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Evolution of oncolytic viruses

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Owing to their replicative capacity, oncolytic viruses (OVs) can evolve under the action of natural selection. Reversion to virulence and recombination with wild-type strains may compromise OV safety, therefore requiring evolutionary risk assessment studies. On the other hand, evolution can be directed in the laboratory to create more potent and safer OVs. Previous work in the experimental evolution field provides a background for OV directed evolution, and has identified interesting exploitable features. While genetic engineering has greatly advanced the field of oncolytic virotherapy, this approach is sometimes curtailed by the complexity and diversity of virus–host interactions. Directed evolution provides an alternative approach that may help to obtain new OVs without prejudice toward the underlying molecular mechanisms involved.

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Introduction

Oncolytic virus (OV) therapy uses replication-competent viruses to selectively target malignant cells, in contrast to virus-based gene therapy which uses replication-deficient viruses. Despite a large number of preclinical studies with impressive results that have led to several OVs being currently tested in human clinical trials, some critical challenges need to be addressed, including insufficient selectivity for tumoral cells, low oncolytic potency, inability to penetrate and spread in tumor tissues, premature viral clearance, and poor stimulation of tumor-specific immunity. Many efforts have addressed these issues. Due to the increasing availability of techniques for the genetic manipulation of animal viruses during the last decades, today the field is dominated by an engineer's view based on the rational modification of viral genomes. Hundreds of genetically engineered OVs have been created in which virulence genes have been deleted; the viral tropism or the ability to escape premature neutralization has been reset by modifying viral envelope proteins; and viruses have been armed with tumor suppressor or suicide genes expressed only in cancer cells, with immunostimulatory genes, or with genes that increase susceptibility of infected cells to chemo and radiotherapy [1–3].

These approaches have undeniably led to important advances, but have also met some difficulties. Virus–host interactions are extremely complex and often still poorly understood, and therefore our ability to manipulate them is limited. This is particularly challenging within the context of virus–tumor cell interactions, as tumor cells vary widely depending on the cancer type and between and within patients [4]. As a result, the rational design of OVs for each specific tumor becomes a formidable task. Additionally, extensive manipulation of viruses often renders them so attenuated, that, in many cases, it abrogates replication. Although strong attenuation is desirable for safety, many OVs have proven to be insufficiently potent against tumors. A unique feature of OV, though, is that, unlike other therapeutic agents, they replicate and mutate, and therefore have the potential to evolve. This has two important implications. First, OVs are amenable to optimization by directed evolution. This approach can help improve the efficacy of previously engineered viruses, and may allow creation of new OVs by acting on still uncharacterized molecular pathways. Second, evolution of therapeutic viruses *in vivo*, once delivered to patients, needs to be seriously assessed and, particularly, the probability of reversion to virulence.

Directed evolution to generate more effective OVs

Directed and experimental evolution has been used for practical applications in various research areas. For instance, pioneer procedures for creating oral polio vaccines included serial passages in non-human hosts [5]. In light of today's knowledge, this probably favored adaptation to the alternate hosts at the cost of reduced fitness in humans and promoted the accumulation of deleterious mutations by random genetic drift, thus making these basic evolutionary processes instrumental in the success of polio vaccines. Evolutionary studies have also been used to predict the appearance of drug resistances in HIV-1 [6], or the pathogenesis and pandemic potential of influenza viruses [7].

Using modern experimental evolution techniques, it is possible to adapt viruses to cells showing the hallmarks of

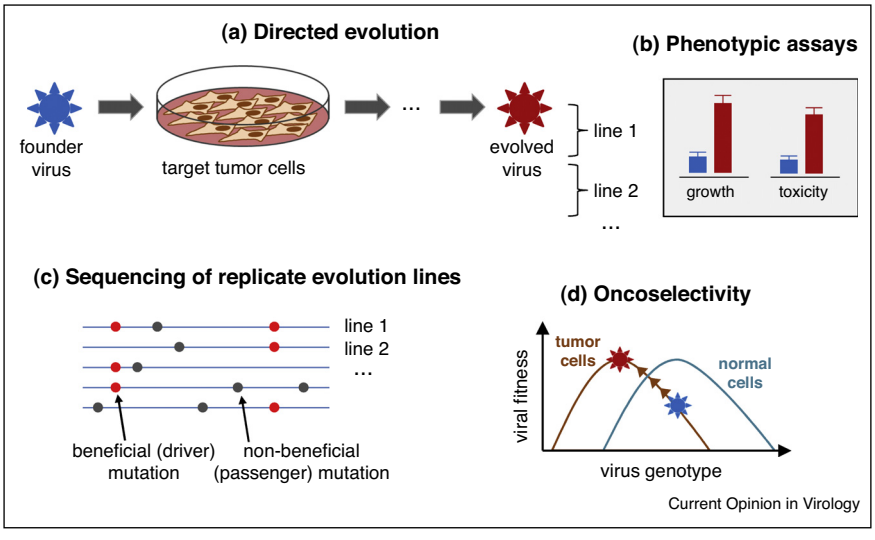
cancer, to characterize the genetic and molecular basis of this adaptation, and to subsequently evaluate the usefulness of these OVs in cell cultures and animal models (Figure 1). However, this approach has not been systematically applied in the field, albeit a few notable exceptions [8,9••,10,11,12••,13,14•]. In one study, pools of different adenovirus serotypes were serially passaged in cultures of human colon cancer cells, favoring among-serotype recombination and the selection of the fittest variants in these cells [9••]. This led to isolation of a recombinant virus (ColoAd1) that outperformed existing oncolytic adenoviruses and was approved for phase I/II clinical trials. A limitation of using directed evolution with double-stranded DNA viruses, though, is their low rates of spontaneous mutations compared to RNA viruses [15] thus making the production of new potentially adaptive variants slower. However, DNA virus variability can be enhanced using chemical mutagenesis [13] or by engineering viral polymerases with reduced replication fidelity [10].

There are also a few examples of directed evolution of RNA viruses in the context of oncolytic virotherapy. In one study, a pseudotyped vesicular stomatitis virus (VSV) was engineered to express a single-chain antibody against the Her2/neu receptor (ErbB2). Although the engineered virus initially showed a very low titer in target mammary cancer cells expressing ErbB2, serial passages in these cells increased viral fitness [8]. Another example is serial passages of wild-type VSV in human glioblastoma cells with the aim of promoting selective attachment to these cells and replication [11]. Interestingly, the evolved virus was later found to be effective also against other tumor cell lines [12••]. Recently, VSV was adapted to MEF p53−/− cells by serial passaging and then tested in isogenic p53+/+ cells, as well as in p53-positive and p53-negative tumors *in vivo* [14•]. This revealed gene-specific adaptation, suggesting that VSV can be selectively adapted to a broad cancer feature such as p53 inactivation.

Relevant factors in directed evolution experiments

Owing to their extremely high rates of spontaneous mutation, RNA viruses are ideal candidates for directed evolution. This, combined with the often high titers achieved under cell culture conditions, increases the efficacy of selection and allows for deterministic evolution of fitness-related traits in the laboratory [16]. Additionally, the genomes of most RNA viruses are less than 20 kb, making it easy to identify mutations responsible for adaptation. However, with the advent of next-generation sequencing, the genetic analysis of large oncolytic DNA viruses such as vaccinia or herpes viruses has been greatly facilitated, allowing direct identification of the molecular basis of adaption in these viruses as well [17]. Using

Figure 1



Directed evolution of oncolytic viruses. **(a)** Starting from a founder virus, which can be the wild-type or a previously engineered virus which has to be optimized, serial transfers can be performed in target tumor cells under conditions that favor the action of selection (high effective population size, low multiplicity of infection). The duration of this process can vary depending on the type of virus (RNA/DNA) and the strength of the selective pressure, but should typically range 10–50 transfers. The repeatability of evolution can be assessed by establishing several replicate lines. **(b)** The growth rate, toxicity, or other interesting phenotypic properties can then be evaluated for the evolved virus and compared against the founder. **(c)** Sequencing of replicate evolution lines can help identify relevant mutations responsible for the observed increases in viral fitness (driver mutations), since these mutations typically appear in more than one line (parallel evolution). **(d)** In many cases, adaptation to a specific cell type (here a given tumor cell line) is accompanied by a loss of fitness in other cell types (here, normal cells). These fitness tradeoffs should increase the oncolselectivity of the evolved viruses.