleagues<sup>77</sup> two cell lines were chosen for culture of SeV variants, 4,647 (African green monkey kidney cells) and HEK293 (human embryonic kidney cells). Both cell lines are certified for viral vaccine production in Russia. Figure 3 shows the scheme of adaptation experiment that included multiple (20–25 times) passaging of SeV in both cell cultures. The passaging caused numerous non-synonymous nucleotide substitutions.<sup>77</sup> Most of these substitutions were in the F and HN genes, which encode surface proteins of the SeV virion.<sup>77</sup> Mutation accumulation was associated with a significant decrease of SeV oncolytic activity toward melanoma (Mel8) and glioma (U87MG) cells.<sup>77</sup> Reverse passaging in



**Figure 3** Changes in Sendai virus phenotype and genotype during adaptation to cell culture. SeV strains adapted to grow in 4,647 or HEK293-cell cultures lost their oncolytic properties. They were subjected to reverse passaging in embryonated chicken eggs for evaluation of their ability to restore their oncolytic potentials.

embryonated chicken eggs of SeV, which had been adapted to grow in the 4,647-cell line, partially restored its oncolytic activity.<sup>77</sup> The reverse changes in the virus phenotype corresponded to reverse changes in its genotype. Some of the acquired non-synonymous SeV nucleotide substitutions were changed back to those in the parental genotype. Similar reverse passaging of SeV, which was adapted to grow in the HEK293-cell line, did not lead to recovery of its oncolytic parental phenotype or genotype.<sup>77</sup> The results of this study highlight the challenging problem that an adaptation towards one desirable virus characteristic might be accompanied by loss of another.

## Picornaviruses

### Coxsackieviruses

Coxsackieviruses belong to the genus *Enterovirus* in the family *Picornaviridae*.<sup>8</sup> This family consists of non-enveloped, linear, positive-sense single-stranded RNA viruses. Their genomes are approximately 7 kb long. Coxsackieviruses are widely distributed in humans. They are most frequently transmitted by the fecal-oral route and less frequently by respiratory aerosols. The genus *Enterovirus* also includes poliovirus and echovirus.<sup>8</sup> Coxsackieviruses were discovered in the late

1940s. Subclinical infection of children with these viruses seemed to interfere with poliovirus infection.<sup>78</sup> Coxsackieviruses are divided into group A and group B based on their pathogenicity in newborn mice. At least 23 serotypes of group A and six serotypes of group B are recognized.<sup>79</sup> Coxsackievirus A13, A15, A18, A21 (CVA21),<sup>80–85</sup> and Coxsackievirus B3 (CVB3)<sup>86,87</sup> are being studied as oncolytic agents.

Human melanoma cells implanted as xenografts into immuno-deficient mice demonstrated high susceptibility to viral oncolysis by CVA21.<sup>80</sup> Tumor burden in these animals was rapidly reduced after a single viral administration.<sup>80</sup> Coxsackieviruses A13, A15, and A18 demonstrated similar anti-melanoma efficacy.<sup>83</sup> It is interesting that intratumoral, intraperitoneal, or intravenous administrations of CVA21 were equally effective in reducing the tumor volume of melanoma xenografts implanted into immuno-deficient mice.<sup>81</sup>

CVB3 demonstrated oncolytic activity against nine human non-small cell lung cancer cell lines.<sup>86</sup> This activity positively correlated with the expression of viral receptors such as coxsackievirus and adenovirus receptor (CAR) as well as decay-accelerating factor (DAF).<sup>86</sup> Moreover, CVB3

injection into one implanted tumor resulted in its durable regression along with the regression of another identical uninjected tumor in the same animal. Furthermore, viral ability to spread from tumor to tumor in an animal was demonstrated by detection of replication-competent CVB3 in the uninjected tumor.<sup>86</sup> This preclinical research drives interest in Coxsackievirus adaptation studies.

## Adaptation of Coxsackieviruses through infectivity broadening

Adaptation studies of Coxsackieviruses, which are summarized in Table 1, have been directed toward broadening the range of different malignancies that could be infected. This process frequently involves a change in receptor usage. Thus, for productive infection, CVA21 needs simultaneously two cell entry receptors: DAF and intercellular cell adhesion molecule 1 (ICAM-1).<sup>88</sup> Some rhabdomyosarcomas, express DAF but not ICAM-1.<sup>89</sup> Consequently, for targeting such tumors, it would be beneficial to have a CVA21 variant that could use DAF alone without ICAM-1. Such a virus variant was created by rounds of selection in a DAF-expressing, ICAM-1-non-expressing rhabdomyosarcoma cell line. The selected virus variant retained its binding ability to ICAM-1 but acquired the ability to infect an ICAM-1-negative rhabdomyosarcoma and one additional ICAM-1-negative cancer cell line.<sup>89</sup> It was shown that the selected CVA21 variant binds DAF with higher affinity than its parental strain. Virus genome sequencing revealed two amino acid substitutions in viral capsid protein VP3, which are believed to be responsible for the acquired virus phenotype.<sup>89</sup>

Coxsackievirus B2 strain Ohio-1 (CVB2/O) uses CAR as a cell entry receptor and is infectious for HeLa cells that express this receptor. However, CVB2/O is unable to infect rhabdomyosarcoma cell lines, even though some other coxsackievirus B strains can do so using DAF as a cell entry receptor.<sup>90</sup> Multiple rounds of selection in a rhabdomyosarcoma cell line promoted CVB2/O ability to infect this cell line using DAF.<sup>90</sup> It is interesting that the selected virus variant retained the CAR binding ability that characterized its parental strain. Nucleotide sequencing revealed a few mutations in viral capsid regions, which most likely were responsible for the acquired virus phenotype.<sup>90</sup>

In another study six different immunotypes of group B Coxsackievirus were passaged using the same rhabdomyosarcoma cell line. After multiple blind rounds of selection,

followed by plaque purification, a single virus variant (CVB3) was isolated. This virus variant gained the ability to use DAF as a cell entry receptor. Sequence analysis revealed that a single amino acid change in the virus capsid was responsible for this acquired phenotype.<sup>91,92</sup>

A similar result was achieved with Coxsackievirus non-lytic B6 strain (CV-B6-Schmitt). The relevant study included multiple blind passages in human pancreatic duct epithelial cells. The selected viral strain gained lytic capability toward these cells and acquired the ability to use DAF as a cell entry receptor. The substitution of a single amino acid in the virus capsid protein VP1 was responsible for the new phenotype.<sup>93</sup>

Another strain of Coxsackievirus B6 (CV-B6) demonstrated a high ability to infect one set of malignant human cell lines (C33A, DU145, AsPC-1, SK-Mel28) and a low or no ability to infect others (A431, A549, RD, and MCF7). To broaden CV-B6 infection ability, the parental virus strain was subjected to up to 15 rounds of selection in cell lines, which were not permissive for viral infection. After such passaging, the new selected virus variants acquired the ability to infect the previously non-permissive cells in which they were passaged, without losing their high replication ability in the original cancer cell lines.<sup>94</sup> Several experiments with murine xenografts demonstrated that the selected viral variants showed significantly improved oncolytic properties in comparison with the parental strain.<sup>94</sup>

## Changes in capsid proteins affect infectivity of Coxsackieviruses

Almost all the studies cited above demonstrate that the main changes responsible for the virus ability to bind the DAF receptor are in genomic regions that code for viral capsid proteins. Thus, these changes seem to be responsible for broadening Coxsackievirus infectivity.

## Poliovirus

Polioviruses, like Coxsackieviruses, belong to the *Picornaviridae* family, and thus they are also represented by non-enveloped, linear, positive-sense single-stranded RNA viruses. The poliovirus genome is about 7.5 kb long.<sup>95</sup> While wild-type poliovirus virus variants cause serious human disease, their derivative attenuated vaccine strains usually do not cause disease in individuals with healthy immune systems. The oral polio vaccine strains (Sabin 1, Sabin 2, and Sabin 3) were selected as

spontaneous mutants of wild-type isolates following repeated passages in simian kidney cells.<sup>96,97</sup> The procedure introduced mutations in the viral internal ribosome entry site (IRES) and attenuated the ability of the virus to infect human nervous tissue.<sup>96,97</sup>

## PVSRIPO

PVSRIPO is a recombinant strain of poliovirus Sabin type 1, in which the IRES part of the genome is replaced with the IRES from human rhinovirus type 2. It was shown that this construct has oncolytic properties.<sup>98</sup> Poliovirus and its derivative construct PVSRIPO both enter a host cell using poliovirus receptor (PVR), also called Nectin-like molecule 5 (Nect5), or CD155 cell receptor. The expression of this molecule correlates with virus infection ability in glioblastoma multiforme (GBM) cells.<sup>98</sup> Thus, GBM expressing PVR is a target for PVSRIPO treatment. GBM is a rare but very aggressive brain malignancy. Treatment options are very limited, while patients have a median survival time of only 15 months.<sup>99</sup> However, patients' survival rate measured two and three years after PVSRIPO intratumoral injections was higher than that of the historical controls.<sup>100</sup> Other brain tumors also express CD155 at high levels and this expression in cell cultures correlated with PVSRIPO oncolytic efficiency.<sup>101</sup> The PVSRIPO construct demonstrated oncolytic efficacy in human breast and prostate cancer xenograft models.<sup>102</sup> Like many other OV, PVSRIPO kills cancer cells both directly (through infection or apoptosis) and indirectly (through broad activation of the immune system).<sup>103–106</sup>

## Testing genotype stability of recombinant poliovirus (PVSRIPO)

Experimental evolution can help in assessing the risk of a virus reversion to its virulent state during cancer treatment.<sup>107</sup> Reversion of vaccine attenuated poliovirus to the parental wild-type neurovirulent phenotype does occur but it is extremely rare.<sup>108,109</sup> Is it possible that PVSRIPO virus could gain neurovirulence during glioblastoma treatment due to its amplification in brain tissue? Dobrikova and coauthors addressed this question by examining the genetic stability of PVSRIPO during its propagation in human glioma xenografts (Figure 4).<sup>110</sup> In the relevant study intratumoral virus inoculations caused tumor regression in all animals with implanted gliomas. The virus isolated from the tumors of sacrificed animals and further amplified in human glioma cell culture retained

the attenuated neurovirulence phenotype. Sequence analyses confirmed that the genetic determinants for this attenuated phenotype were not changed upon *in vivo* passaging in gliomas.<sup>110</sup> This study demonstrated that PVSRIPO did not gain neurovirulence during its propagation in glioma tissue.

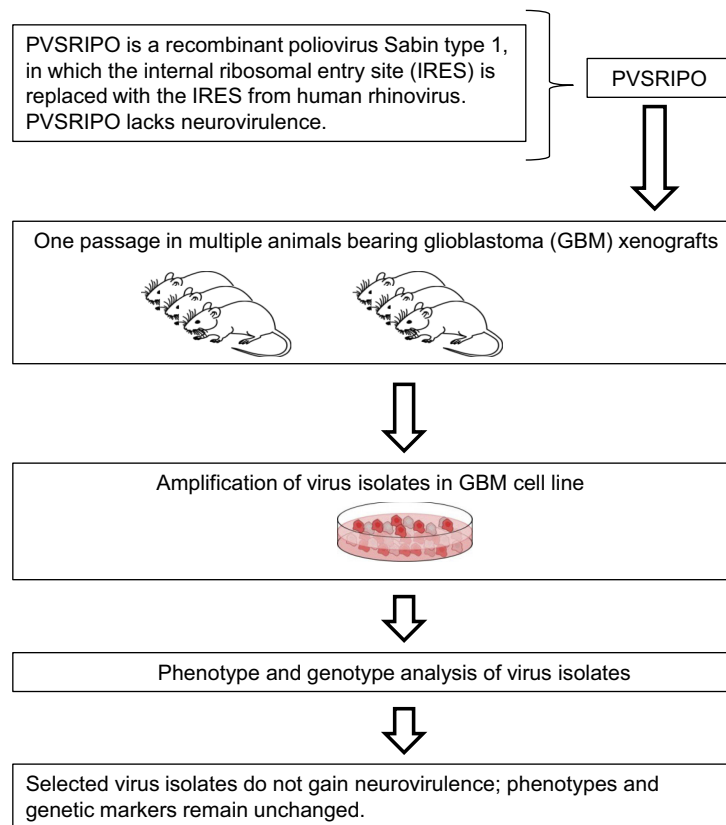
## Rhabdoviruses

### Improved infectivity and selectivity of Vesicular Stomatitis Virus (VSV)

VSV belongs to the family *Rhabdoviridae*. It is a negative-sense, single-stranded RNA virus with a genome of approximately 11 kb. VSV infects some mammals including domestic animals; it usually causes a non-lethal illness, accompanied by fever and ulceration of the epithelium of oral and nasal cavities, feet, and teats.<sup>111</sup> Insects are vectors for VSV, so infection incidence depends on insect activity. VSV infection is usually asymptomatic in humans.<sup>111</sup>

VSV and VSV-based constructs demonstrated oncolytic properties in multiple cell lines and animal models.<sup>112,113</sup> However, in animal models, VSV can be neurotoxic.<sup>112</sup> Numerous attenuated virus constructs were created to avoid this problem and work on further improvement of these constructs is ongoing.<sup>112,113</sup> Higher oncoste selectivity and oncoinfectivity are needed.

An interesting adaptation study was performed with one VSV recombinant construct in pursuit of these goals. The construct had one substituted gene, which expressed a chimeric Sindbis virus glycoprotein and a single-chain antibody directed toward the human Her2/neu receptor. Such substitution allows for very specific targeting of human breast cancer cells expressing ErbB-2 (Her2). However, the recombinant virus demonstrated poor growth characteristics in its target cell line. The problem was easily overcome by using the directed evolution approach. Fifteen passages in the targeted breast cancer cell line generated an adapted virus with significantly improved growth characteristics. Sequencing of the genome of this adapted virus revealed only two mutations, both of which occurred in the gene region that encoded the single-chain antibody. Surprisingly, an additional N-glycosylation site was created by one of the mutations. Along with improved infectivity, the adapted virus showed a higher density of glycoprotein on the viral envelope.<sup>114</sup> Thus, two adaptive mutations made the VSV construct more infectious toward one ERB-2 positive breast cancer cell line. How helpful are these mutations for infecting other ERB-2 positive cell



**Figure 4** Testing phenotype and genotype stabilities of attenuated poliovirus recombinant.

lines? How will these mutations affect virus safety? These questions await further study.

A similar study was done with VSV and glioblastoma cells. As a result of multiple passaging of wild-type VSV, a selected variant, VSV-rp30, was isolated with much higher selectivity and lytic capability toward glioblastoma cells than the wild-type virus. The variant VSV-rp30 was very effective at replicating, spreading within and selectively killing transplanted human glioblastoma cells in a murine model.<sup>115</sup>

Because p53 deficiency frequently characterizes malignant cells, directed VSV evolution was also used to obtain a virus variant with increased selectivity toward p53-deficient cells. After approximately 40 passages, the variant virus became much more infective and cytotoxic against target cells in comparison with the parental virus. Moreover, the virus infectivity increase was specific even for p53-deficient cells that were not used for virus selection. Syngeneic animal models demonstrated that the selected VSV variant significantly delayed the growth of p53-deficient mammary gland tumors compared with its parental strain.<sup>116</sup> This study suggests that gene-specific virus adaptation is possible.

## Conclusion remarks

The directed evolution/adaptation studies described in this review are summarized in Table 1. They demonstrate that oncolytic RNA viruses can acquire desirable characteristics through rounds of selection. The viruses can be selected to have higher infectivity toward a particular type of tumor cell or to infect a broader spectrum of malignant cells. They can be also selected for better intratumoral spreading ability or a better safety profile. Mapping of genotypic changes responsible for the selection acquired phenotype provides valuable insights into the molecular mechanisms that are responsible for viral infection of cancer cells. However, this type of research has problems and limitations.

An adaptation toward one desirable virus characteristic might be accompanied by a loss of another one. It is not always straightforward to map changes of viral phenotype to genotype. Culture conditions for maintaining acquired desirable traits of selected virus variants are not always easy to define. The stability of the viral phenotype acquired through directed evolution is questionable. Theoretically, without selection pressure any new viral trait might be lost through reverse evolution.



The directed evolution studies described in this review involved RNA viruses, whose replication is accompanied by high mutation rates, and which demonstrate major genetic variability. Virus populations are usually not made of a single variant with a defined nucleic acid sequence. Rather, they represented by a broad spectrum of non-identical but related mutants. These mutants called *quasispecies* and they collectively contribute to the characteristics of the population.<sup>117,118</sup> High mutation rates of RNA viruses and rapid increase in the diversity of quasispecies can both be explained by the low replication fidelity of the RNA-dependent RNA polymerase (RdRp) that replicates RNA virus genomes.<sup>119</sup> The low replication fidelity of RdRp causes its high error rate. On the one hand, the high error rate of RdRp is beneficial for directed evolution because it allows performing a comparatively low number of selection cycles for rapid achievement of the bioselection goal. On the other hand, the low fidelity of RdRp could promote a high rate of reverse evolution which can compromise the ultimate bioselection goal.

However, a high mutation rate and corresponding rapid genomic change could represent an advantage for cancer patient treatment. Perhaps oncolytic RNA virus adaptation could occur naturally, within heterogeneous tumors and metastases inside a patient's body. May be such "personalized" targeted evolution could produce multiple optimized virus variants that would be capable of causing complete tumor clearance despite antiviral immunity.

Both directed evolution and genetic engineering are approaches that could generate viral variants optimized for oncolytic applications. Despite the drawbacks of directed evolution, the approach has certain advantages over genetic engineering because it allows achieving a desirable virus phenotype without knowing in advance the genetic determinants, which are responsible for the phenotype. These two approaches complement each other and their combination will pave the way toward more safe and effective OV treatment.

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