Design of Alternative Live Attenuated Influenza Virus Vaccines

Courtney Finch, Weizhong Li and Daniel R. Perez

Abstract Each year due to the ever-evolving nature of influenza, new influenza vaccines must be produced to provide protection against the influenza viruses in circulation. Currently, there are two mainstream strategies to generate seasonal influenza vaccines: inactivated and live-attenuated. Inactivated vaccines are non-replicating forms of whole influenza virus, while live-attenuated vaccines are viruses modified to be replication impaired. Although it is widely believed that by inducing both mucosal and humoral immune responses the live-attenuated vaccine provides better protection than that of the inactivated vaccine, there are large populations of individuals who cannot safely receive the LAIV vaccine. Thus, safer LAIV vaccines are needed to provide adequate protection to these populations. Improvement is also needed in the area of vaccine production. Current strategies relying on traditional tissue culture-based and egg-based methods are slow and delay production time. This chapter describes experimental vaccine generation and production strategies that address the deficiencies in current methods for potential human and agricultural use.

Abbreviations

EID_{50}	Egg Infectious Dose 50
IRES	Internal Ribosome Entry Site
KO	Knockout
LAIV	Live-attenuated influenza vaccine
MDCK	Madin-Darby Canine Kindey Cell
MLD_{50}	Mouse Lethal Dose 50
nts	Nucleotides
Pfu	Plaque Forming Units

C. Finch · W. Li · D. R. Perez (✉)
Department of Veterinary Medicine,
College Park and Virginia-Maryland Regional College of Veterinary Medicine,
University of Maryland, College Park, MD, USA
e-mail: avianflu@me.com

Current Topics in Microbiology and Immunology (2015) 386: 205–235
DOI: 10.1007/82_2014_404

Published Online: 9 July 2014

PSI	Packaging Signal
UTR	Untranslated Region
RG	Reverse Genetics
RISC	RNA-induced Silencing Complex
WT	Wild-Type

Contents

1 Introduction

Influenza virus is an eight-segmented, negative-sense, single-stranded RNA virus in the family *Orthomyxoviridae*. It infects a wide host range including avian, aquatic, and terrestrial species. There are three types of influenza viruses known to infect humans: A, B, and C. Influenza A is capable of infecting the broadest host range of the three influenza types, while influenza B is thought to be a primarily human pathogen. Influenza C is also known to infect humans but infects primarily infants and young children (Calvo et al. 2013, 2006). Each winter, vaccines, both inactivated and live-attenuated, are produced for protection against influenza A and B. The need for seasonal influenza vaccines is a product of the error prone viral polymerase as well as the host's immunological pressure. Since the viral polymerase lacks the ability to correct mistakes made during replication, mutations

accumulate causing a phenomenon known as antigenic drift—a gradual change in antigenicity through the accumulation of mutations (Taubenberger and Kash 2010). The segmented nature of the influenza genome allows it to undergo more drastic changes through another process as well: antigenic shift. This is the product of influenza viruses co-infecting the same cell and swapping segments. This can cause a more dramatic change in antigenicity than is caused by antigenic drift (Taubenberger and Kash 2010).

Influenza epidemics and pandemics are the result of the introduction of a novel influenza virus into a population. Influenza surveillance dictates which viruses to include in each year's vaccine. Currently, the seasonal vaccine includes two strains of influenza A and one strain of influenza B; however, recently, a tetravalent vaccine was approved which contains two strains of influenza A and two strains of influenza B. The 2013–2014's influenza season will see the first approved tetravalent vaccines; both live-attenuated and inactivated available for market consumption. Although including a second strain of influenza B will no doubt improve the coverage and protection conferred by the seasonal vaccine, problems remain, and there is room for significant improvement, both in production time and protection. Currently in the US, the live-attenuated, cold adapted A/Ann Arbor/6/60 and B/Ann Arbor/1/66 backbone vaccine is not licensed for the elderly, women who are pregnant, those who are immunocompromised, or those with pre-existing conditions, like asthma (Alexandrova et al. 1990; DeBorde et al. 1987, 1988; Maassab 1967; Maassab and Bryant 1999; Maassab et al. 1969; Monto and Maassab 1977).

As influenza A virus infects a wide variety of host species, it is, in addition to being a significant human pathogen, a significant pathogen in many agricultural species such as chicken and swine. Improved LAIV vaccines are needed for agricultural animals as well, as influenza can have a devastating economic impact on the agricultural industry and the food supply (Leibler et al. 2009). Although there are many alternative vectors and other genetic approaches that have been tested experimentally as platforms for influenza vaccines, this chapter will exclusively focus on novel methods for the generation and production of live-attenuated influenza virus vaccines. These strategies are aimed at accelerating vaccine efficacy or production by manipulation of the influenza genome via mutations, deletions, insertions, and rearrangement.

2 Production of LAIV Vaccines

2.1 Alternative Strategies to Generate Influenza Virus Vaccines

The emergence of pandemic and epidemic influenza virus strains such as the pandemic H1N1 virus in 2009 as well as various avian influenza viruses with the ability to cause disease and death in humans has prompted the renewal of efforts to

develop alternative or modified virus-generating systems as well as improve virus rescue efficiency to enable a quicker response to outbreaks of novel viruses (Kadota 2013; Schnitzler and Schnitzler 2009).

2.2 *Adoption of Universal or Species-Specific Pol I Promoter for Virus Vaccine Generation*

The traditional DNA-based reverse genetics (RG) system allows for the production of vaccine seed strains of influenza virus via the co-transfection of 8 (or 12 or 17) plasmids into appropriate cells (Fodor et al. 1999; Hoffmann et al. 2000; Neumann et al. 1999). The more common eight plasmid system (Fig. 1a, left and top right panels) is based on a bidirectional transcription vector, which employs an RNA polymerase II (pol II) promoter to synthesize positive-sense mRNA and an RNA polymerase I (pol I) promoter to transcribe negative-sense viral RNA (vRNA) from the same viral cDNA template (Hoffmann et al. 2000). The pol I promoter

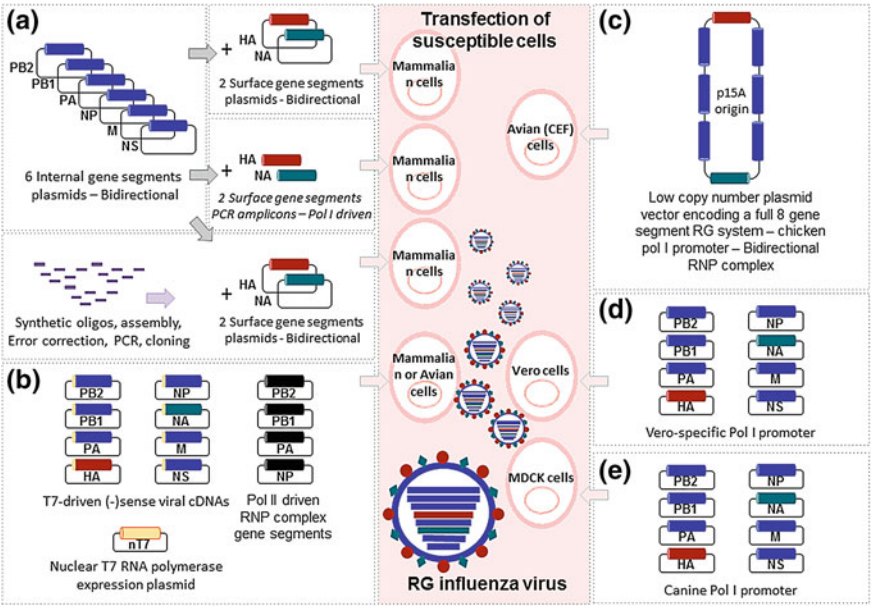

Fig. 1 Variations in reverse genetics systems for influenza virus. Reverse genetics systems and the cells lines required for use of the respective systems are shown. **a** depicts methods to expedite the generation of vaccine seed strains. **b** shows a T7 RNA polymerase reverse genetics system, which can generate virus from cells of multiple origins in the presence of a *nuclear T7 RNA polymerase expression plasmid*. **c**–**e** show alternative reverse genetics systems which are specific to a cell type/species [Based on (Chen et al. 2012; de Wit et al. 2007; Dormitzer et al. 2013; Massin et al. 2005; Murakami et al. 2008; Wang and Duke 2007; Wood and Robertson 2004)]

ensures the generation of vRNA with accurate 3′ ends, whereas a terminator sequence from pol I (tI) or a hepatitis delta virus ribozyme sequence ensures accurate 5′ ends (Fodor et al. 1999; Hoffmann et al. 2000). In contrast to pol II, transcription driven by pol I exhibits stringent species specificity (Heix and Grummt 1995). For example, RG plasmids harboring human pol I promoters work well in primate-origin cells (e.g, 293T cells) but behave less efficiently in cell lines of canine-origin (Madin-Darby Canine Kidney, MDCK) or porcine-origin (PK15) or not at all in cell lines derived from avian species (Murakami et al. 2008; Wang and Duke 2007). Thus, virus rescue by RG in different cell types may require a distinct set of expression plasmids.

In the past decade, numerous strategies have been exploited to resolve this problem. An RG vector containing a universal RNA polymerase promoter (T7 pol I) has been shown to generate recombinant influenza virus from human-origin (293T), avian-origin (QT6) and MDCK cells (Fig. 1b) (de Wit et al. 2007). Since the T7 RNA polymerase does not exist in animal cells, it is necessary to introduce an extra T7 polymerase expression plasmid with a nuclear localization sequence into the same cells (Fig. 1b) (de Wit et al. 2007). Alternatively, species-specific promoters may be used in order to transfect a single cell type (Fig. 1c–d). For instance, transfection of chicken RNA pol I promoter-based plasmids has been shown to result in virus rescue, although the virus titer is relatively low (Fig. 1c) (Massin et al. 2005; Zhang et al. 2009). Likewise, an alternative 8 plasmid RG system using canine RNA pol I promoter was developed for virus rescue in MDCK cells (Murakami et al. 2008; Wang and Duke 2007), a vaccine approved cell line (Fig. 1e) (Brands et al. 1999; Palache et al. 1999). Finally, efficient virus rescue has been shown in African green monkey kidney epithelial (Vero) cells (Wood and Robertson 2004), with the transfection of plasmids carrying Vero pol I promoters (Fig. 1d) (Song et al. 2013).

2.3 Improving the Transfection Efficiency of the RG System in Vaccine-Producing Cells

An important factor affecting vaccine manufacture using RG is transfection efficiency. Although 293T cells are easily transfected with plasmid DNA, they are not yet licensed for use in the vaccine industry. Alternatively, Vero cells are approved for vaccine production (Kistner et al. 1998; Wood and Robertson 2004), but the low-transfection efficacy poses a barrier to robust vaccine production (Kistner et al. 1998, 1999). In order to overcome this, one possible solution is to reduce the number of plasmids required for virus generation. To this end, Neumann et al. placed eight-unidirectional pol I driven vRNA transcription cassettes into one vector. Each cassette corresponded to one of the 8 segments of influenza virus (Fig. 2a). Additionally, three pol II driven transcription cassettes carrying the viral polymerase genes (PB2, PB1 and PA) were cloned into another vector (Neumann

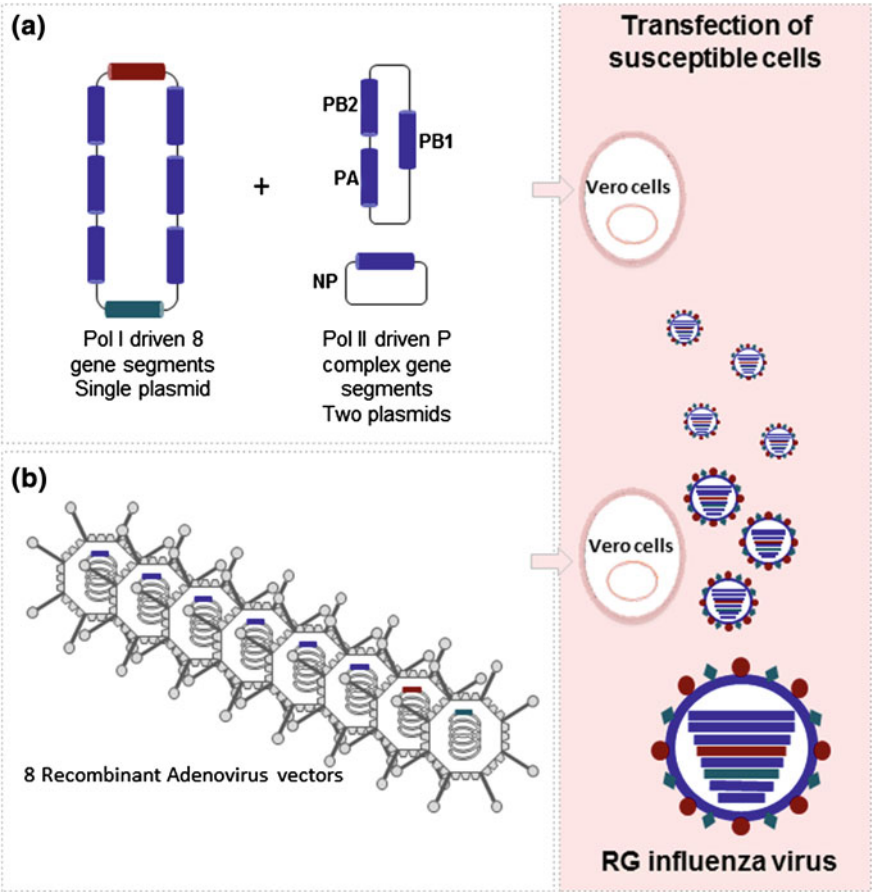

Fig. 2 Variations in reverse genetics systems for influenza virus. Reverse genetics systems designed to increase transfection efficiency of influenza gene segments in Vero cells are shown. **a** depicts a 3-plasmid strategy. **b** describes an adenovirus vector strategy. Each adenovirus is shown carrying 1 segment of influenza virus. Based on (Neumann et al. 2005; Ozawa et al. 2007)

et al. 2005). High titer virus was obtained when these 2 plasmids together with an NP-expression plasmid (3-plasmid system) were transfected into Vero cells (Neumann et al. 2005).

In a separate study, a single plasmid containing all 8 influenza gene segments was constructed, achieving high virus yield in chicken embryo fibroblast (CEF) cells (Fig. 1c) (Zhang et al. 2009). A major challenge inherent to this technology is determining how best to prevent intra-plasmid recombination while maintaining the stability of the construct. In order to address this challenge, the authors used a low-copy-number vector (approximately 15 copies per bacterial cell), minimized the number of CMV promoters (4 CMV promoters were used to initiate the synthesis of the viral RNP complex), and shortened the length of homologous

sequences (Zhang et al. 2009). This novel one-plasmid system can produce the vRNA polymerase complex (PB2, PB1, PA, and NP) and 8 vRNA segments upon transfection of cells, generating recombinant virus.

Compared with DNA vectors, the gene transfer efficiency of viral vectors is generally several orders of magnitude higher. An adenovirus vector-mediated RG system was designed (Ozawa et al. 2007) using the bidirectional transcription cassette for each vRNA segment of influenza virus inserted into a replication-incompetent adenovirus vector (AdV) lacking E1 and E3 genes (Fig. 2b). Eight recombinant AdVs were produced. After 3 days co-infection of these viruses in Vero cells, the amount of influenza virus obtained from AdV-infected cells was about 10,000 fold higher than those cells transfected with 12-plasmids (Ozawa et al. 2007).

2.4 Strategies to Accelerate Vaccine Seed Stock Preparation

During influenza pandemics, a rate-limiting step in vaccine production is the generation of vaccine seed strains. The traditional RG system takes time because it relies on a cloning step to generate plasmids expressing hemagglutinin (HA) and neuraminidase (NA) surface gene segments and a clone identification step done by sequencing. To hasten this process, a partial and full PCR-based RG strategy was designed (Chen et al. 2012). Using overlapping PCR, a pol I promoter sequence and a tI termination signal were incorporated into the 5′ and 3′ ends of cDNA copies of each individual gene of influenza A virus to produce a full set of influenza PCR amplicons (Fig. 1a left and middle right panels). When two PCR amplicons encoding the HA and NA gene segments were transfected into 293T/MDCK cells together with six bidirectional RG plasmids, RG influenza viruses were rescued with efficiencies similar to those of the eight plasmids system. Alternatively, when the eight PCR amplicons were introduced into 293T/MDCK cells together with four helper plasmids (expressing vRNA polymerase and NP protein) or four PCR amplicons harboring the PB2, PB1, PA, and NP genes flanked by CMV promoter and bovine growth hormone (bgh) polyadenylation element, the virus was generated successfully. For the first time, this showed that recovery of virus entirely from PCR products is feasible (Chen et al. 2012). This system would eliminate the cloning steps, particularly of HA and NA gene segments, involved in generating a virus vaccine seed stock, which may be crucial in the early stages of vaccine production during a pandemic.

Another option for vaccine generation during an influenza pandemic event is the use of synthetic HA and NA genes generated either by site-directed mutagenesis of DNA encoding closely related virus strains or by chemical synthesis (Verity et al. 2011). This synthetic DNA strategy has one major disadvantage, in that point mutations invariably appear, especially in the long segments like HA (1.8 kb) and NA (1.6 kb). Thus, this strategy requires correction, delaying vaccine production. Most recently, a new method was developed by Dormitzer et al. to

eliminate this hindrance (Dormitzer et al. 2013) (Fig. 1a left and bottom panels). Hundreds of overlapping oligonucleotides (about 60 bases in length per segment) were synthesized, assembled together, and mismatched bases removed by a commercially available error-correcting enzyme (ErrASE). Following ErrASE, a PCR amplification procedure was employed to generate the full-length HA and NA genes, which were subsequently ligated into a linearized plasmid such that a CMV promoter and pol I terminator flanked one end of the influenza gene and a pol I promoter and a pol II polyadenylation signal flanked the other end. After another round of high-fidelity PCR amplification with primers corresponding to the transcriptional control regions, HA and NA amplicons used for virus rescue were obtained (Fig. 1a). The first seed virus was generated within one week (Dormitzer et al. 2013), suggesting this technology could be a viable option for a faster pandemic response.

3 Improving LAIV Vaccines: Attenuation Through the Modification of Gene Segments

3.1 Modifying the NS Gene Segment

3.1.1 NS1 Truncation Mutants

Considerable effort has been placed in modifying the influenza NS gene, segment 8, as a means of creating a safer alternative to the current LAIV vaccine strategy. The NS gene encodes two proteins: NS1 and NS2. NS1, or non-structural protein 1, acts as an IFN antagonist, aiding in viral evasion of the host innate immune response (Fukuyama and Kawaoka 2011). NS2, also known as the nuclear export protein (NEP), is involved in the export of viral mRNAs from the host nucleus (Palese and Shaw 2007). Such crucial roles in the viral life cycle make segment 8 a good target for attenuation.

Studies have shown that influenza A and B viruses possessing truncated NS1 proteins are immunogenic and attenuated in a range of animal models (Fig. 3a): mouse, swine, ferret, chicken, and equine (Chambers et al. 2009; Pica et al. 2012; Solórzano et al. 2005; Steel et al. 2009; Wressnigg et al. 2009). Several NS1 truncations have been described. Each truncation involves the insertion of stop codons at the 3′ end of the NS1 ORF causing premature termination of NS1 translation at the C-terminus. Several truncations have been tested for influenza A: NS1 1–73, NS1 1–99, and NS1 1–126 (number range indicates amino acids expressed). IFN expression upon infection of a host cell with one of these truncated NS1 viruses has been shown to correlate with the length of the truncation (Solorzano et al. 2005). Although all NS1 truncations were shown to attenuate the viruses both in vitro and in the swine model, the NS1 1–73 virus has been shown to be the most attenuated (Solorzano et al. 2005). Of the NS1 truncated viruses, NS1

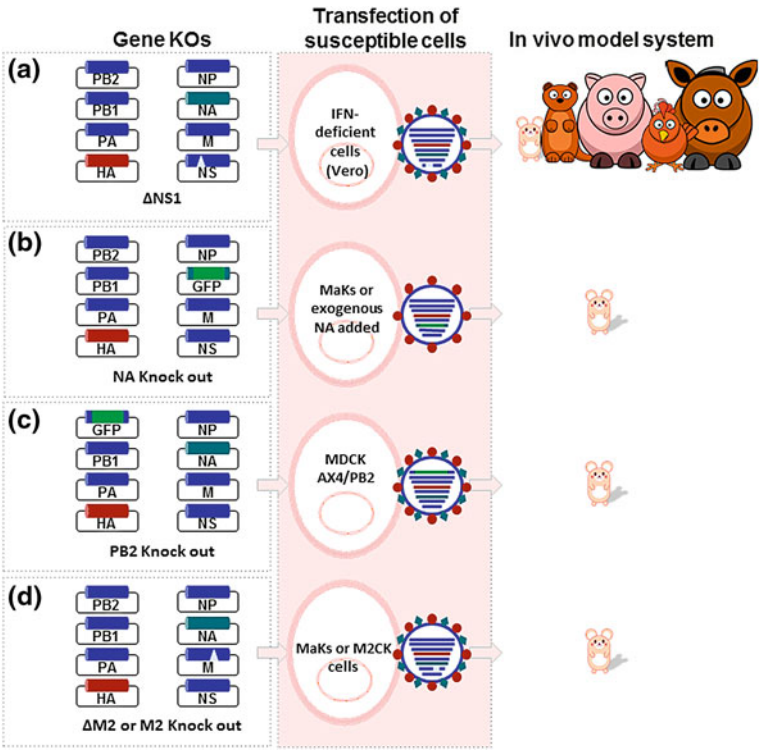

Fig. 3 Live attenuated approaches for influenza viruses—Gene knockout strategies. Four gene knockout strategies are depicted. Cells lines required for growth of the corresponding viruses are also shown as well as the animal models in which each strategy has been tested. **a** describes the NS1 truncation strategy. The *white* Δ in NS denotes the location of the truncation. **b** summarizes the NA knockout strategy. The foreign gene, *GFP*, cloned in placed of NA is shown in *green*. **c** describes the PB2 knockout strategy. As in the NA strategy, the foreign gene, *GFP*, cloned in place of PB2 is shown in *green*. **d** depicts both the M2 truncation and the M2 knockout strategy. As in the NS truncation strategy, the *white* Δ in M denotes the location of the truncation or deletion, in the case of the knockout. Based on (Chambers et al. 2009; Hatta et al. 2011; Pica et al. 2012; Shinya et al. 2004; Solórzano et al. 2005; Steel et al. 2009; Victor et al. 2012; Watanabe et al. 2008, 2009; Wressnigg et al. 2009). Royalty free animal clip art obtained from https://openclipart.org

1–73 is the most deficient in blocking IFN because it contains the longest truncation. However, the NS1 1–99 virus proved more potent from a vaccine efficacy standpoint. Both NS1 1–99 and NS1 1–73 viruses caused significantly fewer lung lesions in swine than did WT virus at the same dose (Solorzano et al. 2005).

The NS1 truncation attenuation strategy has been validated in seasonal viruses as well as highly pathogenic (HP) H5N1 viruses, which have traditionally been a challenge for vaccine developers. Studies have shown that the NS1 1–126 truncation strategy is a potentially excellent LAIV vaccine candidate for high-risk populations unable to receive traditional LAIV vaccines, such as the elderly, based

on studies performed in aged mice (Pica et al. 2012). While aged mice vaccinated with 10^3 Pfu were protected against lethal challenge with 50 mouse lethal dose 50 (MLD_{50}) and had detectable virus specific antibodies, neutralizing antibody titers were not detected (Pica et al. 2012); however, when compared with mice that received 50 ng of formalin-inactivated vaccine, the NS1 1–126 vaccine candidates performed better, providing full protection where the inactivated vaccine did not. Mice vaccinated with formalin inactivated vaccine succumbed to challenge (Pica et al. 2012).

In the case of H5N1, the NS1 truncated (NS1 1–73, NS1 1–99 and NS1 1–126) vaccines were further modified. The polybasic cleavage site was mutated to a monobasic cleavage site, as the presence of this cleavage site is a major determinant of virulence (Steel et al. 2009). Additionally, the virus was mutated in codon 627 of segment 1 encoding PB2. The E627K (glutamic acid to lysine) mutation in PB2 is known as a marker for mammalian adaptation (Palese and Shaw 2007). Steel et al. found that the NS1 1–99 truncated virus with a monobasic cleavage site and E at position 627 provided that best protection in mice against lethal challenge of 1,000 MLD_{50} and caused no weight loss at a vaccination dose of 10^6 EID_{50}. Importantly, these same viruses grow to low titers in human lung epithelial cells, A549, but high titers in embryonic chicken eggs (due to E at position 627) (Steel et al. 2009). Efficacy of the H5 NS1 1–99 vaccine was also assessed in chickens. Results showed that 100 % of vaccinated chickens challenged with 100 CLD_{50} of the parental virus survived.

Influenza B NS1 truncated viruses have also been generated. Similar results have been seen in influenza B as in influenza A; even though, BNS1 RNA binding to IFN is not required for IFN antagonism (Donelan et al. 2004). As in influenza A, a series of truncations were made in the C-terminus of NS1: NS1 1–14, NS1 1–38, NS1 1–57 and NS1 1–80 (Wressnigg et al. 2009). These viruses, like those of influenza A, replicate in Vero cells, which do not produce interferons. While all of these truncated viruses are attenuated, the degree of attenuation corresponds to the length of the truncation (Wressnigg et al. 2009). NS1 1–80 is not sufficient to block IFN and induces an IgG response which is greater than that produced by the WT virus (Wressnigg et al. 2009). Mice were fully protected after just one immunization (at 5×10^5 tissue culture infectious dose 50, $TCID_{50}$) of NS1 mutants against 5×10^5 $TCID_{50}$ homologous virus challenge. Viral titers of lung homogenates revealed that none of the mice immunized with an NS1 mutant had detectable virus titers in the lungs after lethal challenge (Wressnigg et al. 2009).

More recently, an NS1 truncation strategy has been tested in human trials. To date, both safety/immunogenicity and heterosubtypic immunity trials have been completed (Morokutti et al. 2014; Wacheck et al. 2010). Referred to as ΔNS1-H1N1, this monovalent vaccine completely lacks the NS1 ORF and carries H1 and N1 surface glycoproteins from A/New Caledonia/20/99 (H1N1). During the safety and immunogenicity trial, 1 of 5 doses of the vaccine was administered intranasally to healthy seronegative adults in spray form. Adverse effects were mild, ranging from headaches to rhinitis. In the highest dose group, 10 of 12 adults seroconverted to the vaccine. Additionally, neutralizing antibodies to heterologous

variants were detected (Wacheck et al. 2010). Following a successful safety and immunogenicity study, heterosubtypic immunity induced by this vaccine was evaluated. Seronegative healthy adults were similarly immunized with ΔNS1-H1N1, although at only one high dose. Of those vaccinated 8/12 developed a serum IgG response to the vaccine. Importantly, nasal washes were found to neutralize heterosubtypic H3N2 and H5N1 viruses, and this cross-neutralization generated by the vaccine was determined to be a result of IgA, not IgG.

Manipulation of the NS gene has not been limited to NS1. NEP's open reading frame has also been modified. NEP interacts with M1 to export vRNAs from the nucleus of the infected host cell into the cytoplasm where they are packaged into new influenza virions (Palese and Shaw 2007). Akarsu et al. modified the NEP coding sequence to introduce mutations into the M1 binding region, E67S, E74S, and E75S. Two viruses were generated, E67S/E74S and E67S/E74S/E75S. The introduction of these mutations caused the addition of 7 amino acids at the C-terminus of the protein (Akarsu et al. 2011). Vaccine efficacy of the E67S/E74S virus was tested. E67S/E74S immunized mice challenged with 10 MLD_{50} of the WT (A/WSN/33 (H1N1)) virus saw no weight loss. While a decrease in polymerase activity was detected, peak viral titer of this virus was similar to WT (Akarsu et al. 2011). This is particularly important when considering vaccine development.

3.2 Gene Knockout Strategies

3.2.1 Neuraminidase-Deficient Viruses

Segment 6 of the influenza genome encodes for neuramindase (NA), an enzyme responsible for cleavage of the sialic acids (Palese and Shaw 2007). Neuraminidase allows for release of progeny influenza virion from the host cell (Palese and Shaw 2007). NA is also thought to be involved in reaching the target cell surface by cleaving sialic acids in the mucopolysaccharides covering the surface of the target cell (Cohen et al. 2013). Like segment 8, segment 6 is also a target of manipulation.

Shinya et al. showed that a neuraminidase deficient virus is not only attenuated, but also capable of stably carrying a foreign gene in place of the NA ORF, making this strategy a potential vaccine vector (Shinya et al. 2003). The majority of the NA ORF was replaced ($\sim$1,100 nucleotide deletion) with the green fluorescent protein (GFP) ORF (Fig. 3b). Additionally, 185 nucleotides of NA at the 5′ end of the vRNA and 202 nucleotides (nt) of NA on the 3′ end of the vRNA flanked GFP (Shinya et al. 2004), each region corresponding to packaging signals. Rescue of this virus was achieved through growth in modified MDCK cells (MaKs) that express lower levels of sialic acids (Shinya et al. 2004). In MaKs cells, the NA deficient virus grows to WT titers. Alternatively, NA-deficient viruses can be grown in the presence of exogenous NA added to the cell culture medium. Shinya

et al. then demonstrated that, upon vaccination of mice with at least 10^6 Pfu of the NA-deficient virus, complete protection against 100 MLD_{50} of challenge virus was achieved. Seroconversion and neutralizing antibodies were detected at vaccination doses as low as 1.1×10^4 Pfu (Shinya et al. 2004). Additionally, GFP expression was detectable up to five passages in MaKs cells, suggesting that this virus could also be a vaccine vector (Shinya et al. 2004).

3.2.2 PB2-Deficient Viruses

Segment 1 of the influenza genome codes for PB2. The encoded protein interacts with PB1 and PA proteins (encoded by segments 2 and 3, respectively) to form the vRNA-dependent RNA polymerase complex (Palese and Shaw 2007). PB2 binds 5′ cap of the host mRNA, initiating the cap snatching activity of the viral polymerase and allowing for synthesis of viral mRNA (Palese and Shaw 2007). This is a crucial step in the viral life cycle, making PB2 a good target for attenuation.

Like the NA knockout strategy described previously, the PB2 nucleotide sequence of A/WSN/33 (H1N1) (WSN) was deleted, except for the packaging signals. The GFP ORF was cloned in place of the deleted region (Fig. 3c) (Victor et al. 2012). The virus grew to WT levels in AX4/PB2 cells, which have enhanced expression of alpha 2, 6 sialic acid receptors and stably express PB2 (Fig. 3c). A safety profile of the vaccine in mice revealed no weight loss upon vaccination, and tissue collected was virus free. The best protection with this vaccination strategy against a lethal A/Puerto Rico/8/1934 (H1N1) (PR8) challenge of 5 MLD_{50} was seen with 3 doses of the vaccine (at 10^6 Pfu). The best IgG and IgA responses were also seen with 3 immunizations. Post-challenge, no virus was detected in the lungs or nasal turbinates of any mouse that received 2 or 3 doses of the PB2KO vaccine. Interestingly, GFP antibodies were detected in mouse sera suggesting that this strategy may be a viable candidate for a virus vector.

3.3 Viruses Possessing M2 Deletions

Segment 7 of the influenza genome encodes for the M gene. Upon translation in influenza A, it is spliced into M1 and M2. Similarly, segment 7 of influenza B encodes for M gene; however, in the case of influenza B, BM1 and BM2 are derived via a stop–start translation signal separating the ORF of the 2 proteins (Palese and Shaw 2007). M1 is a matrix protein that functions as a structural component of the influenza virion. M2 and BM2 are ion channels that aid in the release of the vRNA into the cytoplasm by facilitating the acidification of the virion (Palese and Shaw 2007). The M2 cytoplasmic tail is also known to be involved in virus assembly and is a determinant of morphology (Palese and Shaw 2007). Viruses possessing deletions of the M2 cytoplasmic tail and complete M2

knockouts have proven to be attenuated in mice in the context of H5N1, pH1N1, and mouse-adapted PR8 (H1N1) viruses (Hatta et al. 2011; Watanabe et al. 2008, 2009).

A series of M2 cytoplasmic tail mutants were created as vaccine possessing deletions of various lengths beginning at the C-terminus in the context of a H5N1 vaccine (Fig. 3d) (Watanabe et al. 2008). Another mutant was constructed which contained a full M2 knockout (Watanabe et al. 2008). Each mutant was constructed by the introduction of premature stop codons in the M2 ORF. Growth kinetics in MDCK cells showed that viruses containing 5 (VN1203M2del5) and 11 (VN1203M2del11) amino acid (aa) deletions grew to similar titers as the WT virus. Titrations of these M2 cytoplasmic tail mutants in mice detected the VN1203M2del5 and VN1203M2del11 viruses in mouse tissues only on day 3 post-infection including lung, nasal turbinates, brain (except for VN1203M2del11), spleen and kidneys, while the longer amino acid deletions of the M2 cytoplasmic tail were undetectable (Watanabe et al. 2008). Both the VN1203M2del5 and VN1203M2del11 viruses were present at lower titers than WT in all tissues assayed.

After modifying the HA cleavage site from a polybasic cleavage site to a monobasic site (ΔHA), the VN1204M2del11 virus was further assessed for protection against WT highly pathogenic A/Vietnam/1203/ 2004 (H5N1) challenge (Watanabe et al. 2008). Mice vaccinated with 100 or 1,000 Pfu of ΔHA VN1203M2del11 virus did not lose weight post-vaccination. Vaccinated mice also showed high levels of IgG in the sera as well as IgG and IgA in lung washes. Little IgA or IgG was found in nasal washes. In each medium, the mice vaccinated with 1,000 Pfu had the highest titers of each immunoglobulin. Viral replication of the vaccine strain was seen in the lungs at both doses and in the nasal turbinate at 1,000 Pfu on day 3 post-vaccination. All vaccinated mice survived challenge of 100 MLD_{50} WT H5N1. None showed weight loss (Watanabe et al. 2008); furthermore, no virus was detected in any immunized mouse tissue at either dose 3 days post-challenge (Watanabe et al. 2008).

Similar findings as those described above in the context of HP H5N1 influenza virus were also found in studies done using the same strategy (11 amino acid deletion in the M2 cytoplasmic tail) with pandemic H1N1 virus, A/California/04/2009 (H1N1) (CA04) (Hatta et al. 2011). As was seen in H5N1, the CA04M2del11 virus grew to WT levels in MDCK cells. Inoculation of mice resulted in little weight loss, although the virus replicated to WT levels in the lungs at 10^4 and 10^5 Pfu. Upon homologous challenge with 10^6 Pfu of WT CA04, none of the CA04M2d3l11 immunized mice lost weight (Hatta et al. 2011). IgG levels in the serum as well as IgG and IgA levels in the nasal wash were comparable to WT; however, IgG and IgA levels in trachea-lung washes were lower than WT levels (Hatta et al. 2011).

Another study analyzed the M2 knockout (M2KO) strategy as an attenuated vaccine in mice in the context of a lethal challenge (100 MLD_{50}) with the A/Puerto Rico/8/1934 (H1N1) virus (Fig. 3d) (Watanabe et al. 2009). Comparable IgG and IgA responses were seen in mice immunized with M2KO virus as those immunized with WT virus. The primary difference between this knockout strategy and

the cytoplasmic tail deletion strategy is growth. While the M2 cytoplasmic tail mutations were grown in MDCK cells to near WT levels, the M2KO virus must be grown in M2CK cells, MDCK cells that express M2 (Watanabe et al. 2009).

3.4 Modifications in the HA Cleavage Site

Segment 4 encodes HA. HA protein must be cleaved in order to generate a productive influenza infection (Skehel and Wiley 2000). It is cleaved upon release of the virus from the host cell (Palese and Shaw 2007). The cleavage reaction is the product of an interaction between a host cell protease and the cleavage recognition site of the HA protein. Monobasic cleavage sites are cleaved by trypsin-like proteases present primarily in the upper respiratory tract of humans and many other mammals and the intestinal tract of avian species (Skehel and Wiley 2000), while polybasic cleavage sites are cleaved by furin-like proteases ubiquitously present throughout many cell types. Cleavage by furin-like proteases allows the virus to cause systemic infection in birds or to allow more efficient lower respiratory tract infection in mammals (at least for some strains, like the Asian H5N1 strains) causing a more virulent infection (Palese and Shaw 2007). Thus, altering the enzyme specificity of the cleavage may be a means of attenuating the virus.

Influenza A and influenza B viruses have been engineered to carry the porcine pancreatic elastase cleavage site in the HA gene segment (Fig. 4a). The typical trypsin-like HA cleavage site of A/WSN/33 (H1N1) was altered to recognize elastase by mutating the HA gene segment at nucleotide positions 1,059 and 1,060 from AG to GT. These mutations resulted in an amino acid change of arginine to valine at position 343. In vitro, with the addition of elastase, this virus grew to WT levels in MDCK cells (Stech et al. 2005). The WSN elastase virus (WSN-E) was completely restricted to growth in the presence of elastase, as it does not plaque and cannot be cleaved in the presence of trypsin. The WSN-E virus also showed restricted growth in the mouse model. Mice vaccinated with WSN-E did not exhibit any signs of disease, showed no weight loss after inoculation with 10^6 Pfu of virus, and had no detectable virus in the lung 1 day post-inoculation. In contrast, WT WSN was detected in the lung at each time point assayed. A vaccine study showed that mice inoculated with at least 10^5 Pfu of WSN-E were fully protected from a WT WSN lethal challenge at 10^6 Pfu. Interestingly, all mice immunized with WSN-E formalin inactivated vaccine succumbed to infection after challenge (Stech et al. 2005). Post-challenge, no virus was detected in the lungs of mice immunized with 10^6 Pfu of WSN-E. These mice had IgG and IgA responses similar to mice immunized with 10^3 Pfu of WT WSN (Stech et al. 2005).

The HA cleavage site of influenza B/Lee/40 was similarly modified to recognize elastase; however, in this instance, two viruses were generated after mutating the HA: B/Lee/40-Val (arginine to valine at position 361) and B/Lee/40-Ala (arginine to alanine at 361). In the presence of elastase, each vaccine candidate was shown to grow to WT levels in MDCK cells, although B/Lee/40-Val grew to

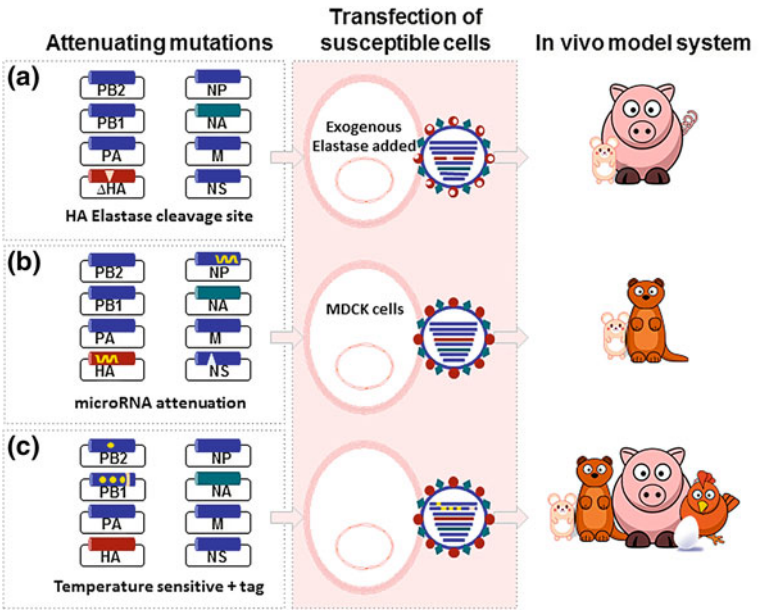

Fig. 4 Live attenuated approaches for influenza viruses—Attenuating mutations. Experimental LAIV strategies based on the mutation of various segments, the cell lines in which the corresponding viruses must be grown, and the animal models in which each strategy has been tested are depicted. **a** describes the elastase HA cleavage site mutation strategy. The *inverted white* Δ represents the mutation site. **b** describes the introduction of microRNAs to attenuate influenza. The sites of microRNA introduction are depicted with a *yellow squiggle line*. **c** illustrates the *temperature sensitive* + *tag* (*att*) strategy of attenuation. Point mutations are depicted with *yellow dots* in the PB2 and PB1 segments. The HA tag is shown as a *pink rectangle* in PB1. Based on (Babiuk et al. 2011; Cai et al. 2011; Hickman et al. 2008; Langlois et al. 2013; Loving et al. 2013; 2012; Masic et al. 2009b, 2010; Pena et al. 2011; Perez et al. 2009; Solórzano et al. 2010; Song et al. 2007; Stech et al. 2005, 2011)

slightly lower titers. Given this finding, in vivo work was completed using B/Lee/40-Ala, which did not cause weight loss in mice. Post-lethal challenge of WT B/Lee/40 at 10^6 Pfu, no virus was detected in the lungs of mice immunized with at least 10^4 Pfu of B/Lee/40-Ala on day 3 post-challenge. Mice immunized with these doses maintained weight post-challenge and survived. In contrast, mice immunized with B/Lee-40-Ala formalin inactivated vaccine had high viral titers detectable in the lungs on day 3 post-challenge. Weight loss was recorded (Stech et al. 2011). This cleavage modification strategy presents major advantages. Elastase vaccines can be grown to WT levels in approved cell lines while providing adequate protection with just one dose; however, this strategy is prone to reversion. Thus, as the authors suggest, combining this strategy with another, such as the A/Ann Arbor and B/Ann Arbor cold-adapted mutations, may be necessary (Alexandrova et al. 1990; DeBorde et al. 1987, 1988; Maassab 1967; Maassab et al. 1969).

Further work has been done to evaluate the elastase cleavage site in the context of its natural host to determine if such a strategy would be suitable as an agricultural vaccine for swine (Masic et al. 2009a). Mutations were introduced at position 345 (arginine to valine and, in a separate virus, arginine to alanine) in the HA of A/swine/Saskatchewan/18789/2002 (H1N1). In vitro, these viruses are strictly dependent on cleavage by elastase; whereas, in vivo, they are attenuated. Swine inoculated with WT virus developed signs of disease; however, no signs of disease were observed in swine inoculated with either of the elastase mutant viruses. Additionally, while WT inoculated pigs shed virus, virus shedding was undetectable in swine inoculated with the elastase mutant viruses. From this study, Masic et al. concluded that these elastase mutant viruses would be good candidates for LAIV swine vaccines. Thus, the protective properties of these vaccines were assessed when administered both intratracheally and intranasally (Masic et al. 2009b, 2010). Two doses of intratracheally administered vaccine induced IgG and IgA responses. The arginine to alanine (R345V) mutant virus was determined to be more immunogenic and was further tested in a vaccine study. Two doses of this virus administered intranasally resulted in complete protection against the homologous virus and partial protection against a heterologous virus upon challenge with 8×10^5 Pfu of either homologous or heterologous challenge virus (Masic et al. 2009b). Protection conferred by the R345V virus was subsequently evaluated intranasally, as this is a more practical route for vaccine administration. Two intranasal inoculations induced strong IgG and IgA responses as well as a specific IFNγ response. Full protection was achieved against homologous parental challenge as well as a homologous challenge with an antigenic variant. Partial protection was achieved with heterologous challenge (Masic et al. 2010). This strain has been shown to protect against pH1N1 via both routes of administration (Babiuk et al. 2011).

3.5 Adding Attenuating Features to the Influenza Genome

3.5.1 MicroRNAs

MicroRNAs (miRNAs) are RNAs generated by RNA polymerase II transcripts of a host cell. Transcripts are cleaved by host endonucleases and used to regulate cell protein expression (tenOever 2013). The miRNA transcripts bind to other host messenger RNA (mRNA) transcripts with partial complementarity and are loaded into the RNA-induced silencing complex (RISC). This process is similar to vRNA silencing (viRNA) in which viRNAs are targeted by the host cell transcripts (with complete complementarity), loaded into RISC, the formation of which is facilitated by DICER (an enzyme that cleaves double stranded RNA), and degraded; however, it is not considered a host defense mechanism. Generally, regulation of protein expression by miRNAs results in a two-fold reduction in protein expression (tenOever 2013). Since vertebrates have miRNAs and there is little reciprocal

interaction between RNA viruses and host miRNAs, it is possible to engineer vaccines by manipulating miRNAs (tenOever 2013).

Manipulation of miRNAs to attenuate influenza virus has been shown. Perez et al. demonstrated attenuation of an influenza A virus carrying an miRNA, miRNA 93, which is present in human and murine cells but absent in chicken, hypothesizing that such a modification to the genome would result in attenuation in murine cells but uninhibited replication in eggs (Perez et al. 2009). Segment 5, encoding NP, was mutated in such a way that did not alter the physical properties of the amino acid coding sequence but modified NP such that it contained miRNA response elements, target sites for miRNA (Fig. 4b). Such modifications resulted in the introduction of point mutations. H1N1 and H5N1 viruses generated in this manner were attenuated in mice, reducing mortality by more than 2 logs. Perez et al. proved that this attenuation was directly related to the introduction of miRNA response elements in NP, showing that, when influenza virus carrying miRNA response elements were administered to DICER knockout mice, the viruses grew to WT levels (Perez et al. 2009).

More recently, it was shown that modification of the HA gene through the addition of a series of miRNAs at the C-terminus could also be used to restrict replication and transmission of influenza to species that do not recognize the miRNA present in the virus (Fig. 4b). Studies were aimed at selecting a miRNA that is abundant in human lung A549 cells but absent in ferret lungs and MDCK cells. Langlois et al. hypothesized that such a strategy would not alter the growth or transmission kinetics of the virus in ferrets or MDCK cells. miRNA 192 was identified as a potential candidate. With the incorporation of miRNA 192, HA protein expression was ablated in A549 cells. Additionally, growth of the virus carrying miRNA 192 was uninhibited in MDCK cells, but in MDCK cells engineered to express miRNA, growth was restricted. To demonstrate restriction of the miRNA192 carrying virus in an animal model, mice were infected. These mice saw little change in weight and survived infection, while WT inoculated mice experienced severe weight loss and succumbed to infection (Langlois et al. 2013). Similarly, ferrets were inoculated with a miRNA 192 expressing virus. As expected, transmission was not hindered by the addition of miRNA 192. Virus sequenced from these ferrets revealed the presence of miRNA 192 in the HA, showing stability of this strategy in vivo (Langlois et al. 2013).

Although neither of these strategies was explicitly tested as a vaccine candidate, each could potentially be incorporated into other LAIV strategies to enhance attenuation without losing replication activity. These studies prove that it is possible to engineer a virus that grows to high titers in approved cell lines but is restricted in the species for which the vaccine would be generated. Thus, miRNAs could have implications for influenza vaccines for many species.

3.5.2 Viruses Carrying HA Epitope Tags

Extensive research on another strategy involving the addition of a feature to the influenza genome has been done. Previously, it was found that the temperature sensitive mutations are not always enough to attenuate animal influenza viruses, in particular avian and swine viruses (Song et al. 2007). With the simple addition of the HA tag, the vaccine becomes significantly safer (Song et al. 2007) (Fig. 4c). This strategy was complemented with the introduction of the cold-adapted mutations found in PB2 and PB1 genes of cold-adapted A/Ann Arbor/6/60: K391E, E581G, and S661T in PB1, and N265S in PB2 (DeBorde et al. 1987; Maassab 1967; Maassab et al. 1969; Snyder et al. 1988). The safety and efficacy of this vaccine strategy has been demonstrated in a variety of animal models including: in ovo, mouse, swine, ferret, and chicken (Cai et al. 2011; Hickman et al. 2008; Pena et al. 2011; Solórzano et al. 2010; Song et al. 2007). This strategy has also been shown to be effective in a variety of different backbones against many viruses including H5, H7, and pandemic H1N1 viruses. With each study, this strategy has been shown to complement the attenuation seen with the cold adapted mutations, making an attenuated virus safer and more effective while maintaining a virus that replicates and grows to WT levels (in MDCK cells and in chicken eggs) at low temperatures. Additionally, this strategy also serves as a Differentiating Infected from Vaccinated Animals (DIVA) vaccine, as the HA tag is easily detected and distinguished from WT virus by RT-PCR and western blot. As an added benefit, the tagged virus is stable through at least 10 passages in eggs and tissue culture cells (Cai et al. 2011; Hickman et al. 2008; Pena et al. 2011; Solórzano et al. 2010; Song et al. 2007).

With each backbone that has been modified to carry these mutations and the HA epitope tag (referred to from here on as an *att* backbone) and in every animal model, significant protection against challenge viruses has been shown as well as reduced viral shedding post-vaccination (depending on the dose, no viral shedding), seroconversion, and strong IgA and IgG responses. Studies in swine are particularly pertinent and encouraging when considering improvements to human live-attenuated influenza vaccines and swine vaccines. Swine immunized with an A/turkey/Ohio/313053/2004 (H3N2) modified backbone, ty/04 *att*, and pandemic H1N1surface (A/New York/18/2009 (H1N1)) and challenged with 10^5 $TCID_{50}$ of WT pandemic H1N1 showed IgG responses comparable to swine immunized with an inactivated adjuvanted A/California/2004 (pH1N1) virus (Pena et al. 2011). IgA responses in these groups, however, were significantly different. Swine vaccinated with the ty/04 *att* vaccine had significantly higher IgA antibodies than the group vaccinated with the inactivated virus (Loving et al. 2012, 2013; Pena et al. 2011).

Efficacy of the *att* strategy has also been shown in ovo, the most convenient time to vaccinate poultry, as the process can be automated. At 19 days old, hen eggs were vaccinated with an *att* vaccine possessing an A/chicken/Delaware/VIVA/2004 (H7N2) surface and an A/guinea fowl/Hong Kong/WF10/99 (H9N2) *att* backbone at 10^6 EID_{50}. Results showed a hatchability rate of about 91 % and a protection efficiency of 70–80 % (depending on age at challenge) of chickens

subsequently challenged with 500 chicken infectious dose 50 (CID_{50}) of WT H7N2 virus (Cai et al. 2011). Thus, this *att* strategy offers an effective means of vaccinating poultry while reducing labor costs and ensuring uniform vaccination of all eggs.

4 Improving Virus Vaccines: Rearrangement of the Influenza Genome and Use of Viral Vectors

Several lines of evidence have demonstrated that influenza viruses carrying a reorganized or rearranged genome might serve as candidate seed strains for vaccine development. All strategies are based upon our understanding of influenza virus packaging signals. These signals are comprised of the 5′ and 3′ non-coding regions as well as partial coding sequences on the 5′ and 3′ ends. Packaging signals have been shown to be essential for efficient and specific incorporation of each segment into virions (Dos Santos Afonso et al. 2005; Fujii et al. 2005; Liang et al. 2008; Marsh et al. 2007; Muramoto et al. 2006; Watanabe et al. 2003).

4.1 Influenza Viruses Containing an Alternate Number of Gene Segments

Many vaccine strategies have taken advantage of the influenza A packaging signals. One study describes the development of a 9-segment experimental vaccine strategy (Fig. 5a). The PB1 gene of PR8 (H1N1) virus was modified through the replacement of its packaging signals with those of NA (Gao et al. 2010). Synonymous mutations were introduced into the PB1 ORF to delete the original packaging signals while maintaining the amino acid sequence. Thus, in the absence of proper PB1 packaging signals, this chimeric PB1 gene must utilize the NA packaging signals. Then, utilizing the PB1 packaging signals, a ninth gene segment was introduced containing an HA ORF from another virus strain (A/HK/1/68 (H3N2)) flanked by the PB1 packaging signals. A bivalent influenza virus encoding both the H1 HA from PR8 (H1N1) virus and the H3 HA from A/HK/1/68 (H3N2) virus was successfully generated. Notably, this virus was significantly attenuated and exhibited decreased growth in eggs compared to the WT virus. Most importantly, mice inoculated with this virus survived lethal challenge with 100 MLD_{50} of recombinant PR8 virus and 33.3 MLD_{50} of X31 virus carrying A/HK/1/68 (H3N2) HA and NA segments (Gao et al. 2010). Using a similar method, the authors further showed that the ninth segment could also be incorporated into the progeny virus particles by the manipulation of specific packaging signals from PB2 or PA genes (Gao et al. 2010).

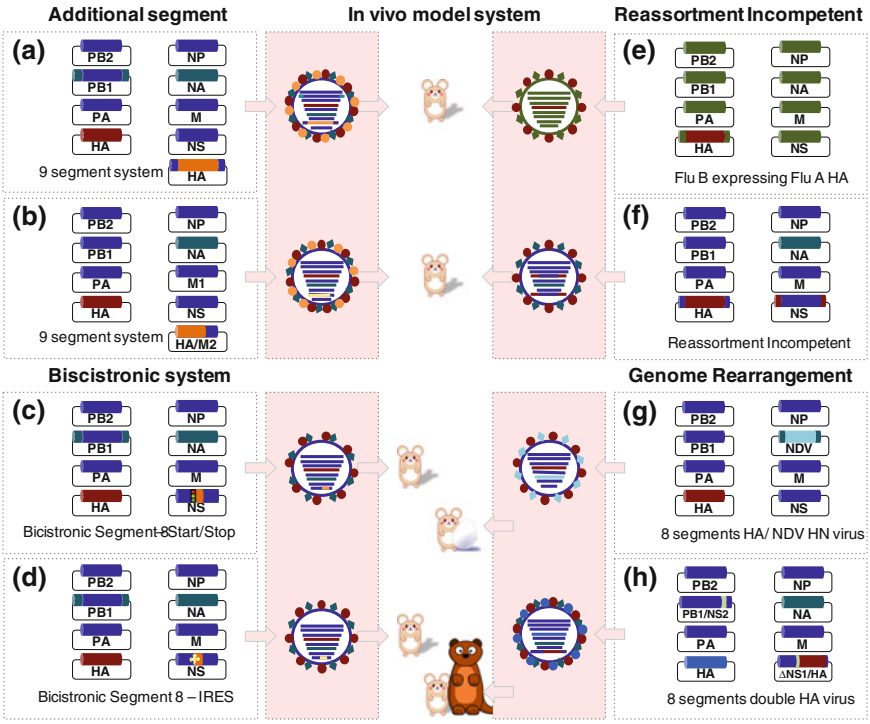

Fig. 5 Live attenuated approaches for influenza viruses—Other strategies. A variety of experimental LAIV strategies and the respective animal models in which each strategy has been evaluated are shown. **a** depicts the 9-segment bivalent strategy in which the PB1 packaging signals were substituted with NA packaging signals. The PB1 packaging signals were then engineered to flank a second HA segment. The second HA segment is shown in *orange*. **b** describes the 9-segment bivalent strategy in which segment 7, M, has been split into 2 plasmids, one carrying *M1* and the other *HA/M2*. **c** illustrates an 8-plasmid bicistronic strategy that makes use of a stop–start element in a truncated NS1 segment, shown as *red, yellow and green dots* (*stop–start signal*), and expresses a foreign gene, shown as an *orange rectangle*. **d** shows another bicistronic strategy that utilizes an IRES in the NS segment. **e** represents the influenza A-influenza B HA chimera strategy. The influenza A HA is shown in *dark red*. **f** depicts the reassortment incompetent strategy in which the HA and NS packaging signals have been swapped. **g** illustrates an influenza virus carrying an NDV gene, shown in *light blue*, in place of the NA ectodomain. **h** describes the double HA, bivalent vaccine cloned by rearranging the PB2 and NS segments and introducing a 2A protease. The 2A is depicted as a *white rectangle*. Based on (Gao et al. 2010; Garcia-Sastre et al. 1994a, b; Hai et al. 2011; Kittel et al. 2004, 2005; Pena et al. 2013; Wacheck et al. 2010; Wolschek et al. 2011; Wu et al. 2010)

To construct an H9N2 virus-based bivalent influenza vaccine expressing the HA antigen from two different subtypes, another study employed a different approach. First, M1 and M2 coding regions (A/Chicken/Jiangsu/11/2002 (H9N2)) were cloned separately into RG vector, pHW2000 (Fig. 5b). Subsequently, the extracellular domain of M2 protein (1–24 aa) was substituted with the antigenic sequences of HA1 (1–344 aa) from PR8 (Wu et al. 2010). The recombinant virus

produced by the co-transfection of modified M1/M2 constructs and 7 other RG plasmids (2 + 7) was shown to be genetically stable after 10 passages in chicken embryos or MDCK cells. In vivo studies demonstrated that this virus is low pathogenic in mice; furthermore, immunization of mice with this virus conferred complete protection against 100 MLD_{50} H1N1 challenge and 40 MLD_{50} H9N2 challenge.

4.2 Generation Influenza Vaccines Containing Bicistronic Expression Cassettes

Mounting an immune response sufficient to protect against influenza infection has been problematic in the elderly and other high-risk groups. However, the immunogenicity of live attenuated influenza vaccines can be improved through co-administration of biologically active molecules such as cytokines and chemokines (Babai et al. 1999, 2001), especially in the elderly (Mbawuike et al. 1990). This was achieved through the construction of a bicistronic mRNA in an influenza virus segment via multiple approaches, such as the insertion of a stop–start sequence (Kittel et al. 2005; Wolschek et al. 2011), an internal ribosome entry site element (IRES) (Garcia-Sastre et al. 1994a), an internal viral promoter (Machado et al. 2003), and protease cleavage sites (Kittel et al. 2004; Manicassamy et al. 2010; Percy et al. 1994). In most studies, GFP or a reporter (such as luciferase) was used to test the feasibility of the particular strategy.

The stop–start sequence implements an overlapping UAAUG sequence in which the first 3 nt serve as the stop codon terminating translation of the upstream gene while the last 3 nt reinitiate translation of the downstream gene (Fig. 5c). Thus, one mRNA template can produce two separate proteins. Using this method, Kittel et al. rescued a virus encoding truncated NS1 (1–125 aa) and full-length human IL-2 (Kittel et al. 2005). This IL-2 expressing virus was genetically stable in cell culture and the mouse respiratory tract. Additionally, it was safe in mice, despite the high replication capacity, and evoked an enhanced T cell response compared to the control virus (Kittel et al. 2005). This method is especially desirable for the production of immunomodulatory molecules because the expression level of the foreign gene from the stop–start sequence is relatively low. To date, recombinant influenza viruses expressing IL-2, IL-24, and CCL-20 have been successfully rescued (Wolschek et al. 2011). There is precedent for the enhancement of immunity by the co-administration of immunomodulatory molecules. A similar strategy using a DNA based bicistronic plasmid expressing an influenza virus NA protein and IL-2, separated by an internal ribosome entry site (IRES), has been show to enhance protection in mice against PR8 challenge (Henke et al. 2006).

Another approach to introduce exogenous genes into influenza virus involves the application of an IRES, as shown by a study performed by Garcia-Sastre et al.

(Fig. 5d). An IRES from the 5′ noncoding region of the human immunoglobulin heavy-chain-binding protein (BiP) mRNA was employed to promote the translation of foreign polypeptides (GP2 or HGP2) which were fused with an NA segment (Garcia-Sastre et al. 1994a). The major problem limiting the use of an IRES is the highly structured nature of the IRES sequence and size constraints. To date, only short polypeptides (91 and 125 aa) are reported to be expressed by the recombinant influenza virus via this method (Garcia-Sastre et al. 1994a).

Based on the finding that the influenza RNA polymerase is capable of recognizing and binding the internally located 3′ viral promoter (Flick and Hobom 1999), Machado et al. explored the possibility of rescuing a viable influenza virus harboring a dicistronic segment with a heterogenous gene under the control of an internal promoter (Machado et al. 2003). To this end, the authors inserted 19 nt derived from viral 3′ promoter sequence as well as foreign genes (chloramphenicol acetyltransferase (CAT) and VP0, from Mengo virus) between the stop codon and 5′ promoter sequence of the WSN NA segment. The transfected viruses replicated efficiently in MDCK cells and proved stable upon serial passage (Machado et al. 2003); nonetheless, they expressed low levels of foreign gene product (e.g., CAT enzyme), indicating that transcription from the internal promoter is inefficient. In a subsequent study, mice were immunized with these dicistronic viruses. Mice mounted an immune response against both the foreign gene and influenza, suggesting that this strategy may be a good LAIV candidate for a dual vaccine (Vieira Machado et al. 2006).

The introduction of an autocatalytic cleavage site (e.g, 2A protease from enterovirus) between a viral gene product and the desired foreign gene may be a more preferable strategy for construction of a recombinant influenza virus. The 2A strategy has two notable advantages. First, the autocatalytic cleavage site is usually short enough to allow for the insertion of long foreign genes. Second, after self-cleavage of 2A, only 18 additional amino acid residues remain at the C-terminus of first protein and 1 additional residue remains at the N-terminus of the second protein (Percy et al. 1994). These residues are not thought to dramatically affect protein function. To date, a number of publications have described the cloning of foreign genes into the influenza virus genome. GFP (Li et al. 2010) and CAT (Percy et al. 1994) have been cloned into NA. Gluc has been successfully cloned into PB2 (Heaton et al. 2013). The HIV Nef protein (Ferko et al. 2001) has been cloned into NS. Other genes have also been incorporated into the NS segment, such as the early secretory antigenic target protein (ESAT-6) from *M. tuberculosis* (Sereinig et al. 2006) and the H5 antigen from A/Vietnam/1203/04 virus (Pena et al. 2013). All strategies have generated replication competent viruses. In addition to the 2A protease, an even smaller peptide sequence, DIDGGETDG, can be recognized and cut by caspase, a cellular enzyme, and has been shown to provide posttranslational separation of recombinant segments which allows the expression of a foreign gene in its native form (just as 2A does) (Kittel et al. 2004).

4.3 Development of Influenza Vaccines Lacking the Ability to Reassort

In comparison to the inactivated vaccine, the intranasally delivered live-attenuated vaccine efficiently elicits both enhanced mucosal immunity and a broader cellular immune response even at low doses (as the above experiments have shown); nevertheless, concern does exist regarding the risk associated with a possible reassortment (or segment swapping) event between vaccine strain and circulating wild-type virus. This concern is perhaps minor when it comes to humans but it is a major issue that prevents the use of live-attenuated influenza vaccines in livestock and poultry.

Several strategies have been developed to address this safety concern. The first is based on the fact that influenza A and B viruses cannot exchange their genes freely to form a novel progeny virus (Ghate and Air 1999; Kaverin et al. 1983; Tanaka et al. 1984). To generate a reassortment-incompetent influenza vaccine, three different chimeric HA genes were constructed in which the signal peptide sequence, the transmembrane region, and cytoplasmic tail from the B/Yamagata/16/88 virus HA segment were fused to the HA ectodomain from an H1-subtype virus (PR8), an H3-subtype virus (A/Hong Kong/6 8(H3N2)), or an H5-subtype virus (A/Vietnam/1203/04 (H5N1)) (Fig. 5e) (Hai et al. 2011). The chimeric HA gene together with a truncated NS1 gene (1–110 aa) and six other intact genes from B/Yamagata/16/88 virus gave rise to a recombinant influenza B virus expressing influenza A HA protein. These chimeric viruses exhibited an attenuated phenotype both in vitro (small plaque size and reduced virus titer in MDCK cells) and in vivo (2 to 3 logs lower viral replication than wild- type influenza B virus in lungs of mice). The chimeras also retained the ability to induce robust antibody response and protected mice against lethal infection with H1 (challenge dose, 1×10^3 Pfu), H3 (challenge dose, 1×10^6 Pfu) or H5 viruses (challenge dose, 5×10^3 Pfu) (Hai et al. 2011). The major advantage of these influenza B virus-based vaccines against influenza A viruses is that they are unable to donate their HA to wild type influenza A viruses due to the restrictions imposed by the packaging signals.

Another strategy designed to avoid reassortment between vaccines and virulent strains is the application of "swapped" influenza A packaging signals. Packaging signals are specific and unique to each segment, both in length and sequence, as they include 5′ and 3′ noncoding regions as well as partial coding sequences at the 5′ and 3′ ends of each ORF (Goto et al. 2013; Hutchinson et al. 2010). In nature, each packaging signal can be used only once to ensure eight vRNA segments are accurately incorporated into the progeny virions. Thus, rewiring the influenza vRNAs by swapping the packaging signals could be a feasible approach in preventing reassortment of a specific vRNA segment. By cloning the 5′ and 3′ packaging sequences from NS gene onto the 5′ and 3′ ends of HA ORF and eliminating the original packaging elements in HA ORF by synonymous mutations, Gao et al. created a chimeric HA segment which must use the NS packaging

signals (Gao and Palese 2009). Conversely, an NS gene flanked by the HA packaging sequence was cloned using the same approach (Fig. 5f). As expected, the recombinant virus encompassing the chimeric HA and NS segments was unable to reassort freely with NS and HA segments from wild-type virus (Gao and Palese 2009). Such a strategy could be expanded to other segments.

Another strategy has shown to be an effective bivalent vaccine against influenza and Newcastle disease virus (NDV), a significant agricultural pathogen in chickens. In two separate studies, Park et al. and Steel et al. modified the NA gene by removing the ectodomain and replacing it with the hemagglutinin-neuraminidase (HN) gene of NDV and H5 avian influenza virus (lacking the polybasic cleavage site) (Park et al. 2006; Steel et al. 2008) (Fig. 5g). HN is the major antigenic protein of NDV. Park et al. demonstrated that the chimeric virus expressed the major antigenic protein of influenza, HA, and of NDV, HN. Additionally, the chimeric virus grew to high titers in eggs. Steel et al. showed efficacy of this vaccine in the mouse model and in ovo. Mice were immunized and boosted with the chimeric virus and challenged with 62.5 MLD_{50} of mouse adapted A/WSN/33 (H1N1) with an H5 surface (lacking the polybasic cleavage site). Post-vaccination, no signs of disease were observed in immunized mice. Post-challenge, all sham-vaccinated mice challenged, succumbed to infection, whereas all vaccinated mice survived with few signs of infection and little weight loss (Steel et al. 2008). The chimeric vaccine was then tested in ovo. Eighteen-day old chicken embryos were vaccinated. Then, at 3-weeks old, these chickens were challenged with either highly virulent NDV AT $10^{5.2}$ EID_{50} or highly pathogenic H5N1 virus at $10^{6.1}$ EID_{50}. Survivability was high for vaccinated chickens challenged with either virus, 80 % for those challenge with H5N1 and 90 % for those challenged with NDV. All sham-vaccinated chickens challenged with either H5N1 or NDV died. Thus, the chimeric virus provided strong protection after in ovo vaccination against lethal challenge (Steel et al. 2008).

Genome rearrangement was used to prevent reassortment and promote attenuation using a bivalent vaccine against both H9 and H5 viruses on an H9N2 backbone (Pena et al. 2013). First, a 2A protease gene and an NS2 gene were cloned at the end of segment 2 (PB1 gene), causing impaired polymerase activity as well as reduced viral replication. Next, a 2A protease gene and an H5 gene were cloned into truncated form of segment 8 (encoding 1–99 aa of NS1 protein) from which the NS2 gene was removed (Fig. 5h). The resulting virus had two HA proteins (H9 and H5) on its envelope. This double HA virus induced protection of mice (after immunization and boost) against lethal H5N1 challenge (Pena et al. 2013). In ferrets, the H5-H9 vaccine (and subsequent boost) provided full protection against H5 challenge (20, 200, and 2,000 MLD_{50}) and significantly reduced viral shedding (Pena et al. 2013). Previous studies showed that NS1 and NS2 are necessary for viral replication. Since these genes are in different segments, any reassortment event will have to encompass both segments (instead of just segments 2 or 8), rendering reassortment less likely. This strategy has the added benefit of being a viral vector. Here, the rearranged H9 virus serves as a vector for the H5

HA surface protein. One can imagine the insertion of other foreign proteins from influenza and other pathogens in this vector, or a similarly constructed vector, to create a dual vaccine.

5 Conclusions and Commentary

As discussed throughout this chapter, there is great need to alternative strategies to the licensed cold adapted, live-attenuated vaccine that provide better protection to all at-risk groups (Alexandrova et al. 1990; DeBorde et al. 1987, 1988; Maassab 1967; Maassab and Bryant 1999; Maassab et al. 1969; Monto and Maassab 1977). There is also great need for better agricultural vaccines and new methods to hasten vaccine production time of a vaccine seed stock during a pandemic. A plethora of candidate strategies exist for both human and agricultural vaccines as well as alternative strategies to the generation of a vaccine seed stock. Many of these strategies show strong efficacy. Indeed, there are many good experimental LAIV candidates when considering both *safety* and *efficacy*. These strategies are numerous but can be summarized into categories: mutations, deletions, insertions, and rearrangement.

Some of these strategies are already being evaluated in clinical trials such as the ΔNS1-H1N1 strategy. Impressively, this monovalent vaccine generated antibody responses, which were cross-reactive with heterosubtypic H3N2, and H5N1 virus. Other strategies, such as the elastase cleavage site strategy and the *att* (HA tag strategy) show great promise for safe and effective agricultural vaccines. Each strategy presents a unique approach to addressing some of the problems that exist in currently licensed vaccines such as immunogenicity in the elderly and the potential for reassortment. The right human strategy must be vetted in many different animal models, be applicable to both influenza A and B, grow to high titers in approved vaccine cell lines, provide safe and efficacious protection to high-risk groups, and be easily grown in large quantities. The right agricultural vaccines must be effective, safe, and most importantly, easy to administer.

Future work should focus on combining LAIV strategies. For example, the microRNA and the *att* strategy could be combined. Combining these strategies would likely enhance stability of the vaccine as well as add a DIVA element to the vaccine without dramatically decreasing replication. Such a vaccine has the potential of being applicable to agricultural animals and humans; furthermore, the selected miRNA could be tailored to the appropriate host without fear of recombination of the vaccine strain with a WT virus in a different host. Research should also be aimed at working within the confines of FDA approved growth medium or closely related growth medium. This will minimize the hurdles involved in seeking FDA approval and will allow manufacturers to use existing vaccine production infrastructure to produce new LAIV vaccines.

Research dollars must also continue to be invested in identifying potential epitopes for incorporation into rearranged genome or bicistronic vaccines in order

to more broadly stimulate the immune response to influenza and enhance cross-protection provided by a given vaccine, particularly in the elderly. These epitopes could be influenza derived or simply better stimulate the immune system as in the delivery of immunomodulatory molecules discussed in this chapter.

Again, while there is more research to be done, the field is advancing in such a way that it is only a matter of time before safer, more efficacious LAIV vaccines become available for administration to the public. Likely many of the vaccine strategies discussed in this chapter or some variation of the same themes will, in time, come to market. Meanwhile, the research continues.

References

Akarsu H, Iwatsuki-Horimoto K, Noda T, Kawakami E, Katsura H, Baudin F, Horimoto T, Kawaoka Y (2011) Structure-based design of NS2 mutants for attenuated influenza A virus vaccines. Virus Res 155:240–248. doi:10.1016/j.virusres.2010.10.014

Alexandrova GI, Maassab HF, Kendal AP, Medvedeva TE, Egorov AY, Klimov AI, Cox NJ (1990) Laboratory properties of cold-adapted influenza B live vaccine strains developed in the US and USSR, and their B/Ann Arbor/1/86 cold-adapted reassortant vaccine candidates. Vaccine 8:61–64

Babai I, Barenholz Y, Zakay-Rones Z, Greenbaum E, Samira S, Hayon I, Rochman M, Kedar E (2001) A novel liposomal influenza vaccine (INFLUSOME-VAC) containing hemagglutinin-neuraminidase and IL-2 or GM-CSF induces protective anti-neuraminidase antibodies cross-reacting with a wide spectrum of influenza A viral strains. Vaccine 20:505–515

Babai I, Samira S, Barenholz Y, Zakay-Rones Z, Kedar E (1999) A novel influenza subunit vaccine composed of liposome-encapsulated haemagglutinin/neuraminidase and IL-2 or GM-CSF. II. Induction of TH1 and TH2 responses in mice. Vaccine 17:1239–1250

Babiuk S, Masic A, Graham J, Neufeld J, van der Loop M, Copps J, Berhane Y, Pasick J, Potter A, Babiuk LA, Weingartl H, Zhou Y (2011) An elastase-dependent attenuated heterologous swine influenza virus protects against pandemic H1N1 2009 influenza challenge in swine. Vaccine 29:3118–3123. doi:10.1016/j.vaccine.2011.02.069

Brands R, Visser J, Medema J, Palache AM, van Scharrenburg GJ (1999) Influvac: a safe Madin Darby Canine Kidney (MDCK) cell culture-based influenza vaccine. Dev Biol Stand 98:93–100 (discussion 111)

Cai Y, Song H, Ye J, Shao H, Padmanabhan R, Sutton TC, Perez DR (2011) Improved hatchability and efficient protection after in ovo vaccination with live-attenuated H7N2 and H9N2 avian influenza viruses. Virol J 8:31. doi:10.1186/1743-422X-8-31

Calvo C, García-García ML, Borrell B, Pozo F, Casas I (2013) Prospective study of influenza C in hospitalized children. Pediatr Infect Dis J 32:916–919. doi:10.1097/INF.0b013e31828fca10

Calvo C, García-García ML, Centeno M, Pérez-Breña P, Casas I (2006) Influenza C virus infection in children, Spain. Emerg Infect Dis 12:1621–1622. doi:10.3201/eid1210.051170

Chambers TM, Quinlivan M, Sturgill T, Cullinane A, Horohov DW, Zamarin D, Arkins S, Garcia-Sastre A, Palese P (2009) Influenza A viruses with truncated NS1 as modified live virus vaccines: pilot studies of safety and efficacy in horses. Equine Vet J 41:87–92

Chen H, Ye J, Xu K, Angel M, Shao H, Ferrero A, Sutton T, Perez DR (2012) Partial and full PCR-based reverse genetics strategy for influenza viruses. PLoS One 7:e46378

Cohen M, Zhang XQ, Senaati HP, Chen HW, Varki NM, Schooley RT, Gagneux P (2013) Influenza A penetrates host mucus by cleaving sialic acids with neuraminidase. Virol J 10:321. doi:10.1186/1743-422X-10-321

de Wit E, Spronken MI, Vervaet G, Rimmelzwaan GF, Osterhaus AD, Fouchier RA (2007) A reverse-genetics system for influenza A virus using T7 RNA polymerase. J Gen Virol 88:1281–1287

DeBorde DC, Donabedian AM, Herlocher ML, Naeve CW, Maassab HF (1988) Sequence comparison of wild-type and cold-adapted B/Ann Arbor/1/66 influenza virus genes. Virology 163:429–443

DeBorde DC, Naeve CW, Herlocher ML, Maassab HF (1987) Nucleotide sequences of the PA and PB1 genes of B/Ann Arbor/1/66 virus: comparison with genes of B/Lee/40 and type A influenza viruses. Virus Res 8:33–41

Donelan NR, Dauber B, Wang X, Basler CF, Wolff T, García-Sastre A (2004) The N- and C-terminal domains of the NS1 protein of influenza B virus can independently inhibit IRF-3 and beta interferon promoter activation. J Virol 78:11574–11582. doi:10.1128/JVI.78.21.11574-11582.2004

Dormitzer PR, Suphaphiphat P, Gibson DG, Wentworth DE, Stockwell TB, Algire MA, Alperovich N, Barro M, Brown DM, Craig S, Dattilo BM, Denisova EA, De Souza I, Eickmann M, Dugan VG, Ferrari A, Gomila RC, Han L, Judge C, Mane S, Matrosovich M, Merryman C, Palladino G, Palmer GA, Spencer T, Strecker T, Trusheim H, Uhlendorff J, Wen Y, Yee AC, Zaveri J, Zhou B, Becker S, Donabedian A, Mason PW, Glass JI, Rappuoli R, Venter JC (2013) Synthetic generation of influenza vaccine viruses for rapid response to pandemics. Sci Transl Med 5:185ra68

Dos Santos Afonso E, Escriou N, Leclercq I, van der Werf S, Naffakh N (2005) The generation of recombinant influenza A viruses expressing a PB2 fusion protein requires the conservation of a packaging signal overlapping the coding and noncoding regions at the 5' end of the PB2 segment. Virology 341:34–46

Ferko B, Stasakova J, Sereinig S, Romanova J, Katinger D, Niebler B, Katinger H, Egorov A (2001) Hyperattenuated recombinant influenza A virus nonstructural-protein-encoding vectors induce human immunodeficiency virus type 1 Nef-specific systemic and mucosal immune responses in mice. J Virol 75:8899–8908

Flick R, Hobom G (1999) Transient bicistronic vRNA segments for indirect selection of recombinant influenza viruses. Virology 262:93–103

Fodor E, Devenish L, Engelhardt OG, Palese P, Brownlee GG, Garcia-Sastre A (1999) Rescue of influenza A virus from recombinant DNA. J Virol 73:9679–9682

Fujii K, Fujii Y, Noda T, Muramoto Y, Watanabe T, Takada A, Goto H, Horimoto T, Kawaoka Y (2005) Importance of both the coding and the segment-specific noncoding regions of the influenza A virus NS segment for its efficient incorporation into virions. J Virol 79:3766–3774

Fukuyama S, Kawaoka Y (2011) The pathogenesis of influenza virus infections: the contributions of virus and host factors. Curr Opin Immunol 23:481–486. doi:10.1016/j.coi.2011.07.016

Gao Q, Lowen AC, Wang TT, Palese P (2010) A nine-segment influenza a virus carrying subtype H1 and H3 hemagglutinins. J Virol 84:8062–8071

Gao Q, Palese P (2009) Rewiring the RNAs of influenza virus to prevent reassortment. Proc Natl Acad Sci USA 106:15891–15896

Garcia-Sastre A, Muster T, Barclay WS, Percy N, Palese P (1994a) Use of a mammalian internal ribosomal entry site element for expression of a foreign protein by a transfectant influenza virus. J Virol 68:6254–6261

Garcia-Sastre A, Percy N, Barclay W, Palese P (1994b) Introduction of foreign sequences into the genome of influenza A virus. Dev Biol Stand 82:237–246

Ghate AA, Air GM (1999) Influenza type B neuraminidase can replace the function of type A neuraminidase. Virology 264:265–277

Goto H, Muramoto Y, Noda T, Kawaoka Y (2013) The genome-packaging signal of the influenza A virus genome comprises a genome incorporation signal and a genome-bundling signal. J Virol 87:11316–11322. doi:10.1128/JVI.01301-13

Hai R, Garcia-Sastre A, Swayne DE, Palese P (2011) A reassortment-incompetent live attenuated influenza virus vaccine for protection against pandemic virus strains. J Virol 85:6832–6843

Hatta Y, Hatta M, Bilsel P, Neumann G, Kawaoka Y (2011) An M2 cytoplasmic tail mutant as a live attenuated influenza vaccine against pandemic (H1N1) 2009 influenza virus. Vaccine 29:2308–2312. doi:10.1016/j.vaccine.2011.01.023

Heaton NS, Leyva-Grado VH, Tan GS, Eggink D, Hai R, Palese P (2013) In vivo bioluminescent imaging of influenza a virus infection and characterization of novel cross-protective monoclonal antibodies. J Virol 87:8272–8281

Heix J, Grummt I (1995) Species specificity of transcription by RNA polymerase I. Curr Opin Genet Dev 5:652–656

Henke A, Rohland N, Zell R, Wutzler P (2006) Co-expression of interleukin-2 by a bicistronic plasmid increases the efficacy of DNA immunization to prevent influenza virus infections. Intervirology 49:249–252. doi:10.1159/000092487

Hickman D, Hossain MJ, Song H, Araya Y, Solórzano A, Perez DR (2008) An avian live attenuated master backbone for potential use in epidemic and pandemic influenza vaccines. J Gen Virol 89:2682–2690. doi:10.1099/vir.0.2008/004143-0

Hoffmann E, Neumann G, Kawaoka Y, Hobom G, Webster RG (2000) A DNA transfection system for generation of influenza A virus from eight plasmids. Proc Natl Acad Sci USA 97:6108–6113

Hutchinson EC, von Kirchbach JC, Gog JR, Digard P (2010) Genome packaging in influenza A virus. J Gen Virol 91:313–328. doi:10.1099/vir.0.017608-0

Kadota J (2013) Emergence of a new strain of the avian influenza A (H7N9) virus. Respir Investig 51:49. doi:10.1016/j.resinv.2013.05.001

Kaverin NV, Varich NL, Sklyanskaya EI, Amvrosieva TV, Petrik J, Vovk TC (1983) Studies on heterotypic interference between influenza A and B viruses: a differential inhibition of the synthesis of viral proteins and RNAs. J Gen Virol 64(Pt 10):2139–2146

Kistner O, Barrett PN, Mundt W, Reiter M, Schober-Bendixen S, Dorner F (1998) Development of a mammalian cell (Vero) derived candidate influenza virus vaccine. Vaccine 16:960–968

Kistner O, Barrett PN, Mundt W, Reiter M, Schober-Bendixen S, Eder G, Dorner F (1999) Development of a vero cell-derived influenza whole virus vaccine. Dev Biol Stand 98:101–110 (discussion 111)

Kittel C, Ferko B, Kurz M, Voglauer R, Sereinig S, Romanova J, Stiegler G, Katinger H, Egorov A (2005) Generation of an influenza A virus vector expressing biologically active human interleukin-2 from the NS gene segment. J Virol 79:10672–10677

Kittel C, Sereinig S, Ferko B, Stasakova J, Romanova J, Wolkerstorfer A, Katinger H, Egorov A (2004) Rescue of influenza virus expressing GFP from the NS1 reading frame. Virology 324:67–73

Langlois RA, Albrecht RA, Kimble B, Sutton T, Shapiro JS, Finch C, Angel M, Chua MA, Gonzalez-Reiche AS, Xu K, Perez D, García-Sastre A, Tenoever BR (2013) MicroRNA-based strategy to mitigate the risk of gain-of-function influenza studies. Nat Biotechnol. doi:10.1038/nbt.2666

Leibler JH, Otte J, Roland-Holst D, Pfeiffer DU, Soares Magalhaes R, Rushton J, Graham JP, Silbergeld EK (2009) Industrial food animal production and global health risks: exploring the ecosystems and economics of avian influenza. Ecohealth 6:58–70. doi:10.1007/s10393-009-0226-0

Li F, Feng L, Pan W, Dong Z, Li C, Sun C, Chen L (2010) Generation of replication-competent recombinant influenza A viruses carrying a reporter gene harbored in the neuraminidase segment. J Virol 84:12075–12081

Liang Y, Huang T, Ly H, Parslow TG (2008) Mutational analyses of packaging signals in influenza virus PA, PB1, and PB2 genomic RNA segments. J Virol 82:229–236

Loving CL, Lager KM, Vincent AL, Brockmeier SL, Gauger PC, Anderson TK, Kitikoon P, Perez DR, Kehrli ME Jr (2013) Efficacy in pigs of inactivated and live attenuated influenza virus vaccines against infection and transmission of an emerging H3N2 similar to the 2011–2012 H3N2v. J Virol 87:9895–9903. doi:10.1128/JVI.01038-13

Loving CL, Vincent AL, Pena L, Perez DR (2012) Heightened adaptive immune responses following vaccination with a temperature-sensitive, live-attenuated influenza virus compared

to adjuvanted, whole-inactivated virus in pigs. Vaccine 30:5830–5838. doi:10.1016/j.vaccine.2012.07.033
Maassab HF (1967) Adaptation and growth characteristics of influenza virus at 25 °C. Nature 213:612–614
Maassab HF, Bryant ML (1999) The development of live attenuated cold-adapted influenza virus vaccine for humans. Rev Med Virol 9:237–244
Maassab HF, Francis T, Davenport FM, Hennessy AV, Minuse E, Anderson G (1969) Laboratory and clinical characteristics of attenuated strains of influenza virus. Bull World Health Organ 41:589–594
Machado AV, Naffakh N, van der Werf S, Escriou N (2003) Expression of a foreign gene by stable recombinant influenza viruses harboring a dicistronic genomic segment with an internal promoter. Virology 313:235–249
Manicassamy B, Manicassamy S, Belicha-Villanueva A, Pisanelli G, Pulendran B, Garcia-Sastre A (2010) Analysis of in vivo dynamics of influenza virus infection in mice using a GFP reporter virus. Proc Natl Acad Sci USA 107:11531–11536
Marsh GA, Hatami R, Palese P (2007) Specific residues of the influenza A virus hemagglutinin viral RNA are important for efficient packaging into budding virions. J Virol 81:9727–9736
Masic A, Babiuk LA, Zhou Y (2009a) Reverse genetics-generated elastase-dependent swine influenza viruses are attenuated in pigs. J Gen Virol 90:375–385. doi:10.1099/vir.0.005447-0
Masic A, Booth JS, Mutwiri GK, Babiuk LA, Zhou Y (2009b) Elastase-dependent live attenuated swine influenza A viruses are immunogenic and confer protection against swine influenza A virus infection in pigs. J Virol 83:10198–10210. doi:10.1128/JVI.00926-09
Masic A, Lu X, Li J, Mutwiri GK, Babiuk LA, Brown EG, Zhou Y (2010) Immunogenicity and protective efficacy of an elastase-dependent live attenuated swine influenza virus vaccine administered intranasally in pigs. Vaccine 28:7098–7108. doi:10.1016/j.vaccine.2010.08.003
Massin P, Rodrigues P, Marasescu M, van der Werf S, Naffakh N (2005) Cloning of the chicken RNA polymerase I promoter and use for reverse genetics of influenza A viruses in avian cells. J Virol 79:13811–13816
Mbawuike IN, Wyde PR, Anderson PM (1990) Enhancement of the protective efficacy of inactivated influenza A virus vaccine in aged mice by IL-2 liposomes. Vaccine 8:347–352
Monto AS, Maassab HF (1977) Use of influenza vaccine in non-high risk populations. Dev Biol Stand 39:329–335
Morokutti A, Muster T, Ferko B (2014) Intranasal vaccination with a replication-deficient influenza virus induces heterosubtypic neutralising mucosal IgA antibodies in humans. Vaccine. doi:10.1016/j.vaccine.2014.02.009
Murakami S, Horimoto T, Yamada S, Kakugawa S, Goto H, Kawaoka Y (2008) Establishment of canine RNA polymerase I-driven reverse genetics for influenza A virus: its application for H5N1 vaccine production. J Virol 82:1605–1609
Muramoto Y, Takada A, Fujii K, Noda T, Iwatsuki-Horimoto K, Watanabe S, Horimoto T, Kida H, Kawaoka Y (2006) Hierarchy among viral RNA (vRNA) segments in their role in vRNA incorporation into influenza A virions. J Virol 80:2318–2325
Neumann G, Fujii K, Kino Y, Kawaoka Y (2005) An improved reverse genetics system for influenza A virus generation and its implications for vaccine production. Proc Natl Acad Sci USA 102:16825–16829
Neumann G, Watanabe T, Ito H, Watanabe S, Goto H, Gao P, Hughes M, Perez DR, Donis R, Hoffmann E, Hobom G, Kawaoka Y (1999) Generation of influenza A viruses entirely from cloned cDNAs. Proc Nat Acad Sci USA 96:9345–9350
Ozawa M, Goto H, Horimoto T, Kawaoka Y (2007) An adenovirus vector-mediated reverse genetics system for influenza A virus generation. J Virol 81:9556–9559
Palache AM, Scheepers HS, de Regt V, van Ewijk P, Baljet M, Brands R, van Scharrenburg GJ (1999) Safety, reactogenicity and immunogenicity of Madin Darby Canine Kidney cell-derived inactivated influenza subunit vaccine. A meta-analysis of clinical studies. Dev Biol Stand 98:115–125 (discussion 133–4)

Palese P, Shaw ML (2007) Orthomyxoviridae: the viruses and their replication. In: Knipe DM, Howley PM (eds) Fields virology, vol 2, 5th edn. Philadelphia, Lippincott Williams and Wilkins, pp 1647–1689

Park MS, Steel J, Garcia-Sastre A, Swayne D, Palese P (2006) Engineered viral vaccine constructs with dual specificity: avian influenza and Newcastle disease. Proc Natl Acad Sci USA 103:8203–8208. doi:10.1073/pnas.0602566103

Pena L, Sutton T, Chockalingam A, Kumar S, Angel M, Shao H, Chen H, Li W, Perez DR (2013) Influenza viruses with rearranged genomes as live-attenuated vaccines. J Virol 87:5118–5127

Pena L, Vincent AL, Ye J, Ciacci-Zanella JR, Angel M, Lorusso A, Gauger PC, Janke BH, Loving CL, Perez DR (2011) Modifications in the polymerase genes of a swine-like triple-reassortant influenza virus to generate live attenuated vaccines against 2009 pandemic H1N1 viruses. J Virol 85:456–469. doi:10.1128/JVI.01503-10

Percy N, Barclay WS, Garcia-Sastre A, Palese P (1994) Expression of a foreign protein by influenza A virus. J Virol 68:4486–4492

Perez JT, Pham AM, Lorini MH, Chua MA, Steel J, tenOever BR (2009) MicroRNA-mediated species-specific attenuation of influenza A virus. Nat Biotechnol 27:572–576. doi:10.1038/nbt.1542

Pica N, Langlois RA, Krammer F, Margine I, Palese P (2012) NS1-truncated live attenuated virus vaccine provides robust protection to aged mice from viral challenge. J Virol 86:10293–10301. doi:10.1128/JVI.01131-12

Schnitzler SU, Schnitzler P (2009) An update on swine-origin influenza virus A/H1N1: a review. Virus Genes 39:279–292. doi:10.1007/s11262-009-0404-8

Sereinig S, Stukova M, Zabolotnyh N, Ferko B, Kittel C, Romanova J, Vinogradova T, Katinger H, Kiselev O, Egorov A (2006) Influenza virus NS vectors expressing the mycobacterium tuberculosis ESAT-6 protein induce CD4+ Th1 immune response and protect animals against tuberculosis challenge. Clin Vaccine Immunol 13:898–904

Shinya K, Fujii Y, Ito H, Ito T, Kawaoka Y (2004) Characterization of a neuraminidase-deficient influenza a virus as a potential gene delivery vector and a live vaccine. J Virol 78:3083–3088

Skehel JJ, Wiley DC (2000) Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. Annu Rev Biochem 69:531–569. doi:10.1146/annurev.biochem.69.1.531

Snyder MH, Betts RF, DeBorde D, Tierney EL, Clements ML, Herrington D, Sears SD, Dolin R, Maassab HF, Murphy BR (1988) Four viral genes independently contribute to attenuation of live influenza A/Ann Arbor/6/60 (H2N2) cold-adapted reassortant virus vaccines. J Virol 62:488–495

Solórzano A, Webby RJ, Lager KM, Janke BH, García-Sastre A, Richt JA (2005) Mutations in the NS1 protein of swine influenza virus impair anti-interferon activity and confer attenuation in pigs. J Virol 79:7535–7543. doi:10.1128/JVI.79.12.7535-7543.2005

Solórzano A, Ye J, Pérez DR (2010) Alternative live-attenuated influenza vaccines based on modifications in the polymerase genes protect against epidemic and pandemic flu. J Virol 84:4587–4596. doi:10.1128/JVI.00101-10

Song H, Nieto GR, Perez DR (2007) A new generation of modified live-attenuated avian influenza viruses using a two-strategy combination as potential vaccine candidates. J Virol 81:9238–9248. doi: 10.1128/JVI.00893-07

Song MS, Baek YH, Pascua PN, Kwon HI, Park SJ, Kim EH, Lim GJ, Choi YK (2013) Establishment of Vero cell RNA polymerase I-driven reverse genetics for Influenza A virus and its application for pandemic (H1N1) 2009 influenza virus vaccine production. J Gen Virol 94:1230–1235

Stech J, Garn H, Herwig A, Stech O, Dauber B, Wolff T, Mettenleiter TC, Klenk HD (2011) Influenza B virus with modified hemagglutinin cleavage site as a novel attenuated live vaccine. J Infect Dis 204:1483–1490. doi: 10.1093/infdis/jir613

Stech J, Garn H, Wegmann M, Wagner R, Klenk HD (2005) A new approach to an influenza live vaccine: modification of the cleavage site of hemagglutinin. Nat Med 11:683–689. doi:10.1038/nm1256

Steel J, Burmakina SV, Thomas C, Spackman E, Garcia-Sastre A, Swayne DE, Palese P (2008) A combination in-ovo vaccine for avian influenza virus and Newcastle disease virus. Vaccine 26:522–531. doi:10.1016/j.vaccine.2007.11.032

Steel J, Lowen AC, Pena L, Angel M, Solórzano A, Albrecht R, Perez DR, García-Sastre A, Palese P (2009) Live attenuated influenza viruses containing NS1 truncations as vaccine candidates against H5N1 highly pathogenic avian influenza. J Virol 83:1742–1753. doi:10.1128/JVI.01920-08

Tanaka T, Urabe M, Goto H, Tobita K (1984) Isolation and preliminary characterization of a highly cytolytic influenza B virus variant with an aberrant NS gene. Virology 135:515–523

Taubenberger JK, Kash JC (2010) Influenza virus evolution, host adaptation, and pandemic formation. Cell Host Microbe 7:440–451. doi:10.1016/j.chom.2010.05.009

tenOever BR (2013) RNA viruses and the host microRNA machinery. Nat Rev Microbiol 11:169–180. doi:10.1038/nrmicro2971

Verity EE, Camuglia S, Agius CT, Ong C, Shaw R, Barr I, Middleton D, Rockman S (2011) Rapid generation of pandemic influenza virus vaccine candidate strains using synthetic DNA. Influenza Other Respi Viruses 6:101–109

Victor ST, Watanabe S, Katsura H, Ozawa M, Kawaoka Y (2012) A replication-incompetent PB2-knockout influenza A virus vaccine vector. J Virol 86:4123–4128. doi:10.1128/JVI.06232-11

Vieira Machado A, Naffakh N, Gerbaud S, van der Werf S, Escriou N (2006) Recombinant influenza A viruses harboring optimized dicistronic NA segment with an extended native 5' terminal sequence: induction of heterospecific B and T cell responses in mice. Virology 345:73–87. doi:10.1016/j.virol.2005.09.050

Wacheck V, Egorov A, Groiss F, Pfeiffer A, Fuereder T, Hoeflmayer D, Kundi M, Popow-Kraupp T, Redlberger-Fritz M, Mueller CA, Cinatl J, Michaelis M, Geiler J, Bergmann M, Romanova J, Roethl E, Morokutti A, Wolschek M, Ferko B, Seipelt J, Dick-Gudenus R, Muster T (2010) A novel type of influenza vaccine: safety and immunogenicity of replication-deficient influenza virus created by deletion of the interferon antagonist NS1. J Infect Dis 201:354–362. doi:10.1086/649428

Wang Z, Duke GM (2007) Cloning of the canine RNA polymerase I promoter and establishment of reverse genetics for influenza A and B in MDCK cells. Virol J 4:102

Watanabe S, Imai M, Ohara Y, Odagiri T (2003) Influenza B virus BM2 protein is transported through the trans-Golgi network as an integral membrane protein. J Virol 77:10630–10637

Watanabe S, Watanabe T, Kawaoka Y (2009) Influenza A virus lacking M2 protein as a live attenuated vaccine. J Virol 83:5947–5950. doi:10.1128/JVI.00450-09

Watanabe T, Watanabe S, Kim JH, Hatta M, Kawaoka Y (2008) Novel approach to the development of effective H5N1 influenza A virus vaccines: use of M2 cytoplasmic tail mutants. J Virol 82:2486–2492. doi:10.1128/JVI.01899-07

Wolschek M, Samm E, Seper H, Sturlan S, Kuznetsova I, Schwager C, Khassidov A, Kittel C, Muster T, Egorov A, Bergmann M (2011) Establishment of a chimeric, replication-deficient influenza A virus vector by modulation of splicing efficiency. J Virol 85:2469–2473

Wood JM, Robertson JS (2004) From lethal virus to life-saving vaccine: developing inactivated vaccines for pandemic influenza. Nat Rev Microbiol 2:842–847

Wressnigg N, Shurygina AP, Wolff T, Redlberger-Fritz M, Popow-Kraupp T, Muster T, Egorov A, Kittel C (2009) Influenza B mutant viruses with truncated NS1 proteins grow efficiently in vero cells and are immunogenic in mice. J Gen Virol 90:366–374. doi:10.1099/vir.0.006122-0

Wu R, Guan Y, Yang Z, Chen J, Wang H, Chen Q, Sui Z, Fang F, Chen Z (2010) A live bivalent influenza vaccine based on a H9N2 virus strain. Vaccine 28:673–680

Zhang X, Kong W, Ashraf S, Curtiss R 3rd (2009) A one-plasmid system to generate influenza virus in cultured chicken cells for potential use in influenza vaccine. J Virol 83:9296–9303