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# A single NS2 mutation of K86R promotes PR8 vaccine donor virus growth in Vero cells

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ABSTRACT

Vaccination is the most effective way to prevent and control infection by influenza viruses, and a cell-culture-based vaccine production system is preferred as the future choice for the large-scale production of influenza vaccines. As one of the WHO-recommended cell lines for producing influenza vaccines, Vero cells do not efficiently support the growth of the current influenza A virus vaccine donor strain, the A/Puerto Rico/8/1934 (PR8) virus. In this study, a single mutation of K86R in the NS2 protein can sufficiently render the high-yielding property to the PR8 virus in Vero cells. Further analysis showed that the later steps in the virus replication cycle were accelerated by $NS2_{K86R}$ mutation, which may relate to an enhanced interaction between $NS2_{K86R}$ and the components of host factor F1Fo-ATPase, FoB and F1β. Because the $NS2_{K86R}$ mutation does not increase PR8 virulence in either mice or embryonated eggs, the PR8-$NS2_{K86R}$ virus could serve as a promising vaccine donor strain in Vero cells.



## Introduction

Influenza A viral infection is one of the most devastating infectious diseases in human history. In the past century, there were four influenza pandemics that caused threats around the world (Yoon et al., 2014). Annually, seasonal influenza viruses infect at least 10–20% of people, and the high-risk populations are recommended to receive vaccines (Belsey et al., 2006). Vaccination is the most efficient way to prevent influenza infection. However, due to antigenic drift and shift, influenza vaccines should be updated annually to best protect against the circulating strains, and scaled-up production is also required, especially during pandemics (Lu et al., 2006). Currently, the production of influenza vaccines is primarily based on an egg-based production system. Although the system has been used for decades, several inherent drawbacks exist, such as labor intensity, lengthy timeline, limited production capacity, antigenic changes and allergenic responses (Kistner et al., 1998; Vepachedu et al., 2012; Wright, 2008). Considering that one to two eggs are required to produce each vaccine dose, millions of eggs are required to produce a given influenza vaccine during the influenza season (Montomoli et al., 2012).

Although egg-based vaccines should be prepared approximately 6 months in advance, vaccine production is often delayed because pandemics cannot be predicted accurately (Ozaki et al., 2004). In contrast to an egg-based production system, a cell-based production system is easier to scale up, produces fewer changes in antigenicity and causes no allergenic responses (Hatz et al., 2012; Mochalova et al., 2003). Many cell lines have now been licensed for vaccine production, including Vero cells from an African green monkey kidney (Barrett et al., 2009; Kistner et al., 1998), MDCK cells from a canine kidney (Genzel et al., 2006) and PER.C6 cells from human retinoblasts (Montomoli et al., 2012; Pau et al., 2001). Among them, only Vero cells have been used to produce multiple human vaccines for decades, including the poliovirus and rabies virus vaccines (Montagnon et al., 1981). The safety of human vaccines derived from Vero cells has been proven, and their production has been easily scaled up for use at the commercial level. Furthermore, the influenza vaccines derived from Vero cells

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have been estimated to induce proportional humoral responses and even higher cellular immune responses compared with those derived from eggs (Bruhl et al., 2000).

When reverse genetic techniques for influenza A viruses were recently applied to generate vaccine candidates, the advantages of this technique became more apparent and were shown to not only save time in generating vaccine seed viruses (Hoffmann et al., 2002) but also improve the viral growth properties and attenuation of the vaccine strain by introducing designed mutations (Lu et al., 2006). Traditionally, the vaccine seed virus was derived by co-infecting eggs with the circulating strain and the egg-adapted vaccine donor virus PR8, followed by screening for progeny viruses containing the hemagglutinin (HA) and neuraminidase (NA) genes from the circulating strain and the remaining genes from PR8 (Ozaki et al., 2004; Wood and Robertson, 2004). When the cell-based production system starts replacing the traditional reassortment method, a problem arises because the vaccine donor virus PR8 is not adapted to Vero cells and grows to a low titer in Vero cells (Lau and Scholtissek, 1995). Although several strategies have been developed to improve the growth of PR8 in Vero cells (Genzel et al., 2010; Kaverin and Webster, 1995), the viral titer for PR8-based vaccine strains in Vero cells is still not satisfactory, which has led to a demand for further improvement.

In this study, we rescue a Vero-adapted PR8 virus by generating reassortant viruses carrying viral gene segments from a Vero-adapted WSN virus. By viral gene screening and mutagenesis, we found that a single amino acid mutation, $NS2_{K86R}$, is capable of making PR8 grow efficiently in Vero cells. More infectious virus particles can be released from PR8-$NS2_{K86R}$-infected Vero cells than from the wild-type PR8-infected cells. Our study suggests that $NS2_{K86R}$ binds more efficiently to F1β and FoB, the components of host factor F-type proton-translocating ATPase membrane protein that facilitates virus budding. Thus, the modified PR8-$NS2_{K86R}$ virus could be a promising vaccine donor virus for Vero-cell-based vaccine production systems.

## Results

### *A PR8 reassortant virus carrying the WSN NS segment grows efficiently in Vero cells*

To develop an efficient vaccine donor strain for the Vero cell culture system, reverse genetic technology and virological analyses have been employed. The wild-type (WT) vaccine donor virus, PR8, grew slowly and formed tiny plaques in Vero cells at 48 h post-infection (Fig. 1A) (Kaverin and Webster, 1995). In contrast, a laboratory-adapted strain, WSN, grew efficiently in Vero cells by forming larger plaques at 48 h post-infection (Fig. 1A). We therefore individually replaced PR8 virus genes with WSN virus genes to promote the growth of PR8 in Vero cells. Because mutations in the HA and NA surface proteins may alter virus antigenicity, we only focused on internal viral genes. The PR8 gene segments were substituted by WSN gene segments in a PR8 background to rescue the following reassortant viruses using reverse genetics: PR8–WSN M (where the M gene was from WSN), PR8–WSN NS (where the NS gene was from WSN), and PR8–WSN 4P (where the three polymerase genes and the NP gene were from WSN) (Ozaki et al., 2004). Plaque formation by these reassortant viruses was analyzed in Vero cells. The data in Fig. 1A show that only the PR8–WSN NS virus can form larger plaques than the WT PR8 virus and that the PR8–WSN M and PR8–WSN 4P viruses showed no growth advantage over the WT PR8 virus. The growth kinetics further confirmed that the NS gene from WSN, compared with other WSN genes, could significantly promote the growth of PR8 in Vero cells (Fig. 1B). The PR8–WSN NS virus grew

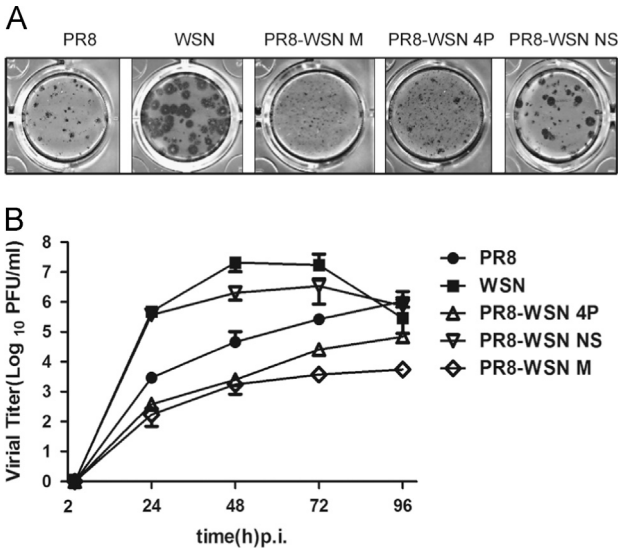


**Fig. 1.** Growth curves and plaque assays for the reassortant viruses between PR8 and WSN. (A) Plaque assays for reassortant viruses in Vero cells. Cellular monolayers cultured in a 24-well plate were infected with the indicated viruses and incubated at 37 °C in 5% $CO_2$. At 48 h post-infection, the monolayer was fixed with 4% PFA, and immunostaining was performed using an anti-NP polyclonal antibody conjugated with HRP, followed by True Blue substrate coloration. (B) Growth curves for the reassortant viruses in Vero cells. Cellular monolayers were infected at an MOI of 0.001 and incubated at 37 °C in 5% $CO_2$. At 2, 24, 48, 72, and 96 h post-infection, the supernatants were harvested, and viral titers were determined by plaque assays in MDCK cells. The viral titers provided for each point are the mean titers with standard deviations calculated from three independent experiments.

much faster than the original PR8 virus, with an approximately 128 and 55 times higher viral titers than that of PR8 at 24 h and 48 h post-infection respectively (the mean viral titers of PR8–WSN NS and PR8 at 24 h post-infection are $3.7*10^5$ and $2.9*10^3$ respectively, about 128 fold, $P<0.01$; the mean viral titers of PR8–WSN NS and PR8 at 48 h post-infection are $1.8*10^6$ and $3.3*10^4$ respectively, about 55 fold, $P<0.05$) (Fig. 1B). In contrast, the PR8–WSN M and PR8–WSN 4P grew even slower and to a lower viral titer than the PR8 virus. Therefore, we conclude that the NS gene from the WSN virus is able to facilitate growth of the PR8 virus in Vero cells.

### *Specific amino acids in the WSN NS gene promote the growth of PR8 in Vero cells*

The NS gene of influenza A viruses directs the synthesis of two mRNAs by alternative splicing, NS1 and NS2 (NEP) (Bullido et al., 2001; Robb et al., 2009), as shown in Fig. 2A. An alignment of the NS genes from WSN and PR8 shows 11 nucleotide differences, resulting in seven amino acid mutations in the NS1 protein (Fig. 2A, NS1 mRNA) and six amino acid mutations in the NS2 protein (Fig. 2A, NS2 mRNA). Among these amino acid mutations, two mutations, F103S and M106I, are known to contribute to the stabilization of NS1 binding with the host cleavage and polyadenylation specificity factor CPSF30 (Das et al., 2008), whereas the functions of the other mutations have not previously been reported. To determine the Vero-adaptive mutations in the NS gene, each of the unique 11 WSN NS nucleotides were introduced into the PR8 NS gene, and 10 reassortant viruses were generated (S103F and I106M were introduced in combination because they function together).

The growth characteristics of the 10 reassortant viruses were evaluated in Vero cells by analyzing growth curves and plaque assays. As shown in Fig. 2B, the viruses harboring mutations at $NS1_{K55E}$, $NS1_{D101H}$, $NS1_{D189M}$/$NS2_{M31I}$, $NS2_{E63G}$ and $NS2_{K86R}$ all grew faster than the WT PR8 virus, although the degrees of