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Development and applications of single-cycle infectious Influenza A Virus (scilAV)

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Abstract

The diverse host range, high transmissibility, and rapid evolution of influenza A viruses justify the importance of containing pathogenic viruses studied in the laboratory. Other than physically or mechanically changing influenza A virus containment procedures, modifying the virus to only replicate for a single round of infection similarly ensures safety and consequently decreases the level of biosafety containment required to study highly pathogenic members in the virus family. This biological containment is more ideal because it is less apt to computer, machine, or human error. With many necessary proteins that can be deleted, generation of single-cycle infectious influenza A viruses (scilAV) can be achieved using a variety of approaches. Here, we review the recent burst in scilAV generation and summarize the applications and findings on this important human pathogen using biocontained viral mimics.

Keywords

Reporter virus; viral vectors; single-cycle virus; influenza vaccine; influenza HA-expressing MDCK cells (MDCK-HA); reverse genetics

1. Introduction

1.1 Influenza viruses as important human pathogens

Influenza virus infections cause both seasonal epidemics and occasional pandemics and remain an enormous clinical and public health problem worldwide. The financial burden in the United States (US) averages more than 87 billion dollars annually, due to prophylactic and therapeutic costs, hospital costs, and missed school or work days (Gasparini et al., 2012; Keech and Beardsworth, 2008; Molinari et al., 2007; Paul Glezen et al., 2013). Influenza viruses belong to the family *Orthomyxoviridae* of which there are six genera, or types: Influenza virus A, Influenza virus B, Influenza virus C, Isavirus, Quarantavirus and Thogotovirus (International Committee on Taxonomy of Viruses. and King, 2012; Shaw and Palese, 2013). Type A and B influenza viruses infect humans regularly, however, only type

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A is believed to possess pandemic potential. The ability to cause pandemics is due to the natural host reservoir of influenza A virus (IAV), wild aquatic waterfowl, and the host's ability to reassort divergent influenza A strains into novel pandemic viruses (Glezen, 1996; Taubenberger and Morens, 2009; Webster et al., 1992). IAV is further classified into different subtypes based on the antigenic major surface glycoproteins: hemagglutinin (HA; 18 subtypes) and neuraminidase (NA; 11 subtypes) (Shaw and Palese, 2013; Tong et al., 2012; Tong et al., 2013). Influenza virus infections are primarily spread by person-to-person transmission via aerosolized droplets. Additionally, zoonoses can occur, as evidenced by the current outbreaks of H7N9 and H5N1, which may contain pandemic potential if acquiring the ability to readily transmit between humans (Uyeki and Cox, 2013; Yen and Webster, 2009). In the 20th century, three documented IAV pandemics have occurred: the Spanish flu (H1N1) of 1918, the Asian flu (H2N2) of 1957, and the Hong Kong flu (H3N2) of 1968 (Kilbourne, 2006; Knipe and Howley, 2013). Of these three, the Spanish flu (1918) was especially fatal and was associated with approximately 50 million deaths worldwide (Taubenberger and Morens, 2006). The first influenza pandemic of the 21st century was declared in 2009 after the emergence of a quadruple-reassortant swine-origin H1N1 virus (Smith et al., 2009) that in less than one year infected more than 600,000 individuals worldwide, causing nearly 16,000 deaths in over 200 countries (CDC, 2009; WHO, 2010).

Current available options to counter the respiratory system attack by influenza viruses include both vaccines and antivirals. Vaccines, due to the induction of sterilizing immunity, are the primary means to prevent infection. However, the three types of vaccines available (inactivated influenza vaccine, IIV; live attenuated influenza vaccine, LAIV; and recombinant influenza vaccine, RIV) have moderate efficacy that changes seasonally due to constant viral evolution (Carrat and Flahault, 2007). Recently, the Advisory Committee on Immunization Practices (ACIP) recommended that each vaccine should be available in quadrivalent formulations, containing two strains of type A (H1N1 and H3N2) and two lineages of type B (Victoria and Yamagata) influenza viruses (Grohskopf et al., 2014). Options to control influenza after infection are limited to two classes of US Food and Drug Administration (FDA)-approved antivirals, targeting either the viral matrix 2 (M2) ion channel (amantadine, rimantidine) (Hay et al., 1985) and subsequently inhibiting viral entry, or the sialidase active site of NA (oseltamivir, zanamivir) (Gubareva et al., 2000) and inhibiting virus release. Similar to the challenges of vaccination, viral mutations can arise that render influenza antivirals ineffective, like in the case of M2 blockers, which are no longer recommended against circulating strains (Pizzorno et al., 2011). Despite our best efforts to prevent (vaccines) and treat (antivirals) infections, influenza annually accounts for upwards of approximately 49,000 deaths in the US and 500,000 worldwide (CDC, 2010; WHO, 2014b). Improved strategies preventing the morbidity and mortality associated with influenza virus infections are warranted, and studies aimed to better understand the biology of and develop new countermeasures against this important human pathogen are desperately needed.

1.2 Influenza virus biology

Influenza viruses contain a segmented single-stranded RNA genome of negative polarity that is packed within spherical or filamentous, lipid-enveloped particles (Shaw and Palese,

2013). The eight RNA segments of IAV encode at least 12 proteins (Table 1), using alternative splicing (nuclear export protein, NEP; and M2), alternative open reading frames (PB1-F2) (Chen et al., 2001), or ribosomal frame-shifting (PA-X) (Jagger et al., 2012) mechanisms.

Infection with IAV begins when HA binds to its cellular receptor, sialic acid (SA)-linked glycoproteins containing SA in alpha 2,3 or alpha 2,6 linkages (Skehel and Wiley, 2000). Following endocytosis, the viral HA undergoes a conformational change, exposing a fusion peptide that inserts into the endosomal membrane and facilitating virion-endosome fusion (Harrison, 2015; Skehel and Wiley, 2000). Next, the proton-conducting M2 ion channel promotes the cation-mediated release of the viral ribonucleoprotein (vRNP) complexes from the virion core to the cell cytoplasm (Pinto et al., 1992; Wise et al., 2012). The vRNPs then translocate to the nucleus, where viral replication and transcription takes place, a unique mechanism compared to other negative-sense, single-stranded, RNA viruses (Lamb and Choppin, 1983).

Influenza negative-sense viral RNA (vRNA) is not recognized by host cell polymerases, thus viral genome replication and transcription requires the virus-encoded RNA-dependent RNA polymerase (RdRp) (Neumann et al., 2004). The influenza RdRp is a trimeric complex consisting of polymerase acidic (PA) and basic 1 and 2 (PB1, PB2) proteins that together with nucleoprotein (NP) are the minimal components for viral replication and transcription (Huang et al., 1990). The IAV vRNA segments are flanked at both termini by non-coding regions (NCRs) that serve as promoters to initiate transcription by the viral polymerase (Flick et al., 1996; Fodor et al., 1995). To coordinate movement of newly synthesized vRNPs from the nucleus to the site of viral release at the plasma membrane of infected cells, NEP and M1 are required, though the mechanism by which eight unique vRNAs are packaged into each budding virion is not completely understood, and is discussed further below (Hutchinson et al., 2010; Rossman and Lamb, 2011). Once assembled at the cellular membrane, nascent viral particles are released from infected cells by the receptor-destroying enzymatic activity of NA (Varghese et al., 1992).

The segmented genome of IAV provides an evolutionary advantage granting the ability to reassort or exchange homologous gene segments between different influenza strains of the same type. Reassortment can allow for co-circulating H1N1 and H3N2 strains to explore fitness landscapes, which can additionally result in antigenic shift (Laver and Webster, 1979; Rambaut et al., 2008). In fact, the emergence of pandemic IAV strains often is the result of reassortment events, as occurred with the quadruple- reassortant swine-origin H1N1 virus in 2009 (Garten et al., 2009). In addition to antigenic shift, influenza viruses can accumulate mutations within antigenic sites of the viral glycoproteins (antigenic drift) (Carrat and Flahault, 2007; Laver et al., 1979; Sandbulte et al., 2011), leading to resistance against existing antivirals or neutralizing antibodies (NAb) that were generated against the original strain (de Jong et al., 2005; Hurt et al., 2009; Krammer and Palese, 2015; Monto et al., 2006).

1.3 Decreasing the biosafety level of containment is beneficial for influenza virus research

Highly virulent IAV have the potential to pose a greater threat to human health than many other pathogens classified as biosafety level (BSL)-3 and BSL-4 agents, due to efficient transmission and limited antiviral therapeutic options (Sandbulte et al., 2011). While efficient human infection of highly pathogenic avian IAV H5N1 is rare, the case fatality rate is high (Fiebig et al., 2011; Horimoto and Kawaoka, 2001; WHO, 2014a). Recent laboratory studies suggest that although transmission of wild isolates is limited, adaptation of avian influenza (H5N1) to acquire aerosol transmissibility can be achieved in ferrets and that just a few mutations in HA are required (Herfst et al., 2012; Imai et al., 2012). The 2013 outbreak of H7N9 influenza in China, responsible for at least 571 human infections and 212 deaths, has reaffirmed the importance of surveillance against these threats (WHO, 2015). Therefore, when novel IAV are introduced into humans, rapid analysis of their virulence would be beneficial to better determine public health impact and the immediacy of implementing countermeasures. Concomitantly, screening current antiviral options against newly emerged viruses, while evaluating novel antivirals for prophylaxis against circulating influenza viruses, is of great importance (Hayden and de Jong, 2011).

Repeat infection with antigenically identical influenza viruses is rare in immunocompetent humans because prior exposure can result in the generation of NAbs. Most NAbs target IAV HA and prevent receptor binding or fusion. Common serologic assays to detect IAV Abs or NAbs targeting the viral HA are hemagglutination inhibition (HI) or virus microneutralization (MN) assays (2003; de Jong et al., 2003; Rowe et al., 1999; WHO, 2011). These assays have traditionally necessitated the manipulation of live-replicating viruses, though inactivated viruses can be used for HI (WHO, 2011). These assays can be used to evaluate potential antivirals that target HA receptor binding, such as HI, or steps in the viral life cycle, such as MN. The HI assay is problematic for detecting NAbs against avian influenza HA due to difficulty with interpreting results, as was found with avian H5 (Beare and Webster, 1991; Rowe et al., 1999; Stephenson et al., 2003). Some improvement in interpreting HI results can be achieved by using horse erythrocytes (Stephenson et al., 2003; WHO, 2013). New methods that overcome the safety concerns that accompany using live forms of IAV are therefore warranted. New approaches should be at least as sensitive as current available techniques while decreasing biosafety concerns associated with highly pathogenic IAV.

1.4 Single-cycle infectious viruses

Single-cycle viruses are defective for one or more essential functions that can be involved in viral genome synthesis, assembly and release of viral particles, or re-infection of new host cells (Dudek and Knipe, 2006). These defective viruses are typically generated in complementing cell lines that provide the missing gene product or its function *in trans*. While permissive infection of normal cells is possible, no infectious viral progeny will be produced. Genetically abrogating one or more steps in the virus life cycle makes these defective viruses very safe, highlighting their potential to serve as effective laboratory tools. Single-cycle virus technology has been broadly used with IAV and also with other viral pathogens (Evans et al., 2005; Gao and Palese, 2009; Hai et al., 2011; Jia et al., 2009; Rodrigo et al., 2011; Suzuki et al., 2009), which we will not discuss in this review. The

continued use and improvement of single-cycle viruses is evidence of their utility in laboratory settings and as potential vaccine candidates.

High-throughput screening (HTS) has been performed using single-cycle viruses by including a reporter gene as a valid surrogate of viral infection (Beylerveld et al., 2013; Hertzberg and Pope, 2000; Rodrigo et al., 2011). For example, screening drugs to identify antivirals, and investigating cellular host protein interactions important for viral replication have also been accomplished utilizing single-cycle reporter-expressing viruses (Beylerveld et al., 2013). Single-cycle viruses have also proven useful to evaluate virus-host interactions and host immune responses during a single round of infection (Hirsch, 2010; Konig et al., 2010). Moreover, since blocking one or more steps in the viral life cycle makes these viruses replication defective, they have the potential to serve as safe vaccine candidates or vaccine vectors, which may prove to be advantageous over classical viral immunization approaches (Dudek and Knipe, 2006). Lastly, single-cycle infectious viruses can also serve as novel delivery vectors modified to express cellular modulators like interleukins or small non-coding RNAs (Chua et al., 2013; Dudek and Knipe, 2006).

2. Single-cycle infectious influenza A viruses (scilAV)

2.1 Influenza plasmid-based reverse genetics techniques

Plasmid-based reverse genetics allows for the simultaneous expression of the IAV RdRp and negative-stranded genome in transiently transfected mammalian cells, which together generate *de novo*, or rescue, recombinant IAVs (Shaw and Palese, 2013). Luytjes et al, described a system that allowed the use of recombinant DNA technology to modify the genome of IAV and to engineer vectors for the expression of foreign genes (Luytjes et al., 1989). Recombinant RNA was expressed from a plasmid, and transfected together with purified IAV polymerase proteins in the presence of a helper virus. Then the recombinant RNA was expressed and packaged into virus particles.

Originally established in 1999, IAV reverse genetics that do not rely on “helper viruses” have revolutionized the influenza research field (Fodor et al., 1999; Neumann et al., 1999), leading to advances in virus biology, vaccines, and therapeutics (Neumann and Kawaoka, 2002; Neumann et al., 2002; Ye et al., 2014). The initial description of IAV reverse genetics required the use of 12 plasmids to rescue recombinant influenza viruses: four protein expression plasmids for vRNP reconstitution plus eight vRNA expression plasmids for the eight genomic vRNA segments. However, it was later described that only eight plasmids were needed, where an ambisense coding strategy generates both polymerase I-driven vRNA transcription and polymerase II-driven mRNA transcription from the same plasmid (Hoffmann et al., 2000a; Hoffmann et al., 2000b).

2.2 Importance of packaging signals for the development of scilAV

The vRNA segments of IAV share a common organization: a long central coding region, sometimes encoding more than one major transcript (Table 1), flanked by relatively short NCRs (Shaw and Palese, 2013). This genome segmentation confers evolutionary advantages, but also poses a problem for the virus. Genetic evidence indicates that IAV virions normally incorporate exactly eight vRNAs, and at least one copy of each of the eight

viral vRNAs must be packaged within a single nascent virion to create productively infectious viral progeny (Chou et al., 2012; Gavazzi et al., 2013b; Hutchinson et al., 2010). If a packaging network exists, selective incorporation of the eight vRNAs requires a minimum of seven inter-segment interactions that could, in principle, be mediated by RNA-binding proteins that interconnect two vRNA segments. However, to date, no such cellular or viral protein has been identified, leaving direct vRNA-vRNA interactions within defined vRNA packaging sequences as an alternative hypothesis supported by *in vitro* observations (Essere et al., 2013; Fournier et al., 2012a; Fournier et al., 2012b; Gavazzi et al., 2013a). Electron microscopy and electron cryotomography of various IAV strains revealed that seven vRNAs are organized around a central segment forming a star-like structure (Essere et al., 2013; Fournier et al., 2012a; Fournier et al., 2012b; Gavazzi et al., 2013a). Surprisingly, evidence suggests that this interaction network, and more specifically, the regions and domains of the vRNAs involved in specific intermolecular interactions is not conserved between H3N2 and H5N2 IAV, suggesting that different vRNA interactions may drive selective packaging across IAV subtypes (Fournier et al., 2012a; Gavazzi et al., 2013a). These studies support previous evidence that conserved codons are important for vRNA packaging in H1N1 IAVs A/WSN/33 (WSN) but not in A/Puerto Rico/8/34 (PR8), indicating that some packaging signals are not conserved even among IAVs of the same subtype (Gog et al., 2007; Marsh et al., 2008).

To generate single-cycle infectious influenza A virus (sciIAV), an essential viral gene segment is removed, which then needs to be provided *in trans* to support viral propagation. However, early attempts to generate seven-segmented IAVs yielded phenotypes with reduced replicative fitness, even when the protein encoded by the deleted gene was provided *in trans* (Marsh et al., 2007). These findings suggested that an intact vRNA packaging network may be important for optimal virus fitness. Engineered IAV segments containing the 3' and 5' NCRs were replicated and transcribed by the viral RdRp but were not incorporated during budding to reconstitute an eight-segmented virus (Luytjes et al., 1989; Noda and Kawaoka, 2010). Incorporation of foreign gene segments into influenza virions would therefore need to hijack the proposed packaging signals.

Reverse genetics helped to identify packaging signals by flanking reporter transcripts with varying lengths of IAV vRNA segments. Packaging signals were first determined for NA (Fujii et al., 2003) by engineering constructs encoding green fluorescent protein (GFP) flanked by the 3' NCR and 183 nucleotides (nt) of the 3' NA; and the 5' NCR and 157 nt of the 5' NA, which were successfully incorporated into budding virions. Similar approaches corroborated the existence of packaging signals for the 7 remaining IAV vRNA segments (Dos Santos Afonso et al., 2005; Fujii et al., 2005; Fujii et al., 2009; Fujii et al., 2003; Gog et al., 2007; Hutchinson et al., 2010; Liang et al., 2005; Marsh et al., 2007; Marsh et al., 2008; Muramoto et al., 2006; Watanabe et al., 2003) (Table 1). Defective interfering (DI) particles generated from vRNA segments during IAV replication gave further evidence of the presence of segment-specific packaging signals within approximately 100 to 300 nt from each end of the vRNAs (Hutchinson et al., 2010). Sequence analysis further supported this hypothesis by determining that the most conserved codons are found to accumulate at the termini of each viral segment (Gog et al., 2007; Hutchinson et al., 2008; Marsh et al., 2008).

Using the incorporation of GFP flanked by varying lengths of vRNA termini, it was found that not all vRNA segments have equivalent proportions of their length dedicated to virion packaging (Table 1). In these studies, the packaging signals were mapped within either PR8 or WSN by providing the deleted gene, or function thereof, *in trans* (Fujii et al., 2009; Fujii et al., 2003; Gao et al., 2012; Hutchinson et al., 2010; Liang et al., 2005; Marsh et al., 2007; Muramoto et al., 2006; Ozawa et al., 2009). However, the packaging signal domains were not completely conserved between these two H1N1 strains, and were even more disparate between different IAV subtypes.

Nucleotide sequences at IAV segment termini have been recently suggested to have two functions, one to control an individual segment's incorporation (packaging signal), and another to orchestrate an entire eight-segmented packaging event (bundling signal) (Goto et al., 2013). Specific vRNA-vRNA interactions have been mapped to these same terminal locations, via *in vitro* transcribed vRNA plus native agarose gel electrophoresis, suggesting a role for these interactions in packaging (Fournier et al., 2012a; Fournier et al., 2012b; Gavazzi et al., 2013a) and potentially serving as reassortment barriers between IAV subtypes (Essere et al., 2013). Furthermore, incompatible packaging signals between IAV and influenza B virus (IBV) are likely to be the bottleneck that prevents intertypic reassortment (Baker et al., 2014). Whether or not similar packaging signals in IBV are present in each vRNA segment, and whether the organization of the eight vRNA segments is similar to that found in IAV, remains to be evaluated.

2.3 Generation of protein-expressing cell lines

Several methods have been described for transfection-based generation of viral protein-expressing cell lines (e.g., calcium phosphate, cationic lipids, and electroporation), allowing for the transient or stable expression of proteins in diverse cell lines. In this review, we will focus on a brief overview of the generation of Madin-Darby canine kidney (MDCK) cell lines that express IAV HA (MDCK-HA) (Fig. 1A). Membrane-bound HA readily complements sciIAV lacking HA (HA) (described in more detail below), and the antigenicity of the HA carried by the cell transfers to the pseudotyped particles produced during budding (Martinez-Sobrido et al., 2010). MDCK-HA cells were generated by transfection with either an HA protein expression plasmid, modified to also encode for a hygromycin resistance gene inserted downstream of an additional IRES element (Marsh et al., 2007), or co-transfected with HA and a hygromycin B resistance vector (Martinez-Sobrido et al., 2010). After transfection, cells are seeded at a low density so that hygromycin-resistant cells can be clonally isolated. To screen for homogenous and functional HA expression between cell clones, the ability to complement sciIAV HA expressing GFP is evaluated at a low multiplicity of infection (MOI), where there are approximately 1,000 cells per infectious particle at the start of the experiment (Fig. 1A). The observable spread of GFP by fluorescence microscopy and concomitant titer increase of infectious or hemagglutinating units (HAU) in tissue culture supernatants qualitatively and quantitatively indicates the functionality of the MDCK-HA cells. To observe the relative quantity and uniformity of HA expression in MDCK-HA clones, immunofluorescence (IFA) or flow cytometry assays that incorporate anti-HA specific Abs are used (Fig. 1A). Analyzing both the percentage and functionality of HA expression in MDCK-HA clones

ensures sciIAV HA growth phenotypes that recapitulate wild type (WT) IAV replication kinetics, provided that the heterotypic HA is compatible with the sciIAV backbone. We have described here one method to generate MDCK stable cell lines, but additional methods that are more suitable for various species or cell types may also be used (Bussow, 2015; Lanza et al., 2013). Moreover, transgenic animals expressing complementing IAV proteins in tissues could also be used, similar to those expressing fluorescent proteins in specific cell types (Abe and Fujimori, 2013). For instance, Shih *et al.* described the generation of transgenic mice expressing the HA from IAV PR8 in different tissues, including lungs (Shih et al., 1997). It is thus feasible to infect these mice with a sciIAV HA, which should be complemented and subsequently propagate *in vivo* but not be transmissible except to identical transgenic mice.

2.4 Generation of sciIAV

IAV requires the presence of eight vRNA segments for efficient virus fitness. Therefore, to reconstitute the full genome when generating sciIAV, the modified gene segment should encode for a *trans* gene flanked by the packaging signals and NCRs of the native segment. For example, sciIAV HA was generated by using GFP flanked by the 3' NCR and 45 nt of 3' HA, and the 5' NCR and 80 nt of 5' HA (Fig. 1B). Compared with other non-infectious or single-cycle infectious virus vectors, sciIAV allows for the expression of foreign genes with nt lengths that are, at least, similar to those of the segments replaced (Dudek and Knipe, 2006), although the size limit for the foreign sequence has not been empirically defined for each gene segment. This represents an advantage over the generation of replication competent reporter-expressing influenza viruses that are limited by nt length insertion (Nogales et al., 2015; Tran et al., 2013). There are numerous sciIAV that differ in the gene deleted, the foreign sequence introduced, as well as the viral backbone used, which to date has been limited to H1N1 viruses WSN, PR8, A/California/04/2009 (pH1N1), and A/Swine/Saskatchewan/18789/02 (SW02) and H3N2 X31 (Table 2). Whether or not different packaging signals need to be used to generate non-H1N1 sciIAVs remain to be evaluated. And this could be a limitation to generate sciIAV using other backbones or genes deleted. In the last decade, this viral system has experienced a notable increase in use, exemplifying their versatility, ease of production, and utility for multiple *in vitro* and *in vivo* applications.

2.4.1 sciIAV HA—To study IAV assembly and the packaging of the HA segment into infectious virions, Marsh *et al.* rescued a sciIAV with the WSN or PR8 backbones and without the WT HA vRNA (sciIAV HA) (Marsh et al., 2007). The modified IAV HA vRNA segment incorporated reporter genes, GFP or RFP, flanked by 45 and 80 nt in the 3' and 5' end of the vRNA, respectively (Fig. 2A) (Marsh et al., 2007). The packaging signals in the 3' end differed from the 9 nt required as previously described for WSN (Watanabe et al., 2003). This sciIAV HA could be efficiently passaged in cells constitutively expressing WSN-HA protein; however, and as expected, it could not spread in parental MDCK cells (Fig. 2B). Upon further investigation, it was found that although an IAV containing seven segments could be rescued, the replication was reduced compared to a virus whose missing segment was replaced with a recombinant reporter segment (containing HA packaging signals) that restored the eight-segmented IAV (Marsh et al., 2007). The seven-segmented HA virus resulted in a 40 to 60% reduction in the packaging of the PA, NP, NA, M, and

NS vRNAs, as measured by quantitative PCR, and the packaging of these vRNAs was partially restored in the presence of GFP/RFP packaging constructs.

Martínez-Sobrido *et al.* demonstrated the application of sciIAV HA for the screening of NAbs against H1 or H5 influenza virus, including the highly pathogenic Spanish flu (A/Brevig Mission/18 [H1N1]) and Bird Flu (A/Vietnam/1203/04 [H5N1]) (Martínez-Sobrido *et al.*, 2010). Because the plasma membrane-bound HA from MDCK-HA cells is incorporated into budding sciIAV, progeny virions from infected MDCK-HA (A/Brevig Mission/18 or A/Vietnam/1203/04) bear the antigenicity of the cellular-expressed virus protein. Therefore, sciIAV HA could be HA-pseudotyped with different IAV HAs by propagation in different MDCK-HA cells (Martínez-Sobrido *et al.*, 2010) (Fig. 3). This antigenicity was demonstrated using monoclonal NAbs, and importantly, these neutralization assays could be conducted at BSL-2 conditions, even when evaluating NAbs against highly pathogenic IAVs (Martínez-Sobrido *et al.*, 2010).

To compare the replication kinetics of sciIAV HA with WT virus, it was first demonstrated that WT virus grew to similar titers in parental and MDCK-HA cells, whereas sciIAV replicated to high titers only in MDCK-HA and failed to replicate in parental MDCK cells (Marsh *et al.*, 2007; Martínez-Sobrido *et al.*, 2010) (Fig. 2B). At the protein level, sciIAV has a similar composition as WT virus, with the exception of the levels of HA that appear to be slightly reduced (Martínez-Sobrido *et al.*, 2010). Electron microscopy analysis of sciIAV and WT virus revealed that the sciIAVs HA have morphology and particle size similar to those of the WT WSN virus (Martínez-Sobrido *et al.*, 2010) (Fig. 2C). Importantly, the vRNA composition of the HA-deficient sciIAV was different than that of WT IAV because the GFP vRNA-like segment was incorporated into viral particles instead of the IAV HA vRNA (Fig. 2D).

Recently, our group has generated GFP-expressing sciIAV HA on the backbone of, pH1N1, X31, and PR8 (Baker *et al.*, 2013; Baker *et al.*, 2014; Guo *et al.*, 2014). The pH1N1 sciIAV HA was used as a proof of concept for the development of safe alternative vaccines to prevent IAV infection. Powell *et al.* generated pH1N1 and PR8-backbone sciIAV HA by suppressing the HA signal sequence and replacing the original ATG start codon with TAG to suppress translation (Powell *et al.*, 2012). A single base at the end of the signal sequence at nucleotide position 83 was removed to ensure that if the original ATG was reconstituted, it would read out of frame. Additionally, this sciIAV HA contained an inactivated HA cleavage site, and the resulting sciIAV was shown to only propagate in MDCK-HA cells. This recombinant virus lost virulence in mice but could still induce heterotypic protection, which lasted for at least four months post-vaccination. Kawaoka's group also demonstrated the ability of sciIAV HA to serve as a safe vaccine by mutating the HA cleavage site, so that the HA1 C-terminal arginine residue was replaced with threonine, rendering host cell proteases incapable of cleaving HA1-HA2 and eliminating infectivity of progeny virus (Katsura *et al.*, 2012).

2.4.2 sciIAV NA—IAV NA glycoprotein is required for virus release during budding, and deleting the gene would be expected to limit IAV to a single cycle of infection. Reporter-expressing sciIAVs NA were developed as vaccine candidates (Shinya *et al.*, 2004), to

evaluate viral attachment to the host cell (Rimmelzwaan et al., 2007), rate of co-infections (Bodewes et al., 2012), as a tool for NAb screening (Rimmelzwaan et al., 2011), or for inhibition during intertypic infection (Wanitchang et al., 2012). Although recombinant sciIAV lacking NA enzymatic activity has similar growth kinetics as WT virus in the presence of exogenous *Vibrio cholerae* sialidase, sciIAV NA grows with reduced replication levels compared with WT virus lacking enzyme supplementation, thus it can be considered a pseudo-sciIAV (our unpublished results) (Fujii et al., 2003; Inagaki et al., 2012). An alternative to complement sciIAV NA viruses has been reported, which is based on the use of modified MDCK cells that express reduced levels of sialic acid on the cell surface (Shinya et al., 2004).

2.4.3 sciIAV PB2—SciIAVs can also be generated by deleting the PB2 gene (sciIAV

PB2) and providing the missing protein *in trans* by generating PB2-expressing stable cell lines (Ozawa et al., 2011). Foreign reporter genes (GFP, DsRed, and renilla or firefly luciferases) were introduced between the NCR and packaging signals of the PR8 PB2 segment. The sciIAV PB2 grows in PB2-expressing cells with similar kinetics compared to WT PR8 (Inagaki et al., 2012; Ozawa et al., 2011). Importantly, the stability of the foreign reporter gene is maintained over 5 serial passages. Reporter-expressing sciIAV PB2 that contained heterologous HA and NA genes from pH1N1 were used to screen for NAb reactivity in convalescent ferret sera (Ozawa et al., 2011). The potential of sciIAV PB2 as a vaccine candidate against influenza was also assessed in mice (Victor et al., 2012). Interestingly, Abs against GFP (expressed in place of PB2) were also detected in vaccinated mouse sera, suggesting the possibility of using PB2-deficient sciIAV as vectors to deliver antigens from disparate pathogens. Indeed, this hypothesis was proven correct when three separate reports detailed bivalent protection conferred by sciIAV PB2 viruses that harbored either heterologous IAV HA, human parainfluenza virus type 3 (HPIV-3) hemagglutinin-neuraminidase (HN), or respiratory syncytial virus (RSV) F glycoprotein sequences instead of the PB2 vRNA segment (Fonseca et al., 2014; Kobayashi et al., 2013; Uraki et al., 2013). Besides inducing Abs against the heterologous immunogens, both PB2-deficient sciIAVs retained their protection efficacy against lethal IAV challenge.

2.4.4 sciIAV PB1—To study the fitness of oseltamivir-resistant IAV mutants, Bloom *et al.* generated a recombinant WSN virus possessing PB1 segments in which most of the coding sequence was replaced by a gene encoding either GFP or mCherry flanked by the PB1 NCRs and packaging signals (Bloom et al., 2010). The PB1 segment was also modified to mutate the ATG start codon of PB1 and destroy the alternative start codon normally leading to PB1-F2 transcription. The WSN sciIAV PB1 grew to high titers in cells constitutively expressing the PB1 protein. The authors used this virus to show that viruses containing the NA H274Y mutation, which alone reduced cell surface expression of NA, evolved compensatory mutations that increased NA surface expression (Bloom et al., 2011). More recently, by utilizing the same sciIAV PB1 platform, the discovery of a new mutation in NA (G147R) was reported that provided a gain-of-function phenotype to the N1-NA protein. The protein acquired the receptor-binding function normally performed by HA (Hooper and Bloom, 2013; Hooper et al., 2015). Interestingly, even though this mutation was generated in the laboratory, it has also been found in several recent H1N1 and

H5N1 natural isolates, suggesting that IAV could potentially switch the receptor-binding function between its two viral glycoproteins (Hooper and Bloom, 2013).

3. SciIAV applications

3.1 Identification of neutralizing antibodies (NAbs)

Abs that neutralize IAV typically bind to HA, and NAb induction serves as a surrogate for seroconversion following infection or vaccination in humans (Belshe et al., 2000; He et al., 2015). New interest has arisen in the identification of monoclonal NAbs due to their therapeutic benefit, with either specific or broad (predominantly HA stalk- reactive) spectrum activity against IAV (see (Laursen and Wilson, 2013) for review). Traditional HI and MN or plaque reduction neutralization test (PRNT) assays used to identify NAbs have various advantages and limitations. The HI assay provides a rapid read-out (same day), but sensitivity is limited because of the large amount of virus required to agglutinate erythrocytes (10^5 – 10^6 egg infectious dose₅₀ (Killian, 2008) and the Abs inhibiting the HI does not necessarily neutralize the virus infection. In addition, HI assays are hampered by inconsistencies between interpretation of results across research personnel (Stephenson et al., 2007), an inability to discriminate between non- infectious and infectious viruses in viral preparations, improper sera preparation to deactivate non-specific virus inhibitors, and restriction of only being able to identify Abs that directly interfere with the binding of HA to its SA receptor on red blood cells (Hirst, 1942; Salk, 1944). Comparatively, MN or PRNT is advantageous because of its increased sensitivity over HI (only 100 – 200 egg infectious doses 50% are required; (Rimmelzwaan et al., 1998). However, the assays are disadvantageous with long protocol times, with results taking several days (2–4 days) when evaluating direct cytopathic effect (CPE). However, new MN methods that combine tissue culture cells and antigen detection by enzyme-linked immunosorbent assay (ELISA) can yield results within 2 days and are more sensitive. However, inter-laboratory discrepancies can arise due to variable assay endpoints such as cell staining by IFA, ELISA or HI assays (Stephenson et al., 2007; WHO, 2011).

To overcome limitations, such as biosafety and the requirement of secondary assays, posed by traditional NAb identification approaches, self-limiting, reporter- expressing sciIAVs are a feasible alternative (Fig. 4A). HA-pseudotyped lentivirus particles expressing reporter genes have been experimentally used to evaluate influenza NAb generation (Nefkens et al., 2007). However, HA-pseudotyped lentivirus particles lack IAV internal proteins, and the altered stoichiometry of HA protein on the surface of the lentiviral particles can produce variability in defining NAbs, including stalk- reactive NAbs (Sui et al., 2009; Temperton et al., 2007). As an alternative to the use of WT IAV or HA-pseudotyped lentivirus particles, we and others have used sciIAV in reporter-based MN assays, which (i) can be performed with BSL-2 containment, (ii) are highly sensitive (100 – 200 FFU required), (iii) use a reporter protein to identify infected cells and eliminate the need for secondary assays while providing quantitative results that limit the error of interpretation, and (iv) can be completed within 24 hours (Hooper and Bloom, 2013; Martinez-Sobrido et al., 2010; Ozawa et al., 2011; Ramon et al., 2014; Rimmelzwaan et al., 2011). In addition, sciIAV represents a

bonafide IAV and therefore can be inhibited at any step of the viral life cycle, including the use of non-HA NAb (e.g. NA NAb).

Martínez-Sobrido *et al.* first described the use of a GFP-expressing sciIAV HA as a viable surrogate to evaluate NAb against different IAVs, including highly pathogenic IAVs (Martínez-Sobrido *et al.*, 2010). The study demonstrated that both monoclonal NAb and polyclonal human sera could be evaluated, and showed comparable, and possibly more sensitive, results than traditional HI assays. In further support, Rimmelzwaan *et al.* used sciIAV NA to evaluate human, rabbit, ferret, sheep and swan sera, and demonstrated that sciIAV NA produces results comparable to the classic MN and HI assay (Rimmelzwaan *et al.*, 2011). Likewise, Ozawa *et al.* showed that both GFP fluorescence and renilla or firefly luciferase luminescence reporter proteins can be used to quantify sciIAV PB2 infection, and that luminescence signals can be used to identify NAb from ferret sera with high sensitivity (Ozawa *et al.*, 2011). Additionally, Hooper and Bloom explored the effect of oseltamivir on neutralization of GFP-expressing sciIAV PB1, when HA had reduced affinity for sialic acid linkages and NA could bind receptor (Hooper and Bloom, 2013). Through these four gene-deficient examples, sciIAV use in immunoassays was demonstrated to have distinct advantages for the evaluation of NAb as compared to the use of replication-competent influenza viruses or pseudotyped lentivirus.

When comparing various sciIAVs for use in NAb screening, sciIAV HA is advantageous because the backbone of the virus only requires one rescue, and the virus can then be pseudotyped with a variety of IAV or IBV HAs by propagation on HA-expressing MDCK cells (Baker *et al.*, 2014; Martínez-Sobrido *et al.*, 2010). The sciIAV HA is also advantageous in that two identical sciIAV varying only in their reporter gene could be quickly pseudotyped with HA to develop flexible multivalent screening assays (Baker *et al.*, 2015). Additionally, the HA cell lines generated could theoretically be used in a virus-free ELISA to determine HA-binding NAb with monoclonal or polyclonal samples (our unpublished results). Drawbacks of using sciIAV HA include nonspecific NAb reactivity to the cell line (MDCK-HA) on which the assay is performed. The Ab can bind to HA expressed on MDCK-HA cells, instead of the viral particles. Since pseudotyped sciIAV is incubated with putative NAb, and the mixture is then added to MDCK-HA cells to allow for multicycle infection and reporter gene expression, there is the possibility of the binding of the Ab to the HA expressed from the cell line. This can be controlled by using irrelevant MDCK-HA cell substrates for infection or parental MDCK cells, although this would decrease the sensitivity of the assay because a higher MOI is needed to enhance signal intensity (Ozawa *et al.*, 2011). Also, in terms of evaluating NAb against newly emerging viruses, one must either quickly generate an MDCK-HA cell line or transiently transfect large batches of cells to generate stocks of novel pseudotyped sciIAV HA. Transfection efficiencies may, however, be a limiting factor for pseudotype virus production and increase the variability between the viral stocks produced. Therefore, although the generation of stable cell lines take more time, it is highly recommendable.

Other gene-deficient sciIAVs are also ideal for NAb identification. However, sciIAV NA are somewhat replication competent (Inagaki *et al.*, 2012; Shinya *et al.*, 2004) (our unpublished results), and the stoichiometry of HA on the virion may be inaccurate in the

absence of NA. Conversely, sciIAV PB2 or PB1 are limited to a single cycle of replication and have appropriate HA and NA gene expression in infected cells, which should lead to accurate surface glycoprotein stoichiometry. All PB2, PB1, and NA sciIAVs would require *de novo* rescue to change the HA antigenicity, which is disadvantageous to screen against diverse isolates. Reporter-based NAb screening using sciIAV can also be utilized to identify stalk-reactive broad spectrum NAb (Baker et al., 2015), which would be advantageous over plaque reduction neutralization assays (Lee et al., 2012). Although sciIAV has been used only to evaluate the presence of NAb, showing a comparable sensitivity to traditional assays, we cannot discard their limitations to detect other inhibitory Abs, such as those that prevent viral egress. However, given that sciIAV mirrored the viral replication of WT viruses when infecting *trans*-complementing cell lines, other kinds of inhibitory Abs could also be detected using sciIAV, although this hypothesis needs to be experimentally evaluated. In the future, the sciIAV platform could be adapted for high-throughput screening (HTS) identification of influenza NAb.

3.2 HTS to identify host factors or compounds affecting IAV replication

The development of new antiviral strategies against IAV would be beneficial to combat the uniform viral resistance in circulating strains to adamantanes (M2 blockers) and previous widespread resistance to NA inhibitors (Bright et al., 2006; de Jong et al., 2005; Garten et al., 2009; Lackenby et al., 2008; Shiraishi et al., 2003). Identifying new viral or host targets for antiviral development would be ideal, since no virus has been described yet to gain resistance against such targets, like the viral RdRp and/or NP (Kao et al., 2010; Su et al., 2010). Current and traditional technologies to detect antivirals against IAV have been extensively reviewed elsewhere (Beyleveld et al., 2013). The following section will focus on the application and limitations of sciIAVs for the screening of new antivirals.

Due to the extremely large size of compound libraries, HTS approaches are the most ideal way to test for viral inhibition, and these approaches commonly employ a reporter protein that is quantifiable with optimal z-score (Hertzberg and Pope, 2000). IAV HTS assays have been reported to identify compounds that inhibit viral NA activity, NS1 function, and host factors that inhibit influenza viral replication and transcription using siRNA libraries (Beyleveld et al., 2013). A major drawback of using single-cycle or WT IAV for drug screens is that specialized cells need to be used that either complement the single-cycle deficiency or that encode for a reporter protein activated by the IAV RdRp, respectively. This consideration is important if host-specific pathways are inadequately recapitulated in the specialized cell lines. Replication-competent, reporter-expressing IAV may be more desirable in this regard, but special safety requirements may need to be considered when using IAV strains that contain genes from highly pathogenic isolates.

The major advantages of using sciIAV to screen for influenza antivirals is their versatility to support multiple reporter genes, which are compatible with HTS (Hertzberg and Pope, 2000) (Fig. 4A). Moreover the use of BSL-2 facilities for screening bypasses the limitations associated with containment when working with highly pathogenic IAV strains. Bottini *et al.* recently used renilla luciferase-expressing sciIAV HA to screen for antivirals in MDCK-HA cells (Bottini et al., 2012). The authors used an HTS approach to evaluate a

library consisting of approximately 14,000 chemical compounds, resulting in the identification of one antiviral (compound 7). The hit was validated by characterizing NP mRNA expression by quantitative real-time PCR in MDCK-HA or A549 cells infected with the sciIAV or WT viruses, respectively. Interestingly, the compound identified by this approach showed promise when it also protected mice against WT IAV challenge. However, the specific function or target of this class of antiviral compounds has yet to be determined. These results provide evidence that application of sciIAV in HTS assays for the detection of novel antiviral compounds is a promising approach capable of identifying antivirals that are active in *in vitro* and *in vivo* models of IAV infection (Fig. 4A).

An HTS approach to identify cellular proteins involved in the IAV life cycle was taken by using a library of small interfering RNA that targets the host cell transcriptome prior to reporter-expressing sciIAV infection (Hirsch, 2010; Konig et al., 2010) (Fig. 4A). Here, Konig *et al.* used sciIAV HA expressing renilla luciferase and a genome-wide RNA interference (RNAi) library to identify key cellular factors required for successful replication of IAV. Using this approach, 295 cellular host factors were found that are required for IAV replication in human lung epithelial (A549) cells, a non-complementing cell line. Reporter-expressing sciIAV was beneficial to initially screen the library consisting of small interfering RNAs that together target 19,000 human genes. The rate of false-positives was acceptable, as 219 of the 295 genes targeted also affected WT virus replication over multiple cycles. It is important to consider that sciIAV in HTS screens can be used to identify targets that affect a single round of replication, which may not be identifiable during multiple rounds of infection due to signal saturation. Perhaps targeting genes that redundantly affect a single-cycle infection may prove advantageous in inhibiting multi-round infection.

3.3 Influenza vaccines and vaccine vectors

Currently available non-replicating virus vaccines (IIV and RIV) induce moderate humoral immunity that depends on the immunologic fitness of the recipient. Elderly and immunocompromised individuals typically have reduced responses compared to young healthy individuals (DiazGranados et al., 2014). IIV and RIV also afford limited protection during years where the vaccine formulation does not match circulating strains, although the vaccines may still offer some level of cross-reactivity (Ohmit et al., 2006; Osterholm et al., 2012; Treanor et al., 2007). On the other hand, LAIV, which replicates slowly due to its temperature-sensitive (*ts*), cold-adapted (*ca*) and attenuated (*att*) phenotype, generates more robust humoral and cellular immunity (Maassab and Bryant, 1999). Due to safety concerns in the young and asthmatics, and limited efficacy in the elderly, LAIV is only recommended for immunocompetent, non-asthmatic patients between the ages of 2–49 (Ambrose et al., 2012; Belshe et al., 2008; Powers et al., 1991). One study has found that combining inactivated and LAIVs is well-tolerated and efficacious in elderly individuals (Tang, 2012; Treanor and Betts, 1998). Therefore, there is great interest and need to improve the immunogenicity of IIV and RIV or the safety of LAIV, and implement new vaccination strategies that prevent infection in susceptible populations.

The safety of sciIAV was shown in a mouse model of influenza infection by delivering virus intranasally that did not produce overt signs of illness, including weight loss, ruffling of the

fur, and hunching (Baker et al., 2013; Katsura et al., 2012; Powell et al., 2012; Shinya et al., 2004; Uraki et al., 2013; Victor et al., 2012). Recent characterization of an LAIV mouse model (Cox et al., 2015) was able to demonstrate mortality at high doses of LAIV, which has not yet been described for any sciIAV, suggesting that sciIAV is more safe than LAIV. Intranasal immunization is a desirable delivery method to prevent infection with IAV because it leads to the generation of a mucosal immune response, creating an immune barrier at the site of potential infection (Kohlmeier and Woodland, 2009). Indeed, sciIAV priming elicits not only a robust systemic humoral response but also a mucosal immune response, as anti-influenza IgG and IgA Abs were found in nasal washes and bronchial alveolar lavage (Katsura et al., 2012; Victor et al., 2012). Furthermore, sciIAV vaccination protected mice against homologous challenge for up to 4 months after vaccination (Powell et al., 2012) (Fig. 4B). Similar to infection with WT IAV, sciIAV immunization also leads to recruitment of influenza-specific CD8 T cells to the lungs (Baker et al., 2013; Guo et al., 2014; Katsura et al., 2012; Powell et al., 2012; Uraki et al., 2013), which is likely to be the main contributor of immunity against heterologous influenza challenge (Baker et al., 2013; Guo et al., 2014). Two doses (prime and boost) of sciIAV are required to elicit sterilizing immunity, as shown by an absence of challenge virus recovered from animals (Baker et al., 2013; Katsura et al., 2012; Powell et al., 2012; Victor et al., 2012). However, one dose of sciIAV HA is sufficient to prevent lethal heterosubtypic challenge (Guo et al., 2014). Moreover ferrets, which have upper respiratory physiology more closely related to humans than mice and can transmit influenza via aerosol droplets, were also protected by sciIAV against infection and transmitting virus (Baker et al., 2013). Vaccination with sciIAV in swine was also shown to be protective and immunogenic (Masic et al., 2013; Pyo and Zhou, 2014).

Infection with influenza leads to a type 1 skewed adaptive immune response (Kohlmeier and Woodland, 2009) so sciIAV can potentially be used as a vector to protect against other upper-respiratory tract pathogens. Evidence for this was first provided by showing the presence of anti-GFP Abs in mice following immunization with a GFP-expressing sciIAV PB2 (Victor et al., 2012). Furthering this data, when a reporter gene from a sciIAV was replaced by an inactive HA gene from a heterologous IAV, both mice and swine were protected against heterologous influenza virus infections (Masic et al., 2013; Uraki et al., 2013). However, no NAbs were detected against the heterologous strain in mice even though they were detected in swine. Whether these bivalent sciIAV had different protective efficacies than reporter-expressing sciIAV was not addressed (Masic et al., 2013; Uraki et al., 2013). Amenability of sciIAV as a bivalent vaccine was further shown when *Streptococcus pneumoniae* surface protein A (PspA), HPIV-3 HN protein, or RSV F protein replaced the reporter gene in sciIAV (Fonseca et al., 2014; Katsura et al., 2014; Kobayashi et al., 2013). All of these vaccines could induce Abs and reduce the burden against the heterologous pathogen while retaining their ability to protect against influenza challenge. The use of sciIAV has also been demonstrated as a vector for the exogenous expression of small RNAs in cellular and *in vivo* systems, and for gene knock-down with therapeutic purposes (Schmid et al., 2014) (Fig. 4B). Schmid *et al.* evaluated the use of a sciIAV as a delivery tool for both coding and non-coding RNA, demonstrating that these small RNAs

are expressed and functional in cultured cells, primary cells and in infected mice, opening the possibility of using sciIAVs as a delivery tool of therapeutic value.

For vaccine or vaccine vector applications, it is important to consider the viral gene replaced when generating sciIAV. Although the polymerase (PB2, PB1 and PA) segments may afford more flexibility to include larger transgenes, the inability of virus to encode for more polymerase during the single round of infection should theoretically decrease total viral gene expression. For example, it was shown that a single dose of sciIAV HA or NA could protect against lethal influenza challenge (Guo et al., 2014; Shinya et al., 2004). However, Victor *et al.* demonstrated that one dose of sciIAV PB2 was only as efficacious as inactivated virus (Victor et al., 2012). Additionally, it was also demonstrated that *in situ* sciIAV replication is required, because inactivated sciIAV did not confer full protection (Baker et al., 2013; Katsura et al., 2012; Victor et al., 2012).

In summary, sciIAV has been used as a safe and effective vaccine in several animal models and can elicit humoral and cellular responses, representing a feasible alternative to current influenza vaccines. In this regard, sciIAVs combine the advantages (safety of the IIV and better immunogenicity of the LAIV) and avoid disadvantages (poor immunogenicity of the IIV and safety of the LAIV) of current influenza vaccination approaches. However, although this virus elicited protective and therapeutic immunity in animal studies, not human trials has been performed yet. It is important to consider the sizable regulatory requirements of developing human vaccines, which would be more severe in the case of sciIAV because complementing cell lines would similarly need to be approved for vaccine production (Perdue et al., 2011). Importantly, MDCK cells have recently been given FDA approval for use in vaccine virus production, but stable virus protein-expressing MDCK cells would need a corresponding approval (Hussain et al., 2010). Moreover, there are other limitations associated to vaccine manufacturing. Given that, a system for large-scale production must be available, and the sciIAV engineered should be genetically stable in multiple passages in order to achieve commercial quantities of vaccine.

3.4 Studying the biology of influenza virus using sciIAV

3.4.1 Co-infections and viral reassortment—Antigenic shift occurs in IAV when a reassortment event generates a human-transmissible strain that contains novel HA or NA segments, against which a majority of humans are immunologically naïve (Shaw and Palese, 2013). This has directly led to at least three IAV pandemics: H2N2 in 1957, H3N2 in 1968, and pH1N1 in 2009 (Garten et al., 2009; Shaw and Palese, 2013). By using transgenes with unique fluorescent protein emission spectra, sciIAVs have been used to study IAV co-infection and segment specific packaging hierarchy (Bodewes et al., 2012; Inagaki et al., 2012). Inagaki *et al.* showed that rescue transfections of sciIAV NA or PB2, when there was equal competition for a GFP or DsRed transgene, resulted in few bi-fluorescent protein-expressing sciIAV plaques. These results suggest that most virions specifically package eight viral segments, because only 0.6–1.6% of plaques were both GFP and DsRed positive. To evaluate the ability of IAV to co-infect cells *in vitro*, GFP or mCherry sciIAV NA viruses were used to infect A549 or MDCK cells (Bodewes et al., 2012). Using flow cytometry and confocal microscopy, the authors found that although an MOI of 0.1 did not

lead to many GFP and mCherry double positive cells, infecting with an MOI of 3 led to a co-infection rate of 2% in A549 and 4–10% in MDCK cells. Both of these studies exemplify the extreme selectivity to incorporate only eight vRNA segments and infrequency of co-infection at low MOIs.

3.4.2 Influenza virus tropism and host range—Receptor-binding of IAV is one of the main determinants of viral tropism and host specificity, where avian-tropic viruses preferentially bind to α 2,3 linked SA receptors and human strains typically bind α 2,6 linkages (Rogers et al., 1983a; Rogers et al., 1983b; Yoon et al., 2014). To investigate attachment and post-attachment events with mammalian and avian cells, GFP-expressing sciIAV NA were used with three different HA subtypes (H1, H3 and H5) (Rimmelzwaan et al., 2007). For virus attachment, the authors used a sciIAV where the transmembrane region of NA is fused to GFP and thus GFP is incorporated into virions. Flow cytometric analysis of cells bound by virus or productively infected with sciIAV showed that H1 and H3 viruses bind human A549 cells better than quail QT6 cells (6.7 and 35.0 compared to 1.9 and 0.8 percent, respectively) (Rimmelzwaan et al., 2007). Furthermore, H5 sciIAV bound QT6 cells better than H1 and H3 viruses (39.2, 1.9, and 0.8 percent, respectively). It is surprising to note, however, that more than 50% of QT6 cells expressed GFP 16 hours post-treatment in all conditions, despite the inability to detect frequent initial human-tropic virus attachment (Rimmelzwaan et al., 2007).

To evaluate the requirement of HA as the receptor-binding protein, Hooper *et al.* used reverse genetics transfections to rescue a sciIAV PB1 in which the receptor binding function of HA was abolished (BindMut) (Hooper and Bloom, 2013). To the surprise of the authors, a virus arose that contained a mutation in NA (G147R) that conferred receptor-binding functionality to NA. In a follow up study, the authors again used sciIAV PB1 in elegant MN assays that included oseltamivir and found that NA G147R's ability to bind receptor is dependent on its sialidase activity, presumably because sialidase activity is dependent on receptor engagement (Hooper et al., 2015).

To identify a bat cell line permissive to H1N1 virus infection, Poole *et al.* utilized GFP-expressing sciIAV HA for single-round infections at various MOIs (Poole et al., 2014). Prior to this work, this important potential reservoir for IAV lacked experimental evidence suggesting permissiveness to human IAVs (Mehle, 2014). The permissive bat cell line, Tb 1 Lu, that was identified to support H1N1 replication and infection did so through binding of sialic acid-linked receptors, as treatment of cells with receptor destroying enzyme prior to sciIAV HA inoculation inhibited subsequent GFP expression (Poole et al., 2014). With reports that continually suggest new host species for IAV, sciIAV can be used to evaluate virus tropism to and permissivity of new species (Yoon et al., 2014).

3.4.3 Inhibition of influenza A and B intertypic reassortment—Despite the continual co-circulation of IAV and IBV in humans and the ability of each virus to reassort within the same type, intertypic reassortments have never been documented in nature or *in vitro* (McCullers et al., 2004; McCullers et al., 1999; Shaw and Palese, 2013; Xu et al., 2004). In fact, a competitive interference at the level of viral transcription between both virus species has been reported (Aoki et al., 1984; Gotlieb and Hirst, 1954; Kaverin et al.,

1983). To demonstrate the interference of IAV mediated by IBV, Wantichang *et al.* co-infected cells with DsRed-expressing sciIAV NA and either of two IBV strains (B/Lee/40 and B/Maryland/2/59) (Wantichang et al., 2012). In agreement with previous studies (Aoki et al., 1984), the authors used flow cytometry to show that DsRed expression was markedly lower in IBV, but not IAV (WT PR8) co-infected cells (Wantichang et al., 2012). Cells that overexpressed IBV NP prior to sciIAV NA infection were refractory to high DsRed expression post-infection, demonstrating that IBV NP was sufficient to interfere with IAV replication. This work, along with follow-up studies, demonstrated the ability of IBV NP to interfere with IAV replication (Jarun-ampornpan et al., 2014; Wantichang et al., 2013), but it does not explain the inability to rescue intertypic reassortant viruses in the absence of influenza B NP. In fact, using GFP-expressing sciIAV HA, Baker *et al.* demonstrated, complementation of sciIAV HA with full-length IBV HA via MDCK-HA cells (Baker et al., 2014). Following the hypothesis that certain homologs of IBV can function in IAV, the authors demonstrated that packaging signal discrepancies were sufficient to limit IAV and IBV intertypic reassortment, rather than incompatibilities in the function of viral proteins. It remains to be determined whether or not IBV uses similar packaging signals to those described to IAV. Similarities and differences between packaging signals of IAV and IBV will also need to be compared.

3.5 Conclusions and future directions

In this review, we summarize and discuss the biology and applications of the sciIAVs described to date. The recombinant viruses and methods described herein do not cover all potential sciIAV uses in influenza laboratories. Mini-replicon systems – co-expression of IAV RdRp and NP that drive transcription of a viral-like RNA encoding a reporter gene – are commonly used to study IAV genome replication and gene transcription (Shaw and Palese, 2013). Reporter-expressing sciIAVs can similarly be used for quantitative analysis of RdRp activity and are advantageous over mini-genome approaches in that cells are reconstituted with all the proteins of IAV. Moreover, the use of sciIAV is also applicable to translational research and has been demonstrated as a screening platform to identify specific or broadly-reactive NABs, antiviral compounds, or host proteins or RNAs involved in IAV replication, including highly pathogenic IAVs normally confined to BSL-3+ facilities. The sciIAV platform can also be used as a next-generation IAV vaccine or vaccine vector for other respiratory pathogens (Fig. 4). It will be interesting to continue research with sciIAV as an efficacious vaccine or vaccine vector because it is capable of expressing several antigens or immune-modulators during a single round of infection. There is also great value in using sciIAV to perform forward evolution studies safely and with a quantitative read-out that is compatible with HTS. Not only can mutants be identified readily that confer a certain growth phenotype, but also these studies can be conducted in a self-contained system, significantly alleviating concerns about escape of so called gain-of-function mutants. Other future areas of sciIAV application include, but are not limited to, virus tropism, the analysis of viral reassortments during replication, or immunological responses to the first round of infection. In conclusion, there are potential advantages and disadvantages associated to sciIAV. However, the abundant safety, economic, and biological advantages of sciIAV highlight their promising future in basic and applied influenza research or as therapies. The

use of these bio-contained viruses is desirable when studying basic *in vitro* biology of highly pathogenic IAV.

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Abbreviations

ACIP	Advisory Committee on Immunization Practices
Ab	antibody
att	attenuated
BSL	biosafety level
ca	cold-adapted
CPE	cytopathic effect
DI	defective interfering
dsRed	Discosoma red fluorescent protein
ELISA	enzyme-linked immunosorbent assay
Fluc	Firefly luciferase
FFU	focus forming units
FDA	Food and Drug Administration
GFP	green fluorescent protein
HA	hemagglutinin
HN	hemagglutinin-neuraminidase
HI	hemagglutination inhibition
HAU	hemagglutinating units
HTS	high-throughput screening
IFA	immunofluorescence
IIV	inactivated influenza vaccine
IAV	Influenza A virus
IBV	Influenza B virus
IRES	internal ribosome entry site
LAIV	live attenuated influenza vaccine
MDCK	Madin-Darby canine kidney

MN	microneutralization
mRFP	monomeric red fluorescent protein
MOI	multiplicity of infection
NA	neuraminidase
NAb	neutralizing antibody
NCR	non-coding regions
NEP	nuclear export protein
nt	nucleotide
PFU	plaque forming units
PRNT	plaque reduction neutralization test
Pol I	polymerase I
Pol II	polymerase II
RIV	recombinant influenza vaccine
RFP	red fluorescent protein
Rluc	Renilla luciferase
RdRp	RNA-dependent RNA polymerase
SA	sialic acid
sciIAV	single-cycle infectious Influenza A Virus
ts	temperature-sensitive
VLP	virus-like particle
vRNP	viral ribonucleoprotein
vRNA	viral RNA
WT	wild type

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Highlights

- Influenza A virus infections cause both seasonal epidemics and occasional pandemics.
- SciIAVs are defective in viral replication or propagation.
- Biocontained sciIAV can be used to study highly pathogenic influenza A viruses at BSL-2.
- Expression of a reporter gene has allowed the use of sciIAVs in HTS settings.
- SciIAVs have been used as potential safe vaccines or delivery vectors in mammals.

Figure 1. Generation of stable MDCK-HA cell lines and rescue of single-cycle infectious influenza A virus (sciIAV)

A) Schematic representation for the generation of stable MDCK-HA cells: Polymerase II (Pol II) driven plasmids encoding IAV HA (top) and hygromycin-B resistance (bottom) are co-transfected (ratio 3:1) into parental MDCK cells. After transfection, cells are seeded at low density (cloning dilution) and hygromycin-resistant clones are individually selected. HA-expressing MDCK clones are screened by HA-deficient sciIAV complementation using microscopic analysis of reporter gene (GFP) expression (top), and by HA protein expression using specific Abs by immunofluorescence (right). **B) Plasmid-based reverse genetics to generate HA-deficient sciIAV:** Ambisense plasmids encoding PB2, PB1, PA, NP, NA, M, NS as well as a Polymerase I (Pol I) driven plasmid encoding the reporter gene (GFP) flanked by the IAV HA NCR and packaging signals and a Pol II driven HA protein expression plasmid are co-transfected into co-cultures of 293T/MDCK-HA cells. Virus containing tissue culture supernatants are subsequently passaged onto MDCK-HA cells for the amplification of the sciIAV HA. pA: polyadenylation signal. RBZ: Hepatitis Delta Virus ribozyme.

Figure 2. Characterization of sciIAV Δ HA

A) Schematic representation of the HA/GFP vRNA: Top, wild-type IAV HA vRNA segment. Bottom, HA(45)GFP(80) vRNA. NCR (thin black lines) at each vRNA termini and the IAV HA packaging signals (Ψ) necessary for the incorporation of vRNA into virions. Nucleotide lengths of NCR, packaging signals and vRNAs are shown. **B) Plaque morphology of sciIAV Δ HA in parental and MDCK-HA cells:** Confluent parental and MDCK-HA cells were infected with ~25 PFU of the sciIAV Δ HA and, at 3 days post-infection, monolayers were fixed and stained with crystal violet. **C) Electron micrographs of WT IAV (left) and sciIAV Δ HA (right).** Scale bar, 100 nm. **D) sciIAV Δ HA RNA composition:** vRNAs of purified WT (left) and sciIAV Δ HA (right) virions are indicated. RNAs were separated by electrophoresis on polyacrylamide gels containing 7 M urea and silver stained. The positions of the IAV vRNA segments are indicated. The black arrow indicates 18S ribosomal RNA (18S rRNA). The green arrow indicates the GFP vRNA segment in the sciIAV Δ HA.

Figure 3. Generation of HA-pseudotyped sciIAV

sciIAV Δ HA can be pseudotyped with various IAV HA proteins by infection of various MDCK-HA cell lines. Infection of parental MDCK cells will result in VLPs released that do not contain HA on the surface. Infection of MDCK-HA cells will produce sciIAV complemented with the cell-derived IAV HA, allowing the generation of HA-pseudotyped sciIAV.

Figure 4. *In vitro* and *in vivo* applications of sciIAV

A) *In vitro* applications: sciIAV can be used for the screening of influenza neutralizing antibodies (NAbs) and the identification of antiviral compounds. Fluorescent/luminescent assays can also be conducted to investigate host-virus factors important for IAV replication as well as to investigate co-infection and reassortment mechanisms of IAV infection. Color code in 96-well plates indicate single (green and red) or double (yellow) infections with sciIAV expressing green or red fluorescent proteins. **B) *In vivo* applications:** sciIAV can be used as vaccines or vaccine vectors to provide protection against subsequent viral lethal challenges, as viral vectors (left) for *in vivo* delivery of miRNA or genes like GFP (center),

and to study the immunological consequences of an IAV single round of infection (right).
For more details, see text.

Table 1

Influenza A virus proteins and their functions

Segment			Primary transcript		Secondary transcript	
N ^o	Segment length	3'/5' Ψ	Protein	Function	Protein	Function
1	2,341	30/120	PB2	Component of viral polymerase complex. Host cell capped mRNA recognition and binding.		
2	2,341	60/120	PB1	Component of viral polymerase complex. RNA-dependent RNA polymerase. Endonuclease activity.	PB1-F2	Induces cell death.
3	2,233	12/21	PA	Component of viral polymerase complex. Involved in cap-snatching.	PA-X	Repression of cellular host gene expression.
4	1,775	45/80	HA	Glycosylated surface protein. Binding to cellular receptor. Major antigenic determinant.		
5	1,565	60/120	NP	Nucleoprotein. Encapsidation of the RNA segments to form the viral Ribonucleoprotein complexes.		
6	1,409	183/157	NA	Glycosylated surface protein. Neuraminidase activity to release newly made virus from infected cells.		
7	1,027	222/220	M1	Matrix protein 1. Mediate viral encapsidation. Involved in export of vRNPs from the nucleus.	M2	Matrix protein 2. Ion channel.
8	890	35/35	NS1	Non-structural protein 1. Inhibits the host interferon response.	NEP	Mediates nuclear export of vRNPs.

N^o, influenza virus segment number.

Ψ , packaging signals; numbers represent nucleotide positions in the negative sense (obtained from www.fludb.org).

Table 2

Single-cycle infectious influenza A viruses (scIAV)

Gene deleted	IAV backbone ⁽¹⁾	Transgene ⁽²⁾	Complementation	Application	References
PB2	PR8	GFP or dsRed	Stable cell line	Segment incorporation	Inagaki et al. (2012)
PB2	PR8*	GFP, Rluc, Fluc	Stable cell line	Neutralization assays	Ozawa et al. (2011)
PB2	PR8	GFP	Stable cell line	Influenza vaccine	Victor et al. (2012)
PB2	PR8	HA from pHIN1 or VN1203	Stable cell line	Bivalent influenza vaccine	Uraki et al. (2013)
PB2	PR8	HPV3 HN	Stable cell line	Bivalent vaccine	Kobayashi et al. (2013)
PB2	PR8	RSV F	Stable cell line	Bivalent vaccine	Fonseca et al. (2014)
PB1	WSN	GFP, mCherry	Stable cell line	Oseltamivir resistance	Bloom et al. (2010)
PB1	pHIN1	GFP	Stable cell line	Oseltamivir resistance	Bloom et al. (2011)
PB1	WSN	GFP	Stable cell line	NA virus attachment	Hooper et al. (2015)
PB1	WSN	GFP	Stable cell line	Oseltamivir resistance	Hooper and Bloom (2013)
HA	WSN	GFP, mRFP	Stable cell line	Packaging signals	Marsh et al. (2007)
HA	WSN	GFP	Stable cell line	NAb screening	Martinez-Sobrido et al. (2010)
HA	pHIN1	GFP	Stable cell line	Influenza vaccine	Baker et al. (2013)
HA	X31	GFP	Stable cell line	Influenza vaccine	Guo et al. (2014)
HA	PR8	HA	Stable cell line	Influenza vaccine	Powell et al. (2012)
HA	pHIN1	HA	Stable cell line	Influenza vaccine	Katsura et al. (2012)
HA	PR8	PspA	Stable cell line	Bivalent vaccine	Katsura et al. (2014)
HA	PR8	GFP	Stable cell line	HA complementation	Baker et al. (2014)
HA	pHIN1	GFP	Stable cell line	NAb screening	Ramon et al. (2014)
HA	WSN	GFP	Stable cell line	Virus entry into bat cells	Poole et al. (2014)
HA	WSN	Rluc	Single infection	Genome-wide RNAi screen	Konig et al. (2010)
HA	WSN	Rluc	Stable cell line	Antiviral screen	Bottini et al. (2012)
HA	PR8	GFP, miR-124	Single infection	siRNA delivery	Schmid et al. (2014)
HA	PR8	NEP, scramble	Single infection	Viral vector	Chua et al. (2013)
NA	PR8	GFP	Exogenous NA	Virus attachment	Rimmelzwaan et al. (2007)
NA	PR8**	GFP	Exogenous NA	NAb screening	Rimmelzwaan et al. (2011)

Gene deleted	IAV backbone ⁽¹⁾	Transgene ⁽²⁾	Complementation	Application	References
NA	PR8	GFP, mCherry	Single infection	Co-infection	Bodewes et al. (2012)
NA	PR8	GFP or dsRed	None	Segment incorporation	Inagaki et al. (2012)
NA	WSN	GFP	None	Packaging signals	Fujii et al. (2003)
NA	WSN	GFP	None	Influenza vaccine	Shinya et al. (2004)
NA	SW02	HA from SW98	Exogenous NA	Bivalent influenza vaccine	Masic et al. (2013), Pyo et al. (2014)

⁽¹⁾PR8, A/Puerto Rico/8/34 (H1N1); WSN, A/WSN/33 (H1N1); pH1N1, A/California/04/2009 (pH1N1); X31, PR8 internal segments with HA and NA from A/Hong Kong/2/1968 (H3N2); SW02, A/Swine/Saskatchewan/18789/02 (H1N1).

PR8*: HA/NA from PR8, pH1N1 or VN1203; A/Vietnam/1203/04 (H5N1).

PR8**: HA from PR8, Neth178: A/Netherlands/178/95 (H3N2), VN1194: A/Vietnam/1194/04 (H5N1), VN1203, Tur05: A/Turkey/Turkey/1/05 (H5N1), HK97: A/Hong Kong/156/97 (H5N1).

⁽²⁾GFP, Green fluorescent protein; DsRed, Discosoma red fluorescent protein; Rluc, Renilla luciferase; Fluc, Firefly luciferase; mRFP, monomeric red fluorescent protein; HIPV3 HN, Human parainfluenza virus type 3 hemagglutinin-neuraminidase; RSV F, Respiratory syncytial virus F glycoprotein; PspA, *Streptococcus pneumoniae* surface protein A; SW98, A/Swine/Texas/4199-2/98 (H3N2).