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Studies on genetically engineered chicken DF-1 cell for efficient production of influenza virus

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SUMMARY

Influenza virus has been known to cause pandemics in poultry species leading to mortality and massive economic losses in the poultry industry. Therefore, methods have been devised for quick isolation, amplification, and identification of influenza subgroups. Several strategies have been used for quick and abundant amplification of the virus, including an egg-based system and cell-based system. The egg-based system is a well-established system; however, it offers several notable disadvantages especially in the process of vaccine production. Therefore, a cell-based system has been developed as a viable alternative strategy for the amplification of the virus.

The establishment of a cell-based system is critical because the virus replicates efficiently in species-specific host cells. This system provides several advantages as follows: there is no need for virus adaptation to the cell for proliferation, and virus-host factors can be studied in a species-specific manner. Also, the engineering of cells could provide an improved system that could mimic the conditions for the replication of the virus, similar to *in vivo* conditions. Moreover, an added advantage of a cell-based system is that it could reduce the cost during the process of viral amplification for vaccine production. In this study, plasmid-based reverse genetics was established for the rescue of influenza virus in cell culture. After that, it was demonstrated that the influenza virus

codon usage bias between influenza and the host species affects the viral polymerase activity. Furthermore, genetically engineered cell lines were established that could be used for the amplification of the influenza virus in the absence of trypsin.

In the first study, chicken polymerase I promoter was identified, amplified, and inserted into a plasmid to produce the virus in the avian cells. The viral genomes were first codon optimized for the chicken codon usage bias and inserted into the plasmid harboring chicken polymerase I promoter. Then, viral polymerase activity was evaluated by establishing a luciferase assay based system. It was confirmed that the influenza virus could be rescued and that the polymerase activity was correlated to the translation of the proteins of the polymerase complex based on the codon usage of the chicken. Interestingly, compared to the wild-type virus, the codon optimized virus polymerase complex showed significantly higher activity at 24h post-transfection.

In the next study, the expression of host factors, such as TMPRSS2 and TMPRSS4 involved in the cleavage of the hemagglutinin protein of the virus and ST3GAL1 involved in the cell receptor recognition by the hemagglutinin protein, in various chicken tissues that are routinely infected with the influenza virus were evaluated compared to wild-type DF-1 cells. From the results, it was revealed that the expression levels of TMPRSS2 and TMPRSS4 were very low in wild-type DF-1 cells.

Therefore, a piggyBac transposon vector was constructed for integration and overexpression of each host factor in the wild-type DF-1 cells. After that, the expression levels of TMPRSS2, TMPRSS4, and ST3GAL1 were evaluated by qRT-PCR of the transfected cells. Interestingly, TMPRSS2 and ST3GAL1 showed increased expression in the transfected cells, while TMPRSS4 had a relatively low expression. The proliferation of engineered cells was further analyzed, and it was found that the overexpression of host factors TMPRSS2, TMPRSS4, and ST3GAL1 had no negative effect on the cells. Each engineered cell line was challenged with the influenza virus (PR8-H5N8 PB2-627E), and the results showed an increased viral titer in the engineered cells, even in the absence of trypsin compared to wild-type DF-1 cells.

Furthermore, a cell line expressing both TMPRSS2 and ST3GAL1 was established, and this cell line was challenged with the influenza virus (PR8-H5N8 PB2-627E). The results showed an increased viral titer in the cells co-expressing TMPRSS2 and ST3GAL1 compared with the WT DF-1 cell line treated with trypsin.

Collectively, these results indicate that this system could be used for the rescue and amplification of the influenza virus even in the absence of trypsin, which is critical in cell-based systems used for the propagation of the influenza virus. This system and the engineered cells

could be applied to other fields of research, such as the study of species-specific host factors that interact with the influenza virus during infection and in vaccine production without the addition of trypsin, thus reducing the cost of production.

Keywords: chicken, influenza virus, genetically engineered DF-1, host factors.

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LIST OF ABBREVIATIONS

CEF	Chicken embryonic fibroblast
CRM1	Chromatin maintenance 1
cRNA	Complementary ribonucleic acid
HA	Hemagglutinin
HAT	Human airway trypsin-like protease
HEK	Human embryonic kidney
HPAIV	High pathogenic avian influenza virus
IRES	Internal ribosome entry Site
LPAIV	Low pathogenic avian influenza virus
M	Matrix protein
M1	Matrix protein 1
M2	Matrix protein 2
MDCK	Madin-darby canine kidney
mRNA	Messenger ribonucleic acid
MSPL	Mosiac serine protease large-form
NA	Neuraminidase
NEP	Nuclear export protein
NLS	Nucleus localization signal
NP	Nucleus protein
NS	Nonstructural protein
NS1	Nonstructural protein 1

NS2	Nonstructural protein 2
ST3GAL1	ST3 beta-galactoside alpha-2,3-sialyltransferase 1
TMPRSS2	Transmembrane serine protease 2
TMPRSS4	Transmembrane serine protease 4
O/E-ST3	Overexpression of ST3 beta-galactoside alpha-2,3-sialyltransferase 1
O/E-ST3T2	Overexpression of ST3 beta-galactoside alpha-2,3-sialyltransferase 1 and transmembrane serine protease 2
O/E-T2	Overexpression of transmembrane serine protease 2
O/E-T4	Overexpression of transmembrane serine protease 4
PA	Polymerase acidic
PB2	Polymerase basic 1
PB2	Polymerase basic 2
rDNA	Ribosomal deoxyribonucleic acid
RdRP	RNA-dependent-RNA-polymerase
RNP	Ribonucleoprotein
SA	Sialic acid
tRNA	Transfer ribonucleic acid
UTR	Untranslated region
vRNA	Viral ribonucleic acid
vRNP	Viral ribonucleic nuclear protein

CHAPTER 1

GENERAL INTRODUCTION

The influenza virus is part of the *Othomyxoviridae* family and is classified as either type A, B, or C. Influenza A virus is an enveloped virus with a genome made up of negative-sense, single-stranded, segmented RNA. The Virus encodes for thirteen well-known viral proteins and several other strain-dependent accessory proteins (Dou et al. 2018; Samji 2009). Each protein has a specific function in the viral life cycle. The HA, NA and M2 proteins form the lipid viral envelope. The HA protein is the most abundant envelope protein, followed by NA and, lastly, M2 (Nayak et al. 2009; Schroeder et al. 2005). Underneath is the matrix 1 (M1) that supports the envelope protein. Inside the viral envelope is the nuclear export protein (NEP) and ribonucleoprotein (RNP) complex that consists of viral RNA segments coated with nucleoprotein (NP) and the heterotrimeric RNA-dependent RNA polymerase (RdRP). The RdRP is consists of polymerase basic 1 and 2 (PB1 and PB2) and polymerase acidic (PA) (Lee and Saif 2009; Mahy 1983).

The influenza virus does not replicate on its own unless inside a host cell. Therefore, the virus has developed to adapt and replicate in different species host. Among the viral proteins, the HA has evolved to recognize different cell surface receptors as a mode of adaptation. The HA of influenza virus recognizes N-acetylneuraminic (sialic) acid of the cell surface. Avian-adapted influenza virus has shown to

preferentially bind to the α-2,3 linkage while mammalian-adapted influenza virus binds with a high affinity to the α-2,6 linked sialic acid receptors (van Riel et al. 2007).

Attachment to the cell surface receptor is the first step that initiates the viral replication cycle. The HA precursor, HA0, is made up of two domains HA1 and HA2 that has to be cleaved by proteases to render the virus infectious. Various studies have demonstrated that endogenous proteases such as miniplasmin, tryptase Clara, Mast cell tryptase, type II transmembrane serine proteases (including transmembrane protease, serine 2 [TMPRSS2] and transmembrane protease, serine 4 [TMPRSS4]), human airway trypsin-like (HAT) protease, and matriptase can cleave the HA of low pathogenic avian influenza viruses (LPAIVs) *in vitro* (Baron et al. 2013; Bertram et al. 2010; Böttcher-Friebertshäuser, Klenk, and Garten 2013). Nevertheless, the function of these proteases in different cell lines may be strictly limited in sufficiently activating influenza HA. On the other hand, unlike LPAIVs, highly pathogenic avian influenza viruses (HPAIVs) containing multiple basic amino acids are endogenously cleaved by ubiquitously expressed proteases with a polybasic specificity such as Furin and thus leads to systematic tissue infection (Luczo et al. 2018a; Horimoto et al. 1994).

However, for the propagation of the influenza virus, two systems

are used, mainly an egg-based and cell-based system. The egg-based system has been a long-established way to propagate the virus. However, viruses produced in eggs have unintended mutations in the HA. High pathogenic strains grow poorly in eggs, and some reassorted viral strains have reduced growth in eggs. Other drawbacks of using the egg-based system include limited flexibility for expanded vaccine manufacturing and interruption of quality eggs for vaccine production in the case of disease outbreaks in poultry (Wu et al. 2019; Audsley and Tannock, 2008).

On the other hand, cell cultured-based influenza propagation is an alternative system because it offers various advantages (Soema et al. 2015b). The advantages of cell culture-based systems are as follows: they are easier to scale up; they produce a purer vaccine, and they do not pose a threat to people with an allergy to egg proteins (Soema et al. 2015a). Additionally, cell engineering could result in improved viral titer (Yamayoshi and Kawaoka, 2019a). However, during virus replication, trypsin is added to the culture media to cleave the HA and support the viral replication cycle. To overcome the dependence on trypsin during virus propagation, cell lines have been engineered to cleave the HA by endogenous expression of proteases. Some studies with MDCK cell lines expressing proteolytic enzymes such as TMPRSS2, HAT, and Mosaic serine protease large form (MSPL) have

been reported to cleave the HA in the absence of trypsin supplementation (Böttcher et al. 2009; Wen et al. 2015).

Codon usage differs in a variety of organisms. The use of different codons has been suggested to depend on the abundance of tRNA and the cognate codon frequencies of the mRNA (Kurland 1991; Quax et al. 2015). In animals, most amino acids are encoded by two to six different codons, and synonymous codons are used with different frequencies. Among organisms, even though the genetic code is conserved, there is a shift in codon bias (Hershberg and Petrov, 2008). However, in the case of the influenza virus, transcription of the vRNA is performed by the polymerase complex, while the translation of the mRNA is by the host cell. Therefore, the codon usage of the influenza virus is affected by the codon bias of the host cell (Goñi et al. 2012; Nogales et al. 2014; Plotkin and Dushoff 2003). Studies have shown that influenza can adapt to the codon usage of host cells (Ahn and Son, 2010). In vaccine production, DNA vaccines utilize this phenomenon to optimize for vectors that stimulate a higher innate immune response. Optimization of viral proteins could be used as a strategy for viral production that can stimulate a high immune response.

This study demonstrates that the genetic engineering of cells that enhances host factors for viral replication could support the propagation of influenza virus in the absence of trypsin. CHAPTER 2 reviews the

general structure, replication cycle, and host factors of the influenza virus. It also reviews the system of reverse genetics and codon optimization with its applications to the rescue of the influenza virus. CHAPTER 3 describes how a chicken system was established that could be used for the reverse genetics of influenza virus and how codon optimization of synonymous codons of the virus to the chicken could affect protein translation and viral polymerase. CHAPTER 4 describes the genetic engineering of cells with the piggyBac transposon that stably overexpress TMPRSS2, ST3GAL1, and a combination of both. It shows that TMPRSS2 was able to cleave the HA of influenza, thus supporting the replication cycle and that ST3GAL1 enhanced viral uptake.

CHAPTER 2

LITERATURE REVIEW

1. The biology of influenza A virus

The influenza A virus is part of the *Othomyxoviridae* family which structurally consisting of 8 segmented negative-strand RNA genomes enveloped by glycoproteins the hemagglutinin (HA) and neuraminidase (NA). The influenza A virus is categorized in subtypes based on the antigenic relationship of HA and NA surface glycoproteins. Currently, sixteen antigenically different HA subtypes and nine different NA subtypes have been identified (Lee and Saif 2009; Fouchier et al. 2005; Palese and Schulman 1976). Over the past years, distinct subtypes of influenza A virus circulate in various hosts including humans, bats, poultry, horses and migratory birds (Robert G. Webster 1992).

1.1 Virion structure

The influenza virus shape is spherical with forms on the order of 100nm in diameter, and the filamentous forms often over 300nm in length. The envelope of the virion is made up of lipid bilayer derived from the host's plasma membrane that contains three integral membrane proteins HA, NA, and matrix 2 (M2) (Scheiffele et al., 1999). The three integral membrane proteins overlay matrix 1 (M1) that encloses the virion core. The M2 forms ion channels that transverse the lipid membrane critical for triggering the viral uncoating in the endosome. The internal of the virion core comprises eight subunits, of

which three subunits: polymerase basic protein 2 (PB2), polymerase basic protein 1 (PB1) and polymerase acidic protein (PA). The three subunits together form the viral RNA-dependent RNA polymerases (vRdRP) that are responsible for RNA synthesis and replication in the infected cells. All the single-stranded negative-sense viral RNA eight segments are tightly associated with the nuclear protein (NP) and the three polymerase proteins which form the ribonucleoprotein (RNP) complex (Bouvier and Palese 2008; Lee and Saif 2009; Mahy 1983).

1.2 Viral genome structure and proteins

The eight negative-stranded viral RNA, whose segments are numbered in order of decreasing length, encode thirteen known proteins. Segments 1, 4, 5, and 6 encode just one protein per segment: PB2, HA, NP, and NA proteins. Segment 2 encodes polymerase subunit PB1 and in a +1 alternate reading frame, an accessory protein PB1-F2 of 87 amino acid protein associated with the pro-apoptotic activity (Chen et al., 2001). Furthermore, from segment 2, PB1 codon 40 directs the translation of an N-terminally truncated version of the polypeptide (N40) essential for virus replication (Wise et al., 2009). Segment 3 encodes two proteins the polymerase acidic protein and by alternative splicing of RNA, PA-X which functions as a suppressor of the antiviral and immune response of host cell (Hayashi et al., 2015;

Gao et al. 2015; Shi et al. 2012; Hu et al. 2015). Segment 7 encodes for two proteins M1 and M2, while the latter is expressed by RNA splicing (Lamb et al., 1981). Segment 8 encodes three proteins by alternative splicing of RNA. The first protein is a nonstructural protein 1 (NS1) that expresses interferon-antagonist activity while the nuclear export protein (NEP) and nonstructural protein 2 (NS2) are implicated in the viral RNP export from the host cell nucleus (García-Sastre 2001; Lamb et al. 1980). Each vRNA possesses at 3' and 5' ends untranslated regions which act as promoters for transcription and translation, and are highly conserved among all influenza genomes. The ends of each vRNA segment form a helical hairpin which is bound by the heterotrimeric RNA polymerase complex and the remainder of the segment is coated with arginine-rich NP, the net positive charge that binds the negatively charged phosphate backbone of the vRNA (Baudin et al., 1994; Compans et al., 1972; Bouvier and Palese 2008).

1.3 Virus life cycle

For influenza virus to replicate requires a host cell and undergoes multiple steps in order to produce progenies. The first step is viral entry to the host cell, followed by transcription and replication in the nucleus of the host cell, translation of viral proteins, final assembly, and budding of new progeny.

1.3.1 Virus entry to host cell

The influenza virus life cycle can generally be divided into the following stages: entry into host cells; entry of the vRNPs into the nucleus; transcription and replication of the viral genome; export of the vRNP from the nucleus, assembly and budding at the host cell plasma membrane. During the first stage, the HA receptor-binding site attaches to the cell surface glycoconjugates that contain terminal sialic acid (SA) residues, which trigger endocytosis of the virion (Weis et al. 1988; Hamilton et al., 2012). Endocytosis of influenza virions occurs through a clathrin-dependent manner which involves dynamin and the adaptor protein Epsin-1 or by micropinocytosis (Rust et al. 2004; Sieczkarski and Whittaker 2002; De Vries et al. 2011).

In the cytoplasm, the endosome acquires a low pH through the M2 ion channel leading to the acidification of the endosomal, therefore, triggering the fusion of the viral fusion peptide and endosomal membranes. The change in pH triggers a conformational change in the HA exposing the fusion peptide inserting itself into the endosomal membrane. Once the two membranes are brought close together, they form a lipid stalk and subsequently fusion of both membranes (Stegmann 2000; Pinto, Holsinger, and Lamb, 1992), thus releasing their vRNPs into the cytoplasm.

1.3.2 Nuclear transport of vRNPs

The released vRNPs are then transported to the nucleus wholly dependent on the cellular import machinery. The viral proteins are known to have nuclear localization signals (NLS) that bind to karyopherins. It is known that vRNPs bind to importin-α, which in turn is recognized by the importin-β transport receptor directing this complex to the nuclear pore complex through which is transported into the nucleus (Wang et al., 1997; Cros et al., 2005). It has been established that avian adapted influenza viruses depend on importin-α3 while mammalian adapted viruses show adaptation to importin-α7 (Nadler et al., 1997; Gabriel et al., 2011).

1.3.3 Transcription and replication of the viral genome

In the nucleus, the viral RNA-dependent RNA polymerase (RdRP) comprising of PB2, PB1, and PA complex is responsible for the transcription and replication of the vRNA. The first step involves the transcription of negative-sense vRNA templates to synthesis capped polyadenylated messenger RNA (mRNA) and uncapped complementary RNA (cRNA). The mRNA acts as a template for the translation of viral proteins by host-cell translation machinery, while the cRNA acts as the template for subsequent transcription of more copies of negative-sense genomic vRNA. During the cap snatching

process, influenza RdRP binds to the nascent host RNAs using a cap-binding domain on the RdRP PB2 subunit and cleaves 10-13 nucleotide downstream of the 5' cap using an endonuclease domain at the N-terminus of the RdRP PA subunit. The 5' short capped RNA fragment is used as a primer to initiate transcription of the viral genome. The RdRP adds the 3' polyA tail during the stuttering on a short uridine tract present near the 5' terminus of al viral genome segments resulting in polyadenylation of the viral mRNA and transcription termination (Walker and Fodor 2019).

1.3.4 Export and translation of viral mRNA

The export of viral mRNA and vRNP is dependent on the cell export machinery through the chromosome region maintenance 1 (CRM1) pathway. During the export of vRNP, the M1 proteins play a critical role by binding to the vRNP therefore, masking the nuclear localization signal (NLS), thus enabling direct interaction with NEP, which possesses a nucleus export signal (NES). Subsequently, the vRNP-M1-NEP complex is recognized by CRM1 protein, thus mediating the export of the complexes from the nucleus (Fukuda et al., 1997). The export of mRNA is tightly coupled to polymerase II transcription and pre-mRNA maturation, acquiring a myriad of RNA binding proteins and poly (A) binding proteins (York and Fodor, 2013).

The translation of the viral mRNAs is divided between cytosolic ribosomes that translate PB2, PB1, PA, NP, NS1, NS2, and M1 protein while for membrane proteins HA, NA, and M2 are translated by endoplasmic reticulum (ER)-associated ribosomes. The newly synthesized proteins of PB2, PB1, PA, and NP target the importin-α-importin-β pathway to be trafficked back into the nucleus. In the nucleus, the newly synthesized proteins assist in the viral mRNA transcription and vRNA replication. The HA, NA and M2 proteins are folded and trafficked to the Golgi apparatus for post-translational modification and subsequently transported to the cell membrane for virion assembly (Bouvier and Palese, 2008; Dou et al., 2018). The NS1 protein is synthesized in the early stages of replication to act as interferon signaling inhibitor and contributes to viral mRNA export from the nucleus by linking the viral transcripts to the cellular nuclear export components (Dou et al. 2018).

1.3.5. Packaging of vRNP and virus budding

The packaging of RNP is a selective process that ensures that vRNA segments containing discrete packaging signals are fully incorporated (Fujii et al. 2003). It has been observed that the RNP is packaged in a "7+1" pattern (Chou et al. 2012). Reverse genetics experiments confirmed that all eight influenza vRNAs have bipartite packaging

signals located at the 5' and 3' termini (Ozawa et al. 2009; Marsh et al., 2007). Budding of the virus particles occurs at the apical side of polarized cells where the HA, NA, and M2 proteins are transported. M2 is vital in the formation of viral particles, while M1 which present underneath the lipid bilayer, is essential in the final step of closing and budding of the viral particle. Before the release of the virus from the membrane, NA removes sialic acid residue from glycoproteins, therefore, ensuring the release of the virus particle. This process is essential, without which the particle cannot be released from the plasma membrane (Nayak et al., 2009).

2. Host-virus interaction

Various factors have been implicated in the host-virus interactions. For example, the interaction of the HA with binding receptors is an essential step for the virus to infect the host cells. It has been established that aquatic waterfowl can host all influenza A subtypes and they are believed to the natural reservoirs of influenza viruses (Yoon et al., 2014). However, some viruses have adapted to bird hosts such as chicken, quail, turkey, and mammalian hosts such as humans, swine, equine, dogs and marine mammals (Webster and Hulse, 2004). This adaptation has also been attributed to viral polymerases that are error-prone and have higher mutation rates. The adaptation to the

transmission and host cell infection is multifactorial and could involve amino acid substitution in the HA that can lead to optimal receptor binding and HA and NA optimal balance (Mitnaul et al. 2000).

2.1 Sialic acid receptors

Sialic acid belongs to a diverse family of sugars terminally linked to different carbohydrates. The influenza virus recognizes different sialic acid with a different kind of linkage. Avian-adapted influenza virus preferentially binds to α2,3 while human-adapted HA binds more favorably to α2,6 (Rogers et al. 1983; Matrosovich et al. 1997). Equine virus HAs preferred the α2,3-linkage but cannot bind 4-O-acetyl sialic acid. Human-adapted HA, such as the H2N2 (1957) and N3N2 (1968), required changes in the H2, H3, and N2 specificity to change from α2,3 to α2,6. The mutations in the 220-loop of the receptor-binding site Q226L and G228S and mutation in the N2 I275V resulted in pandemics (Kobasa et al., 1999). The changes in the HA may be a prerequisite for highly effective replication and spreading, which characterize epidemic strains (Matrosovich et al. 2000). Matrosovich et al. demonstrated that human-adapted viruses preferentially infect non-ciliated cells, on the other hand, avian-adapted viruses and egg-adapted human influenza virus variant with an avian-like receptor specificity mainly infected ciliated cells. There is a correlation with the predominant localization

of receptors for human viruses on non-ciliated cells and of receptors for avian viruses on ciliated cells (Matrosovich et al. 2004).

2.2 Cleavage of the hemagglutinin and virus infectivity

The HA is translated as a precursor molecule (HA0) of a 75kDa, which assembles to homotrimers. The crystallographic analysis identifies the cleavage site located in the loop formed by nineteen amino acids, of which eight amino acids protrude from the surface of the membrane-proximal third of the HA trimer (Wilson et al.,1981). This loop contains the cleavage site of the HA. Cleavage of the HA precursor molecule HA0 into HA1 and HA2 is vital to render the virus infectious to host cells. Besides, the distribution of activating proteases in the host is one of the determinants of tropism and, as such pathogenicity. The cleavage site of low pathogenic avian influenza viruses (LPAI) contains monobasic amino acid while the loop of highly pathogenic avian influenza viruses (HPAI) have an insertion of multibasic amino acids at the cleavage site. The amino-terminal cleavage fragment HA1 (50kDa) contains the receptor-binding site while the carboxyl-terminal cleavage fragment HA2 (25kDa) is membrane-anchored and responsible for fusion (Lazarowitz and Choppin 1975; Steinhauer 1999; Klenk et al., 1975). The HA proteins are cleaved by activating proteases within a membrane-proximal

surface loop at R (or K) residue located adjacent to the G residue that constitutes the N-terminus of the newly generated and highly conserved fusion peptide of the HA2 subunit. Recently, data shows that the HA0 of LPAI is cleaved by TMPRSS2 in the secretory pathway within the cell (Böttcher et al., 2010). On the other hand, cleavage of HPAI is achieved by ubiquitous intracellular furin-like serine proteases such as furin and PC6. As a result, this increases the likelihood of systemic infection in contrast to HA proteins that require cleavage by trypsin-like proteases restricted to specific sites of expression (Bosch et al., 1981; Horimoto et al., 1994; Galloway et al., 2013).

3. Virus amplification for vaccine production

Seasonal influenza virus epidemics cause about 3 to 5 million cases of severe illnesses and about 290 000 to 650 000 respiratory deaths to humans (W.H.O, 2019) and massive economic losses to the poultry industry (Wiethoelter et al., 2015). The reemergence of a pandemic H1N1strain in 2009 (Neumann, Noda et al., 2009) and the emergence of highly pathogenic avian H5N1 and H7N9 influenza viruses of avian origin (Gao et al., 2013; De Jong et al., 1997) reaffirmed the need of global vigilance and need of finding means of containing the virus. Vaccination is a primary means of controlling seasonal influenza infections and a core strategy in pandemic preparedness. Also,

vaccination is the most cost-effective way against influenza pandemics. Currently, there are two systems for influenza vaccine production – egg-based and cell-based systems.

3.1 Egg-based system

The egg-based system was the first system to be approved for vaccine production. This system offers several advantages because it is a well-established, validated process and regulated egg production facilities are already in place (Matthews, 2006). During vaccine production in an egg-based system, six months before the identification of strain, eggs are massively prepared at selected production plants. After preparation of eggs is the reassortment of wild-type influenza strain with the egg-adapted strain (PR8) for inoculation and adaptation. Then follows the other steps of vaccine production. For a period ranging from nine to ten months, 60 to 100 million doses are produced for the US market, and about 300 million doses worldwide. However, for each dose, one to two fertilized specific pathogen-free eggs are required for vaccine production (Matthews, 2006).

3.2 Cell-based system

The cell-based system is steadily being developed as an alternative to eggs-based and to overcome some of the various disadvantages

offered by egg-based technology. Cell-based has several advantages such as cost-cuts for eliminating then need to manage biosecure flocks, reduction of potential contamination by viable and nonviable particulates, elimination of then need to prepare eggs months before initiation of vaccine production, faster high-volume start-up times for production, higher initial purity of vaccine and could supplement seasonal vaccine supplies when multiple strain changes are necessary. In this regard, several cell lines have been engineered as an alternative to the use of embryonated eggs. Cell lines that have developed include the Madin-Darby canine kidney cells, Vero cells (African green monkey), Per.C6 cells (human), human embryonic kidney (HEK-293T) and CAP cells have been tested and established for vaccine production (Yamayoshi and Kawaoka 2019b; Lohr et al., 2010; Genzel et al., 2010; Pau et al. 2001; Genzel et al., 2013). The cell-based system has been found to offer additional benefits over the egg-based system. Influenza viruses propagated in cell culture have been found not to have mutations at the antibody binding sites of the hemagglutinin. As a result of no antigenic mutation, vaccines are more effective (Wu et al., 2019). Also, there is no need for adaptation of wild-type strain through reassortment with laboratory strains such as PR8 (Skowronski et al., 2014).

4. Reverse genetics system for influenza virus

Numerous methods have been developed for the rescue of influenza viruses. Reverse genetics has proven a useful tool in the study of influenza viruses. Because the influenza virus has negative-sense RNA segments, each segment can be engineered according to the goal of the study. Viral RNA of influenza by themselves are not infectious. However, the vRNA has to be transcribed into a positive sense mRNA by the viral polymerase complex and then the mRNA can be translated into proteins. After that, the virus will package to form infectious virions. Due to these stages of replication in the life cycle of the influenza virus, different methods have been developed for the rescue of the virus.

4.1 Helper virus-dependent methods

Luytjes et al. (Luytjes et al., 1989) were the first group to establish a system for the generation of influenza that contained vRNA derived from cloned cDNA, also known as the RNP transfection method. The NS coding gene was replaced by a reporter gene chloramphenicol-acetyltransferase (CAT) while the 5' and 3' noncoding region was not altered. The cassette is flanked by T7 RNA polymerase promoter sequences (Neumann and Kawaoka 2002). The *in vitro*-reconstituted RNP complexes were transfected into helper influenza virus-infected

cells. RNP complexes were made by incubating synthetic RNA transcripts with purified NP and polymerase proteins (PB2, PB1, and PA). The helper virus was used as an intracellular source of viral NP and polymerase proteins and the other vRNAs (Pleschka et al., 1996). The same was achieved for site-directed mutagenesis of a single influenza virus by the RNP transfection technique in combination with a selection method against the corresponding RNA segment of the helper virus (Pleschka et al,. 1996). Using this principle, instead of *in vitro* reconstitution, the RNP complex could also be reconstituted within cells by transfecting with a plasmid expressing a viral like-RNA. Within the cell, vRNA was achieved using promoter recognized by cellular DNA-dependent RNA polymerase I (pol I). The 3' end of expressed vRNA occurred by the activity of ribozyme encoded immediately downstream of the cDNA of the copy of vRNA or through the use of a polI terminator sequence (Engelhardt, 2013).

Another method required the transfection of linear expression constructs instead of plasmids into the cells using a polI promoter to drive the expression of vRNA. Moreover, the linear constructs also contain a polII promoter and polyadenylation site flanking the polI transcription unit (Muster et al., 2014). Nevertheless, all systems that use the helper virus-dependent reverse genetics requires a selection strategy.

4.2 Helper virus-independent methods

By using this method, no helper virus is required as all vRNA segments are expressed inside cells by using plasmids or other vector systems.

4.2.1 Promoters used for the expression of vRNA.

Different promoters have been used to generate vRNA. The first plasmids contained the human polI promoter to express vRNA and the 3' end of the vRNA was expressed by the activity of ribozyme that generates the desired 3' end by autocatalytic cleavage, or the murine polI terminator sequence was used (Fodor et al., 1999; Neumann et al. 1999). Despite it has been generally known that polI promoters are species-specific (Heix and Grummt, 1995; Paule and White, 2000) in their activity, Suphaphiphat et al. (Suphaphiphat et al., 2010) reported the successful generation of the virus with human polI in canine cells.

Furthermore, polI promoters from canine and chicken have been used for transfection in MDCK (Wang and Duke, 2007) and chicken embryo fibroblasts (CEF) cells (Massin et al., 2005; Zhang et al., 2009). In addition to that, a T7 promoter to express vRNA was established for transfection in 293T and MDCK cells. This system was used to generate influenza virus in 293T, MDCK and QT6 cells (de Wit et al.,

2007). However, despite having developed a system entirely based on plasmids, the number of plasmids transfected in each system was different. Initially, a seventeen plasmid system was developed and a high virus titer could be generated. This consisted of eight plasmids for vRNA segments and nine plasmids for protein expression (Neumann et al., 1999). Hoffmann et al. (Hoffmann et al., 2000) developed a bidirectional eight plasmid system for the generation of vRNA and mRNA from one template and could successfully rescue the influenza virus (Hoffmann and Webster, 2000). Furthermore, Koudstaal et al. (Koudstaal et al., 2009) developed a ten plasmid system that expressed four essential viral helper proteins from only two plasmids using a polII promoter and an internal ribosomal entry site (IRES). The bidirectional system has been adopted, and it is used as a strategy to generated viruses. Neumann et al. (Neumann et al., 2005) developed a one plasmid system that encoded eight vRNA and all mRNA necessary for viral protein synthesis within the cell. This system was developed for vaccine production in Vero cell that cannot be highly transfected.

Consequently, the development of reverse genetics systems has helped in the study of influenza virus replication and the development of vaccines in the cell-based systems.

4.3 Mini-genome reporter assay in reverse genetics

The reporter virus has been developed to accurately study the virus *in vivo*. *In vivo* localization of virus helped to highlight the virus interaction with cellular host factors. Different reporter genes have been used such as NanoLuc Luciferase (Tran et al., 2013) and Green fluorescence protein (GFP) (Manicassamy et al., 2010). The NanoLuc reporter virus was used to perform *in vivo* imagine and to truck the viral load and dissemination of influenza virus infections in mice lungs. Manicassamy et al. (Manicassamy et al., 2010) generated a reporter virus that replicated efficiently in cells and *in vivo*. This reporter virus was used to trace viral spreading in mice lungs and could trace the effect of antiviral drugs on the replication of the virus *in vivo*. Reuther et al. (Reuther et al., 2015) generated a stably expressing virus by inserting GFP in the NS segment of influenza to produce NS1 and NEP separated by two porcine Teschovirus-1 2A peptides. They showed that the virus stably expressed fluorescence and could be used to trace the infected parts of the mouse lungs. Reporter viruses have shown to be a promising method to quantify virus titers, to assess antiviral sensitivity, rapidly detect virus replication and the antigenic subtype (Lutz et al., 2005).

5. Codon usage bias and codon optimization

In animals, there are twenty different amino acids encoded from sixty-one codons and three stop codons. In animals, most amino acids

are encoded by two to six different codons. However, synonymous codons are used with different frequencies a phenomenon known as codon usage bias. Codon usage bias shifts direction among organisms even though the genetic code has generally remained conserved (Hershberg and Petrov, 2008). In the case of the influenza virus, transcription of the vRNA is performed by the polymerase complex, while the translation of the mRNA is by the host cell. Therefore, the codon usage of the influenza virus is affected by the codon bias of the host cell (Goñi et al. 2012; Nogales et al., 2014; Plotkin and Dushoff 2003). Studies conducted by Ahn and Son (Ahn and Son, 2010) revealed that the H1N1 viruses capable of spreading from human to human that were detected in 2009 had swine-host codon usage patterns. In another study, they observed that the H3N2 virus displayed directional changes in HA, NA, PB1, and PB2 (Ahn and Son, 2012). Lindstrom et al. also reported similar findings in the ten RNA that were analyzed (Lindstrom et al., 1998).

5.1. DNA based vaccines and codon bias

To take advantage of codon usage bias, DNA vaccines have been developed as alternative forms of vaccines. They are economical and do not require a complicated process during vaccine production. Furthermore, they do not require the constant supply of eggs nor

reassortment in the case of the outbreak of the highly pathogenic strain (Stachyra et al., 2016). Codon optimization of rare codons within the synonymous codons preferred by the host organism and avoidance of RNA secondary structure motifs have been applied in order to improve the effectiveness of the DNA vaccine (Stachyra et al. 2016; Chen et al. 2018). A vaccine is developed that can trigger an immune response targeting different proteins of the influenza virus. Among the surface proteins, NA has been targeted though they are not as abundant as the HA protein. Studies have shown that targeting NA does not solicit a high level of antibodies and usually, the antibodies are suboptimal compared to that of HA (Chen et al., 2018). M2 is the third influenza virus surface transmembrane protein that is a target for vaccine development. The M2 is present in very low copy numbers on virions but is abundant in infected cells. M2 targeting vaccines do not induce sterilizing immunity but passive studied in humans demonstrated a reduction in clinical signs and nasal wash virus titer upon challenge with human H3N2 influenza virus isolate (Krammer and Palese, 2015).

5.2. DNA vaccine and codon optimization of HA

The HA is the most targeted protein for DNA vaccine development because antibodies against the HA can neutralize the virus and that it is more prevalent and uniform than the NA of the virus particle (Plotkin

and Dushoff 2003; Tenbusch et al., 2010). Advances have been made by creating a chimeric hemagglutinin with high levels of reactivity. The chimeric hemagglutinin consists of H1, H3 and influenza B hemagglutinin stalk domains, mostly of avian origin (Hai et al. 2012; Krammer and Palese, 2013). In humans, chimeric hemagglutinin consisting of H5N1 and H7N1 induced an increased reactive antibody (Krammer and Palese, 2015). Due to the ease of construction and production of DNA vaccines, the HA has been the target, not only for humans but also for poultry vaccines. Jiang et al. demonstrated that by codon optimizing the HA chickens where able to produce antibodies against the H5N1 and were completely protected from lethal virus challenge (Jiang et al., 2007). Another study in mice demonstrated that the use of Codon optimization could induce substantially higher antibody response by the administration of codon optimized DNA vaccines (Tenbusch et al., 2010).

CHAPTER 3

CHICKEN SYSTEM FOR INFLUENZA VIRUS AND THE IMPACT OF CONDON OPTIMIZATION ON VIRAL POLYMERASE ACTIVITY

1. Introduction

Influenza virus pandemics cause economic loss and mortality to humans and the poultry industry (Claas et al. 1998; Yuen et al. 1998). Therefore, identification and amplification of the virus strain is the first step to develop strategies such as vaccines to prevent outbreaks. Therefore, various methods have been established for the rescue of the influenza virus, which is, the initial step towards the amplification of the virus (Luytjes et al., 1989; Fodor et al., 1999; Neumann et al., 1999). However, the recombinant influenza virus has been used for viral rescue and numerous influenza-related studies.

Influenza A virus, unlike other positive-strand viruses such as poliovirus, consists of eight negative-sense viral RNA (vRNA) segments, and on their own, they are not infectious but depend on the host cellular system. When the vRNAs have been released into the cytoplasm, they are transported to the nucleus for transcription and replication. In the nucleus, the viral RNA-dependent RNA polymerase (RdRP) comprising of PB2, PB1, PA, and NP form a complex that transcribe the vRNA in mRNA and complementary RNA (cRNA). The mRNA serves as a template for protein translation, while the cRNA is a template for the synthesis of new vRNA (Hoffmann et al., 2000; Walker and Fodor, 2019).

To capitalize on this replication system of the influenza virus,

reverse genetics has been established by using bidirectional plasmids. Hoffman et al. reported an eight plasmid-based transfection system instead of a twelve plasmid-based system to rescue the influenza A virus entirely from the cloned cDNA under human polymerase I for the production of negative-sense viral RNA. This system showed improved viral titer and reduced the number of plasmids required for transfection (Hoffmann et al., 2000). However, the transcription activity of polymerase I is species-specific dependent (Hempel et al., 1996; Heix and Grummt, 1995). For this reason, different polymerase I reverse genetics systems have been established to adapt transcription activity in specific host species.

Moreover, influenza A virus also replicates efficiently in the adapted host cell. However, Suphaphiphat et al. reported that human RNA polymerase I driven reverse genetics system could rescue the influenza A virus in canine cells (Suphaphiphat et al., 2010). Furthermore, it was reported that canine polymerase I and swine polymerase I were able to rescue the influenza virus in their respective cell lines (Wang and Duke 2007; Moncorgé et al. 2013).

On the other hand, the translation of mRNA for viral proteins is achieved by the host translation machinery. However, the use of synonymous codons for encoding amino acids vary from species to species. In the case of the influenza virus, transcription of the vRNA is

done by the polymerase complex, while the translation of the mRNA is done by the host cell. Therefore, the codon usage of the influenza virus is affected by the codon bias of the host cell (Goñi et al. 2012; Nogales et al. 2014; Plotkin and Dushoff 2003). Studies conducted by Ahn and Son (Ahn and Son, 2010) revealed that the H1N1 viruses capable of spreading from human to human that were detected in 2009 have swine-host codon usage patterns. It was observed that the H3N2 virus displayed directional changes in HA, NA, PB1, and PB2 (Ahn and Son 2012). Lindstrom et al. also reported similar findings in the ten RNAs that were analyzed (Lindstrom et al., 1998). Therefore, codon optimization of rare codons within the synonymous codons preferred by the host organism and avoidance of RNA secondary structure motifs have been used to improve the translation efficiency and thus increase the number of desired proteins (Stachyra et al., 2016; Chen et al., 2018).

In this study, a plasmid-based reverse genetics system was developed for the rescue of the influenza virus in the chicken cells under the promoter of the chicken polymerase I for vRNA production. Furthermore, it was demonstrated that by implementing codon bias usage of chickens through codon optimization, could increase the polymerase activity of the viral polymerase complex.

2. Materials and methods

Cell culture

Chicken DF-1 fibroblast cells (CRL-12203, ATCC) were maintained in DMEM with high glucose (Hyclone, Logan, UT, USA), supplemented with 10% fetal bovine serum (Hyclone) and 1x antibiotic antimitotic (ABAM) (Thermofisher Scientific, Waltham, M, USA) at 37°C, 5% CO2 and with 60-70% relative humidity.

Cloning of chicken polymerase I

Primers were designed to amplify 250 base pair region upstream of chicken polymerase I from the origin of the rDNA transcription start site. The target amplicons were extracted by Wizard SV Gel and PCR clean-up system (Promega, Wisconsin, USA). Three μl of PCR products were incubated in a total volume of 10 μl containing 2X rapid ligation butter, 1μl of T-easy vector (Promega) and 1 μl of T4 DNA ligase at room temperature for 3h. PCR amplicons were ligated into the T-easy vector and the ligated plasmids were incubated on ice for 30 min with competent E. coli. The mixture was then heat-shocked at 42°C for 30 sec and incubated in a shaker with LB broth (Duchefa Biochemie, Haarlem, Netherlands) for 1h. The mixture was spread on the ampicillin (Sigma-Aldrich, MO, USA) and X-gal (Bioneer, Seoul,

Korea) added Agar LB plate and then incubated at 37°C overnight. White colonies were collected and incubated in a shaker with ampicillin added to LB broth for 16 h. The E. coli was harvested, and plasmid DNA was extracted using a mini-prep kit (Favorgen, Wembley, WA, Australia) according to the manufacturer's protocol. Finally, the plasmid was analyzed by sequencing service (Bionics, Korea). The sequenced PCR product was analyzed by pairing with a representative sequence to verify for mutations. The correct amplified PCR was digested with SmaI and HindIII enzymes and then ligated in a vector between the CMV promoter and SV40 polyA.

Construction of Plasmids containing PB2, PB1, PA, and NP

The protein-coding region of PB2, PB1, PA, and NP was derived from a plasmid under a human polymerase I. The target region of each sequence was sequenced, analyzed and annotated. By using a codon optimization tool (Thermo Fisher Scientific), the protein-coding region of each segment was optimized to chicken codon usage. The resulting sequencing was aligned and analyzed for the similarity of sequence. The resulting sequence was synthesized (Bionics, Korea) and cloned to plasmid between the CMV promoter and chicken polymerase I promoter by using Takara In-Fusion Ligation mix (Takara, Kasatsu,

Japan) according to manufacturer's instructions. The resulting plasmid was amplified and purified using a Plasmid Maxi kit (Qiagen, Hilden, Germany). The correct insert was confirmed by sequencing (Bionics, Korea).

Construction of Luciferase Reporter Vector

The protein-coding region of Luciferase was designed in reverse direction flanked by Untranslated Region (UTR) of the NS segment of the influenza virus and synthesized (Bionics, Korea). Then the fragment was ligated into the plasmid containing only chicken polymerase promoter I and terminator to produce only negative-sense viral RNA using Takara In-Fusion Ligation mix (Takara, Kasatsu, Japan) according to manufacturer's instructions. The resulting plasmid was amplified and purified using a Plasmid Maxi kit (Qiagen, Hilden, Germany). The correct insert was confirmed by sequencing.

Minigenome assays

1 x 10^5 cells wild-type DF-1 were seeded on a twelve well plate and 24h later were co-transfected with plasmids encoding the PB1, PB2, PA, and NP proteins (200 ng each), together with a plasmid expressing negative-sense luciferase flanked by NS segment noncoding sequences

under the control of chicken polymerase I promoter (50 ng), and the 50ng of pGL-4.53 plasmids expressing Renilla luciferase used as the internal reference by using Lipofectamine 2000 transfection reagent (Invitrogen) and the transfected cells were incubated at 37°C, 5% CO2 and with 60-70% relative humidity. Twenty hours after transfection, the cells were collected and polymerase activity was determined by luciferase assay. Cells were lysed and samples were assayed for firefly and Renilla luciferase activity using the NanoGlo Dual-Luciferase Reporter Assay System (Promega). Polymerase activity was normalized to Renilla luciferase activity. All reporter assays were repeated at least three times.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software (GraphPad Software 8, San Diego, CA). Significant differences between two groups were examined statistically using Student's t-test. A P value <0.05 was considered to indicate statistical significance (***P<0.001, **P<0.01, and *P <0.05).

3. Results

Cloning of chicken polymerase I

Because the transcription activity of the polymerase I promoter is species-specific, 250 base pairs of the promoter from the rDNA transcription start site was cloned. Using RT- PCR, the target region was amplified, and the results from the gel electrophoresis showed the correct band size of the intended PCR product (**Fig. 3-1A**). Next, the amplified sequence was analyzed by pairwise alignment, and the results indicated the correct amplification with no mutations (**Fig. 3-1B**). Next, the verified promoter target was cloned into a vector that could express unpolyadenylated RNA in a negative direction with a murine terminator.

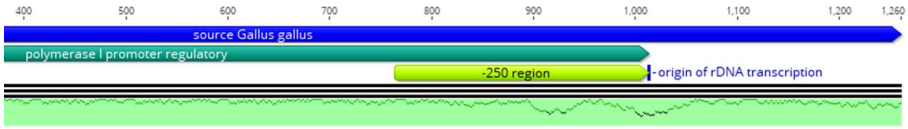
Codon optimization of PB2, PB1, PA, and NP

After confirming that the plasmid had correctly inserted chicken polymerase I promoter, a codon optimization tool was used to optimize the codon usage of chicken, and each segment was analyzed by pairwise alignment. It was revealed that codon usage in PB2, PB1, PA, and NP between virus and chicken was 76.26%,76.52%,76.38%, and 75.21% respectively (**Fig. 3-2**). Next, the plasmids that expressed PB2, PB1, PA, and NP were constructed under the promoter of the chicken polymerase I for vRNA production.

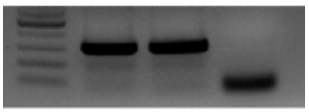
Establishment of a luciferase reporter system

The effect of codon optimization on polymerase activity was then investigated by the luciferase assay system. The four plasmids that encode each subunit of the virus polymerase complex were transfected to WT DF-1 cells together with Renilla as a control reporter for polymerase activity (**Fig. 3-3A**). Twenty-four hours after transfection, the cells were analyzed, and a significant difference in polymerase activity was observed between the wild-type virus and chicken codon optimized virus (**Fig. 3-3B**).

A



- 250 bp



B

```
chPOLI-250      1 ----------------------------------------------GCGGCGGCGGCTAGAG   16
                                                               GCGGCGGCGGCTAGAG
DQ112354      721 AACGGCGGAACGGTCTACCCGGGTGCTACCGACTCGCGCTCTCCGCGGCGGCGGCTAGAG  780

chPOLI-250     17 GTCGCTGCCGGGGCGGCTTGCGATCCGCGTCCAGGTCTACCCCGTTTCGGATTGTCTTGG   76
                  GTCGCTGCCGGGGCGGCTTGCGATCCGCGTCCAGGTCTACCC GTTTCGGATTGTCTTGG
DQ112354      781 GTCGCTGCCGGGGCGGCTTGCGATCCGCGTCCAGGTCTACCCGGTTTCGGATTGTCTTGG  840

chPOLI-250     77 CCGCTCTGGCTGTGGGGGGGGGCGCTACAGCTCCGGAGCTGCCAGAGGCGTCGCTGTAAT  136
                  CCGCTCTGGCTGTGGGGGGGGGCGCTACAGCTCCGGAGCTGCCAGAGGCGTCGCTGTAAT
DQ112354      841 CCGCTCTGGCTGTGGGGGGGGGCGCTACAGCTCCGGAGCTGCCAGAGGCGTCGCTGTAAT  900

chPOLI-250    137 TTTGTACCTCCAGTTACGTCGAGGTAAACCTCGGCTGCCGTCGGAGCCGCTGCCGGTAGT  196
                  TTTGTACCTCCAGTTACGTCGAGGTAAACCTCGGCTGCCGTCGGAGCCGCTGCCGGTAGT
DQ112354      901 TTTGTACCTCCAGTTACGTCGAGGTAAACCTCGGCTGCCGTCGGAGCCGCTGCCGGTAGT  960

chPOLI-250    197 CGGCGCCTATGGGACTAGAACGTTTTTTTCGGATGCCTTATATGTTCGTCTGTA------  250
                  CGGCGCCTATGGG CTAGAACGTTTTTTTCGGATGCCTTATATGTTCGTCTGTA
DQ112354      961 CGGCGCCTATGGGGCTAGAACGTTTTTTTCGGATGCCTTATATGTTCGTCTGTAGGAGCG 1020
```

C

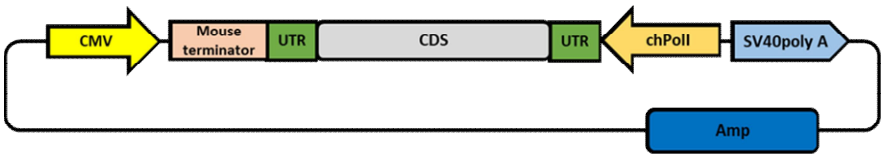
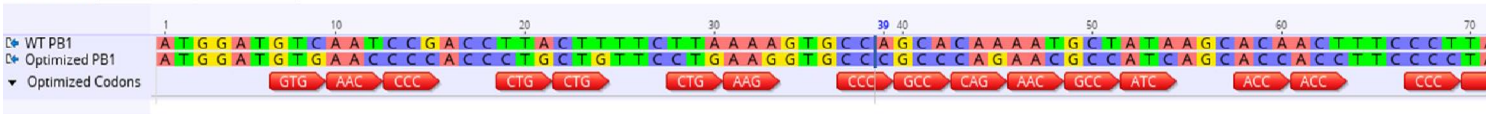


Figure 3-1. Cloning of chicken polymerase I, and construction of expression vector under chicken polymerase I. (A) Target region of amplification of chicken polymerase I for cloning and RT-PCR amplicon of 250bp. (B) Sequence alignment of chicken polymerase I. (C) Schematic representation of vector under CMV promoter and chicken polymerase I.

	WT PB2	Optimized PB2
WT PB2		76.259%
Optimized PB2	76.259%	

	WT PB1	Optimized PB1
WT PB1		76.517%
Optimized PB1	76.517%	



	WT PA	Optimized PA
WT PA		76.383%
Optimized PA	76.383%	

	WT NP	Optimized NP
WT NP		75.217%
Optimized NP	75.217%	

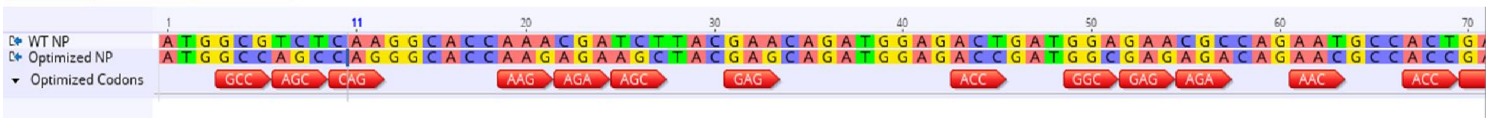
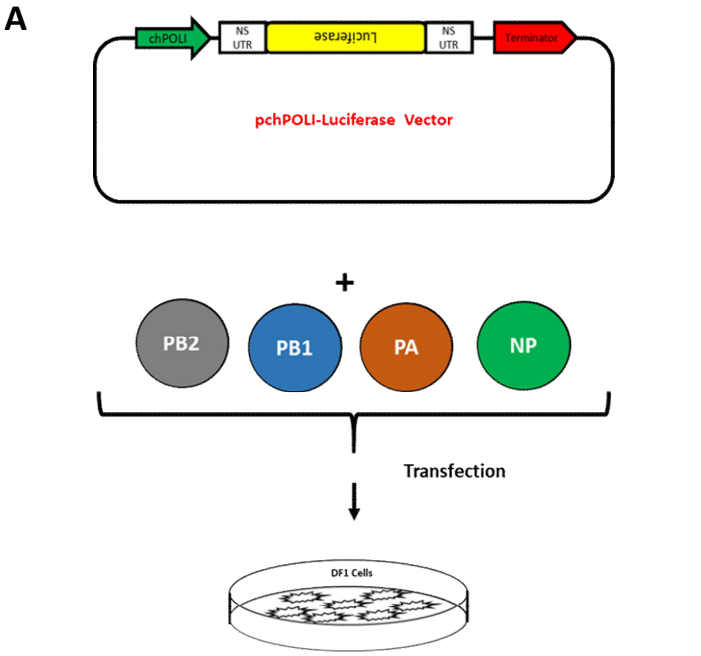


Figure 3-2. Codon optimization and sequence alignment. Representative image of Codon optimization of viral genome PB2, PB1, PA, and NP, and alignment with wild-type virus. Identification of sequence similarity between wild-type virus and codon optimized genome.


A
chPOLI
NS UTR
Luciferase
NS UTR
Terminator
pchPOLI-Luciferase Vector
+
PB2
PB1
PA
NP
Transfection
DF1 Cells

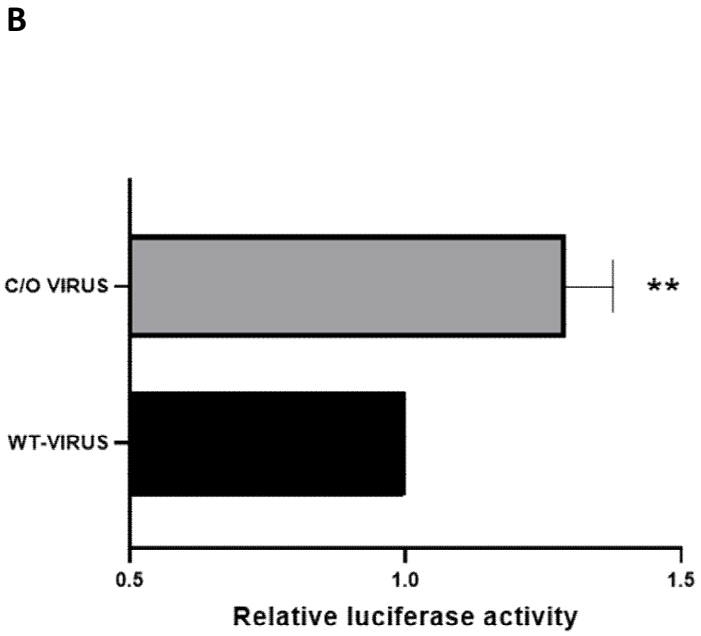

B
C/O VIRUS
WT-VIRUS
**
0.5
1.0
1.5
Relative luciferase activity

Figure 3-3. Mini genome assay. (A) Schematic representation of transfection of Plasmids PB2, PB1, PA, NP and NS-Luciferase to wild-type DF-1 for the Luciferase assay system. (B) Relative luciferase activity of wild-type virus compared to codon optimized virus to indicate polymerase activity.

Table 3-1. List of primers used for RT-PCR

Region	Forward Primer	Reverse Primer	Product Size, bp
-250 Promoter	TCTACCCGGGTGCTACCGAC	ACAGACGAACATATAAGGCATCC	**250**

4. Discussion

Influenza A virus has evolved to replicate efficiently in species-specific host cells. This evolution enables the study of influenza viruses in either mammalian or avian cells. Because the influenza virus is a negative-stranded RNA, different methods have been developed to isolate, propagate, and rescue the virus. Among the methods that have been developed, helper virus-independent methods have proven to be more efficient in the generation and rescue of influenza virus. In this regard, plasmid-based reverse genetics has been well established.

However, reverse genetics for mammalian cell systems using mammalian cells have been well developed for the study and amplification of influenza viruses. On the other hand, the development of an avian-based system has been mostly lacking. Therefore, the development of reverse genetics for avian cells would be beneficial for the study of an avian-adapted influenza virus.

In this study, a plasmid-based system for influenza rescue for the chicken system was successfully established. The chicken polymerase I from genomic DNA was successfully cloned consistent with Massin et al. (Massin et al., 2005). The region above 250 bp had a higher GC content making it challenging to amplify. Therefore, only 250 bp was considered adequate for plasmid construction. A plasmid containing chicken polymerase I was constructed that produced non-

polyadenylated negative sense RNA transcripts. By transfecting vectors containing chicken polymerase I promoter into wild-type DF-1 cells, it was demonstrated that negative sense RNA could be generated.

Next, to investigate that not only vRNA was generated but also viral proteins, viral polymerase activity was examined together with the effect of codon usage bias on viral polymerase activity. It was shown that the production of viral proteins depended on the codon bias usage of each organism. A significant percentage difference was observed in codon usage after optimization between the genomic sequence of the wild-type virus and that of the codon optimized by the codon usage of chickens.

Furthermore, it was demonstrated that under the chicken polymerase I, vRNA was successfully transcribed to complementary RNA (cRNA) and the cRNA used as a template by viral polymerase proteins to generate messenger RNA which was later translated into proteins. As expected, luciferase activity from the codon optimized virus showed significantly higher activity than that of the wild-type virus indicating that translation of mRNA to proteins was faster due to codon bias usage.

In conclusion, it was shown that the chicken polymerase I for the generation of non-polyadenylated RNA transcripts could be used in a chicken cell system to efficiently rescue and propagate influenza A virus in the chicken system.

CHAPTER 4

ESTABLISHMENT OF GENETICALLY ENGINEERED CHICKEN DF-1 CELL LINE FOR EFFICIENT AMPLIFICATION OF INFLUENZA VIRUSES IN THE ABSENCE OF TRYPSIN

1. Introduction

The influenza virus surface protein hemagglutinin (HA) plays two major roles during the early life cycle of the virus: it binds to cell surface receptors and facilitates the fusion of viral and endosomal membranes to release viral RNA (vRNA) into the cytoplasm (Böttcher et al. 2009). The HA protein is translated as an uncleaved HA0 precursor protein; it is folded as a trimer that is both glycosylated and acylated. Because uncleaved HA0 is unable to initiate membrane fusion, lack of cleavage means no infection (Galloway et al. 2013; Russell et al., 2018). Therefore, cleavage of HA0 into HA1 and HA2 subunits is critical for membrane fusion with the endosome and subsequent release of viral segments into the cytoplasm prior to nuclear transport, transcription, and replication. Highly pathogenic avian influenza (HPAI) viruses harbor a polybasic amino acid sequence at the cleavage site, which is cleaved endogenously by ubiquitously expressed subtilisin-like proteases such as furin and proprotein convertases with polybasic specificity, resulting in fatal systemic infection (Luczo et al., 2018b; Horimoto et al. 1994; Stieneke□Gröber et al. 1992; Walker et al. 1994). By contrast, low pathogenic avian influenza (LPAI) viruses are cleaved by trypsin-like proteases such as miniplasmin, tryptase Clara, Mast cell tryptase, type II transmembrane serine proteases such as

TMPRSS2 and TMPRSS4, and human airway trypsin-like protease (HAT) (Baron et al. 2013; Bertram et al. 2010; Böttcher et al., 2013). However, recent studies show that Madin-Darby canine kidney (MDCK) cell lines expressing proteolytic enzymes such as TMPRSS2, HAT, and Mosaic serine protease large form cleave HA in the absence of trypsin (Böttcher et al. 2009; Wen et al. 2015).

Influenza viruses are propagated for vaccine production and for studies of the viral life cycle, interactions with host cellular factors, and host immune responses. Egg-based and cell-based systems are used to generate influenza vaccines. However, viruses produced in eggs often harbor undesired mutations in HA that render the vaccine less effective. In addition, some reassorted viral strains grow poorly, and highly pathogenic strains are difficult to propagate, in eggs. Other drawbacks of egg-based systems include limited flexibility for expanded vaccine manufacture and interruption of vaccine production/quality during disease outbreaks in poultry (Wu et al. 2019; Audsley and Tannock, 2008).

Cell culture-based propagation of influenza virus is an alternative system that offers various advantages, including easy scale-up for cell engineering systems, increased vaccine purity, and utility for people with allergies to egg proteins (Soema et al., 2015b). However, propagation of LPAI viruses in cell-based culture systems requires

supplementation with trypsin to cleave the HA protein and drive viral replication (Klenk et al., 1975). In some cell lines, high trypsin concentration showed also variation in the resulting yield of virus, thus requiring optimization (Le Ru et al., 2010). This may be overcome by removing the reliance on exogenous trypsin. In addition, removing trypsin will reduce the costs of production. Moreover, using species-specific cell lines will remove the need of viruses to adapt to host cells (Kim et al., 2018; Le Ru et al., 2010).

Avian derived cell lines such as chicken DF-1 cells can be used to propagate influenza viruses because they express α-2,3-linked sialic acid receptors, which are targeted preferentially by avian-adapted viruses (Lee et al., 2008); also, immortalized cell lines provide a suitable platform for generating stable cell lines that can be used for virus propagation. Engineering cells such that they can support faster viral replication will also be a great advantage to the vaccine industry.

Here, genetically engineered chicken DF-1 cells were developed that stably overexpress ST3 beta-galactoside alpha-2,3-sialyltransferase 1 (ST3GAL1), which catalyzes transfer of the sialic acid Neu5Ac from CMP-Neu5Ac to Galβ1,3GalNac on glycoproteins or glycolipids with an α-2,3 linkage. A cell line overexpressing type II transmembrane protease, serine 2 (TMPRSS2) was developed, which is required for cleavage of HA. Finally, a cell line expressing both ST3GAL1 and

TMPRSS2 was developed. The engineered cell lines allowed efficient propagation of influenza virus in the absence of exogenous trypsin. These engineered cells may provide a platform for viral amplification even in the absence of trypsin, thereby allowing the development of a vaccine for poultry and study of virus replication in avian cells.

2. Materials and methods

Experimental animals and tissue collection

The management and experimental use of White Leghorn (WL) chickens was approved by the Institutional Animal Care and Use Committee, Seoul National University (SNU-190401-1). The experimental animals were cared for at the University Animal Farm, Seoul National University, in accordance with standard management programs. Tissue samples used in this study were collected from adult WL chickens aged 18 weeks.

Viruses and biosafety

The PR8-H5N8 (PB2-627E) virus was generated from eight bidirectional pHW2000 plasmids using a reverse genetics system, as previously reported (Park et al. 2019). Viruses were rescued by co-transfection of the eight bidirectional plasmids into a co-culture of

MDCK cells (ATCC, CCL-34) and human 293T embryonic kidney cells (293T; ATCC, CRL-11268). Generated viruses were grown in MDCK infection medium comprising Dulbecco's Modified Eagle's Medium (DMEM) (Hyclone, Logan, UT, USA) supplemented with 0.3% bovine serum albumin (BSA), 1× antibiotic antimycotic (ABAM), and 1 μg/ml L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)-treated trypsin (Sigma-Aldrich, MO, USA), and then incubated at 37°C for 48 h. Virus stocks were further propagated in 10-day-old embryonated chicken eggs. Aliquots of infectious virus were stored at −80°C until required. All work with low pathogenicity viruses was conducted in a biosafety level 2 facility approved by the Institutional Biosafety Committee of Seoul National University.

Construction of overexpression plasmids

The piggyBac plasmid (Addgene plasmid no. 92078, Addgene, MA, USA) containing enhanced green fluorescent protein (eGFP) was digested with AgeI and BsrGI enzymes to create a linearized vector. A synthetic protein-coding region of chicken TMPRSS2 (NCBI Gene ID 418528), chicken TMPRSS4 (NCBI Gene ID 770454), chicken ST3GAL1 (NCBI Gene ID 396140), or chicken ST3GAL1-T2A-TMPRSS2 (Bionics, Korea) was cloned into the linearized vector using Takara In-Fusion Ligation mix (Takara, Kasatsu, Japan), according to

the manufacturer's protocol. The resulting plasmid was amplified and purified using a Plasmid Maxi kit (Qiagen, Hilden, Germany). The correct insert was confirmed by sequencing.

Cell culture and establishment of overexpressing DF-1 cell lines

Chicken DF-1 fibroblast cells (CRL-12203, ATCC) were maintained in high glucose DMEM supplemented with 10% fetal bovine serum (Hyclone) and 1× ABAM. Cells were maintained at 37°C at 5% CO_2 under 60–70% relative humidity. To establish cell lines that stably overexpress the genes of interest, 1.2 μg of overexpression vector and 0.8 μg of piggyBac transposon (pCyL50) were transfected into DF-1 cells using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA). After 6 h, transfection mixtures were replaced with DF-1 culture medium supplemented with puromycin (Thermo Fisher Scientific). Cells were maintained in culture medium supplemented with puromycin for 1 week to recover overexpressing cells.

RNA isolation and qRT-PCR analysis

Total RNA from cells or tissues was extracted using Tri-reagent (Molecular Research Center Inc., Cincinnati, Ohio, USA). RNA quantity was determined by spectrophotometry at 260 nm, and 0.5–1 μg of each sample was reverse-transcribed using the Superscript IV

First-Strand Synthesis System (Thermo Fisher Scientific). The cDNA was diluted five-fold and the concentrations standardized for amplification by PCR. Quantitative real-time PCR (qRT-PCR) was conducted to examine changes in expression of candidate genes in test samples and in overexpressing DF-1 cells. The PCR reaction mixture contained 2 μl of PCR buffer, 1 μl of 20× EvaGreen qPCR dye (Biotium, Hayward, CA, USA), 0.5 μl of 10 mM dNTP mixture, 10 pmole each of target gene-specific forward and reverse primers (Table 1), 1 μl of complementary DNA (cDNA), and 1 U of *Taq* DNA polymerase (final volume, 20 μl). qRT-PCR was conducted in a StepOnePlus real-time PCR system (Applied Biosystems, CA, USA). Each test sample was assayed in triplicate. Relative quantification of target gene expression in the cells was performed using the following formulae: $2^{-\Delta Ct}$, where ΔCt = (Ct of the target *gene*–Ct of *ACTB*), or $2^{-\Delta\Delta Ct}$, where ΔΔCt = (Ct of the target *gene*–Ct of *ACTB*) group–(Ct of the target *gene*–Ct of *ACTB*) control.

Viral infection of cells

ST3GAL1 overexpressing (O/E-ST3), TMPRSS2 overexpressing (O/E-T2), both ST3GAL1 and TMPRSS2 overexpressing (O/E-ST3T2), or wild-type (WT) DF-1 cells were seeded on 12-well plates and grown to confluence. The culture medium was removed and the cells were

washed twice with PBS prior to incubation at 37°C for 20 minutes in DMEM containing 1% penicillin/streptomycin. Next, DMEM containing 1% penicillin/streptomycin containing PR8-H5N8 (PB2-627E) virus at a multiplicity of infection (MOI) of 0.1 or 0.01 was added for 50 minutes. Finally, cells were washed with PBS, and incubated with DMEM containing 1% penicillin/streptomycin for 24 h, 48 h, and 72 h until harvesting the medium for viral titration as stated below.

WST-1 assay of overexpressing cells and virus-infected cells

The Premix WST-1 Cell Proliferation Assay System (Takara Bio, Kusatsu, Japan) was used to measure cell proliferation. Briefly, cells (0.15×10^4 cells per well) were seeded in a 96-well plate in which each well contained 0.1 ml of culture medium. At 2 h before each time point (24 h, 48 h, and 72 h) post-seeding, 10 μl of WST-1 Premix solution was added to the cells and incubated at 37°C. Next, optical density was measured at an absorbance of 450 and 690 nm (A450–A690). Data were analyzed to determine proliferation and viability. Similarly, infected O/E-ST3, O/E-T2, and O/E-ST3T2 cells (1×10^4 cells per well) were seeded in a 96-well plate in which each well contained 0.1 ml of culture medium. One day later, confluent DF-1 cells were infected with PR8-H5N8 (PB2-627E) at a MOI of 0.1. Percentage

survival was calculated as the ratio of the optical absorbance at 450 and 690 nm (A450–A690) of infected DF-1 cells and non-infected control DF-1 cells. All experiments were performed in triplicate, with three independent samples.

Viral titration

The virus titer in infected O/E-ST3, O/E-T2, O/E-ST3T2 DF-1 cells, or WT DF-1 cells was determined by calculating the median tissue culture infectious dose ($TCID_{50}$). MDCK cells were cultured in Eagle's Minimum Essential Medium (EMEM) (ATCC, Manassas, VA, USA) supplemented with 10% fetal bovine serum (Hyclone) and 1x ABAM. For viral titration, MDCK cells (2.5×10^4 cells per well) were seeded in 96-well plates until confluence in culture media. Subsequently, the confluent layer of MDCK cells was washed with PBS, and treated with DMEM supplemented with 1% penicillin/streptomycin and infected with PR8-H5N8 (PB2-627E) from the O/E-ST3, O/E-T2, O/E-ST3T2 DF-1 cells, or WT DF-1 cells for 50 minutes at 37°C. Then, the MDCK cells was washed with PBS, and finally incubated with DMEM supplemented with 0.3% BSA, 1% penicillin/streptomycin, and 1 μg/ml TPCK-trypsin at 37°C. After 72–96 h, the plate was stained with crystal violet (Sigma-Aldrich) to observe cytopathic effects. The $TCID_{50}$ values per milliliter were calculated using the Spearman-

Karber formula (Gilles 1974).

Statistical analysis

Statistical analysis was performed using GraphPad Prism software (GraphPad Software 8, San Diego, CA). Significant differences between two groups were determined using Student's t-test. Significant differences between groups were determined using ANOVA with Bonferroni's multiple comparison. A P value <0.05 was considered significant (****P<0.0001, ***P<0.001, **P<0.01, and *P <0.05).

3. Results

Establishment of TMPRSS2- and TMPRSS4-overexpressing cell lines and subsequent viral challenge

The presence of trypsin-like proteases that cleave HA means that influenza viruses preferentially infect the respiratory and gastrointestinal tracts. Therefore, the distribution of TMPRSS2 and TMPRSS4 was compared in lung, trachea, liver, small intestine, and large intestine samples from WL chickens aged 18 weeks and WT DF-1 cells by qRT-PCR. It was found that compared with WT DF-1 cells, TMPRSS2 was expressed at high levels in liver, large intestine, and lung, whereas TMPRSS4 was expressed only in liver and trachea (**Fig. 4-1A**). The low expression of TMPRSS2 and TMPRSS4 by WT DF-1 cells suggests that they would not support viral replication efficiently.

Therefore, a piggyBac transposon vector was contructed that contained the protein-coding region for either chicken TMPRSS2 or TMPRSS4 (**Fig. 4-1B**). This vector was used to drive overexpression in DF-1 cells. Then the effects on cell viability and proliferation of the influenza virus was measured. Firstly, the expression of mRNA in genetically engineered cells was analyzed. The qRT-PCR results showed that overexpression of TMPRSS2 mRNA in O/E-T2 cells was 350-fold higher than that in WT DF-1 cells, whereas expression of TMPRSS4 mRNA in O/E-T4 cells was about 6-fold higher than that in

WT DF-1 (**Fig. 4-1C**). To assess whether overexpression of TMPRSS2 and TMPRSS4 had an antagonistic effect on cell proliferation and viability, a cell proliferation assay was conducted. The results showed that the proliferation of genetically engineered cells was comparable with that of WT DF-1 cells (**Fig. 4-1D**).

Subsequently, it was investigated whether the proteolytic activity of TMPRSS2 and TMPRSS4 supports viral infectivity and the viral life cycle. Engineered cells were infected with PR8-H5N8 (PB2-627E) (MOI = 0.1) in the absence of trypsin and the $TCID_{50}$ was calculated to determine the viral titer. Notably, the viral titer in O/E-T2 cells was 35-fold higher than that in WT DF-1 cells, indicating proteolytic activation of HA by TMPRSS2 and subsequent support of viral replication. Thus, the cell line is suitable for amplification of influenza virus. However, there was no significant difference in the viral titer between O/E-T4 cells overexpressing TMPRSS4 protease and WT-DF1 cells (**Fig. 4-1E**).

Establishment of ST3GAL1-overexpressing cells and determination of viral titer

Sialic acid residues on cell surface receptors are important for binding and endocytosis of influenza virus. Therefore, the expression of ST3GAL1 was examined in lung, trachea, liver, small intestine, and large intestine samples from WL chickens and compared it with that by

WT DF-1 using qRT-PCR. The results revealed that the expression of ST3GAL1 in trachea and lung was significantly higher than that by WT DF-1 cells (**Fig. 4-2A**).

Since WT DF-1 cells expressed lower levels of ST3GAL1, a piggyBac transposon vector was constructed containing the protein-coding sequence of chicken ST3GAL1 (**Fig. 4-2B**) and transfected it into WT DF-1 to engineer cells that express high levels of ST3GAL1. Subsequently, the expression of ST3GAL1 in O/E-ST3-overexpressing cells by qRT-PCR was analyzed. The results showed a 1500-fold increase in expression compared with that in WT DF-1 cells (**Fig. 4-2C**). To access whether cells overexpressing ST3GAL1 had an antagonistic effect on cell proliferation and viability, a cell proliferation assay was performed. The results revealed that proliferation of genetically engineered cells was comparable with that of WT DF-1 cells (**Fig. 4-2D**).

Finally, to examine whether increased expression of ST3GAL1 correlates positively with viral titer, O/E-ST3 cells were infected with PR8-H5N8 (PB2E-627E) at MOI of 0.1 in the presence of trypsin. The $TCID_{50}$ was calculated to determine the viral titer. The results showed the viral titer in O/E-ST3 cells was significantly higher than that in WT DF-1 cells (**Fig. 4-2E**).

Establishment of cell lines expressing both ST3GAL1 and TMPRSS2 and their effect on virus titers

To further investigate whether overexpressing both ST3GAL1 and TMPRSS2 (O/E-ST3T2) generates higher viral titers, a new piggyBac transposon vector was constructed containing the protein-coding sequences of ST3GAL1 and TMPRSS2 linked by the self-cleaving peptide T2A **(Fig. 4-3A**). Cells were transfected with the overexpression vector and analyzed by qRT-PCR. The results showed a 120-fold increase in expression of both ST3GAL1 and TMPRSS2 (**Fig. 4-3B**). To assess whether overexpression increased viral titers to levels comparable with those in WT DF-1 cells supplemented with trypsin, O/E-ST3T2 cells were infected with PR8-H5N8 (PB2-627E) at an MOI of 0.1 in the absence of trypsin. Then calculated the $TCID_{50}$ was calculated. Combined overexpression of ST3GAL1 and TMPRSS2 generated viral titers significantly higher than those in WT DF-1 cells in the absence of trypsin (WT DF-1(-)); however, the titers were not significantly different from those in WT cells treated with trypsin (WT DF-1(+)) (**Fig. 4-3C**).

To understand the effect of trypsin on cell viability after infection by the influenza virus, a cell proliferation assay was performed. The results revealed a significant difference in viability between WT DF-

1(+) and O/E-ST3T2 cells at 24 h and 48 h post-infection, but not at 72 h post-infection (**Fig. 4-3D**).

Finally, viral titers at different time points post-infection were examined. Intriguingly, the viral titer in O/E-ST3T2 cells was significantly higher than that in WT DF-1(+) at 72 h post-infection (**Fig. 4-3E**). Taken together, the results indicate that engineered O/E-ST3T2 cells were able to produce high viral titers in the absence of trypsin due to prolonged viability.

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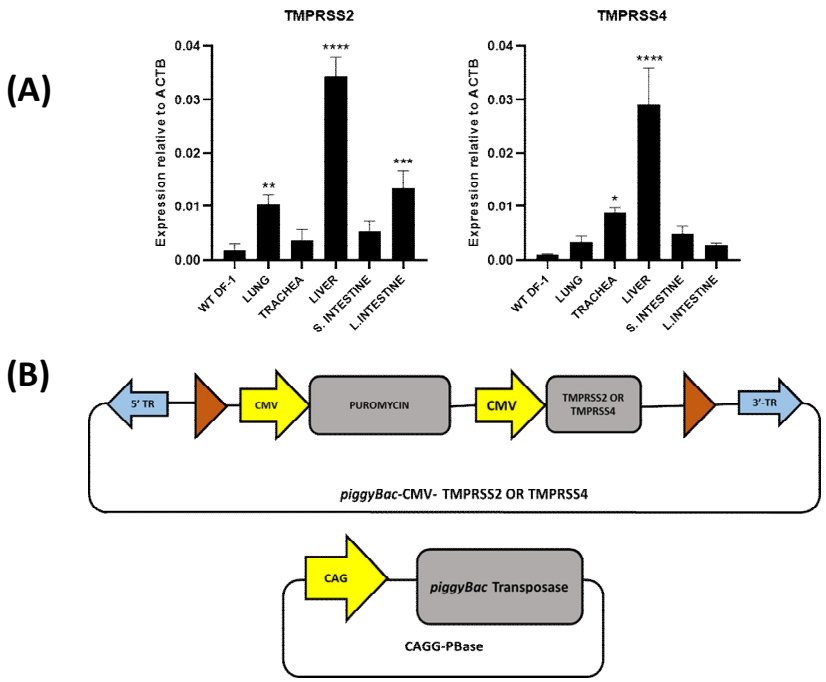

Figure 4-1. Establishment of TMPRSS2- and TMPRSS4-overexpressing cell lines and challenge with viruses. (A) Expression of TMPRSS2 and TMPRSS4 in various chicken tissues and WT DF-1, as measured by qRT-PCR. Data are normalized to the expression of chicken ACTB (beta-actin) and are expressed as the mean ± standard deviation (n = 3). Significant differences (compared with WT DF-1 cells) were determined by one-way ANOVA (****P<0.0001, ***P<0.001, **P<0.01, and *P <0.05). (B) Schematic representation of the piggyBac transposon based expression vector harboring TMPRSS2 or TMPRSS4. The vector was used to express either TMPRSS2 or TMPRSS4 in WT DF-1 cells, termed O/E-T2 or O/E-T4.

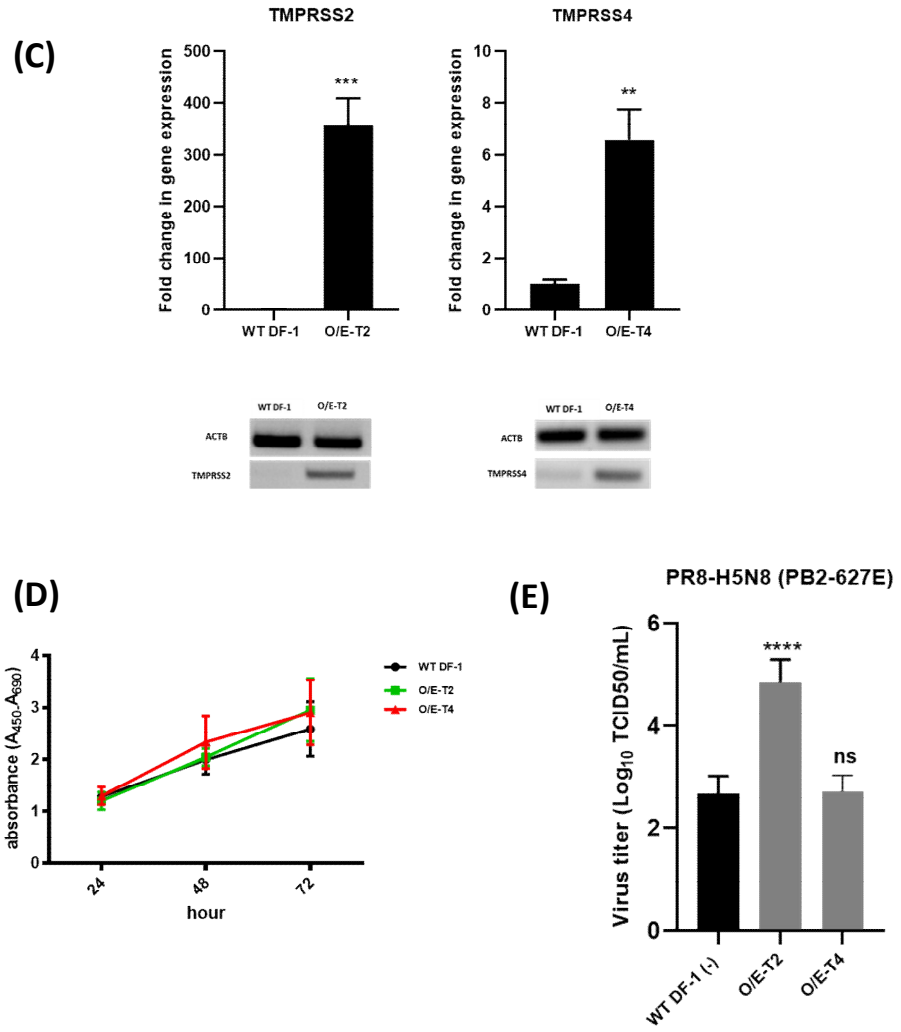

Figure 4-1. Establishment of TMPRSS2- and TMPRSS4-overexpressing cell lines and challenge with viruses. (C) Expression of TMPRSS2 and TMPRSS4 in O/E-T2 and O/E-T4 and in WT DF-1 cells, as measured by qRT-PCR. Data are normalized to the expression of chicken ACTB and are expressed as the mean ± standard deviation (n = 3). Significant differences (compared with WT DF-1 cells) were determined by Student's t-test (***P<0.001, **P<0.01, and *P <0.05). (D) Cell proliferation at 24 h, 48 h, and 72 h after seeding. Error bars

indicate the mean ± standard deviation of triplicate analyses. (E) Viral titer of PR8-H5N8 (PB2-627E) from O/E-T2 and O/E-T4 relative to that of WT DF-1 cells in the absence of TPCK-trypsin (WT DF-$1_{(-)}$) at 24 h post-infection. Significant differences (compared with WT DF-1 cells) were determined by one-way ANOVA (****$P<0.0001$, ns = no significant difference). Data are expressed as the mean ± standard deviation (n = 7).

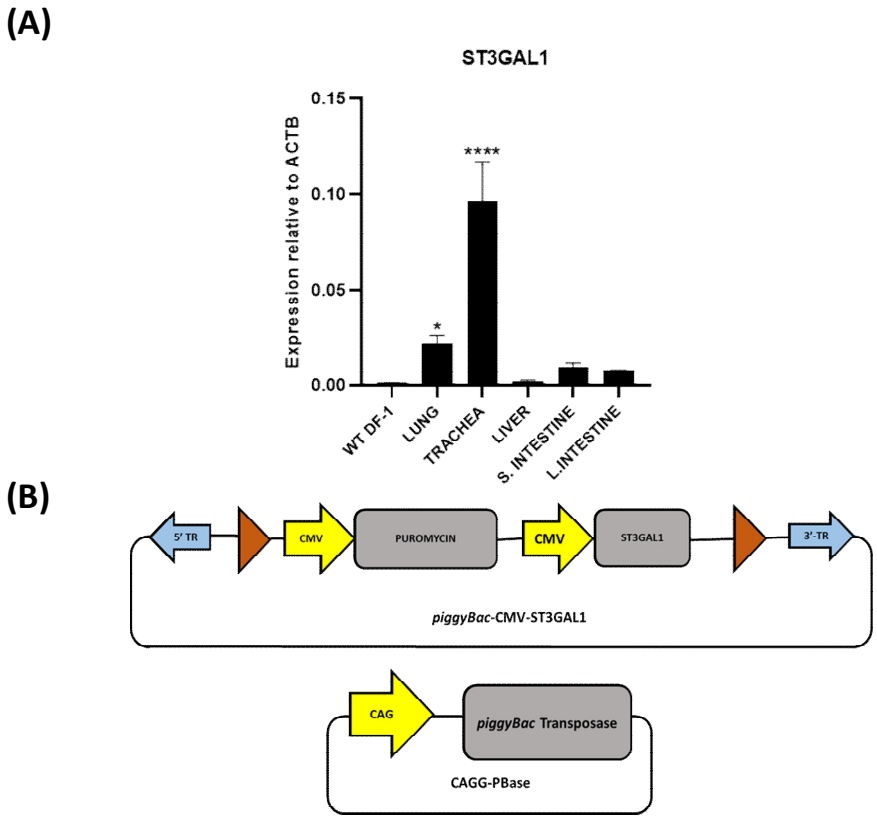

Figure 4-2. Establishment of ST3GAL1-overexpressing cell lines and challenge with viruses. (A) Comparison of ST3GAL1 expression in chicken tissues and WT DF-1 cells by qRT-PCR. Data were normalized to the expression of chicken ACTB and are expressed as the mean ± standard deviation (n = 3). Significant differences (compared with WT DF-1 cells) were determined by one-way ANOVA (****P<0.0001, and *P <0.05). (B) Schematic representation of the piggyBac transposon based expression vector harboring ST3GAL1. The vector was used to express ST3GAL1 in WT DF-1 cells, termed O/E-ST3.

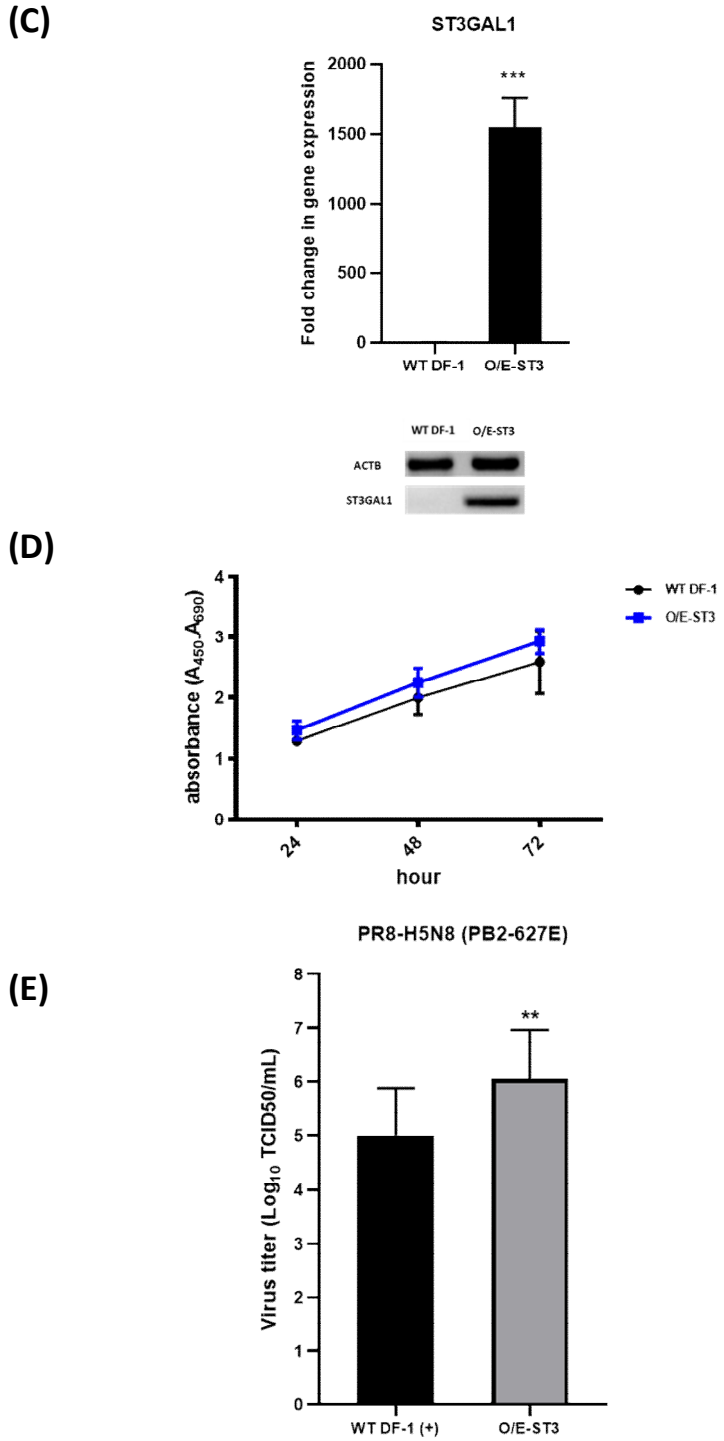

Figure 4-2. Establishment of ST3GAL1-overexpressing cell lines and challenge with viruses. (C) Expression of ST3GAL1 in O/E-ST3

and WT DF-1 cells, as detected by qRT-PCR. Data were normalized to the expression of chicken ACTB and are expressed as the mean ± standard deviation (n = 3). Significant differences (compared with WT DF-1 cells) were determined by Student's t-test (***P<0.001, **P<0.01, and *P <0.05). (D) Cell proliferation at 24 h, 48 h, and 72 h. Error bars indicate the mean ± standard deviation of triplicate analyses. (E) Relative titer of PR8-H5N8 (PB2-627E) in O/E-ST3 compared with that in WT DF-1 cells treated with trypsin at 24 h post-infection (WT DF-1$_{(+)}$). Significant differences were determined by Student's t-test (**P<0.01 and *P <0.05). Error bars indicate the mean ± standard deviation of triplicate analyses.

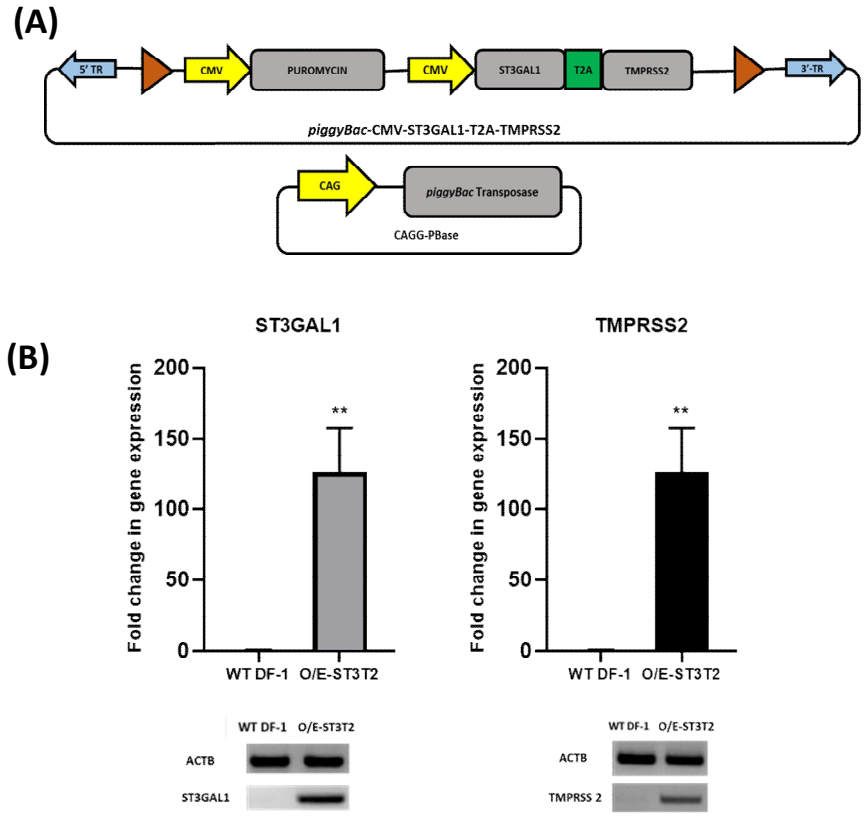

Figure 4-3. Combined overexpression of ST3GAL1 and TMPRSS2 and the resulting viral titer in cells. (A) Schematic representation of the piggyBac transposon based expression vector harboring ST3GAL1-*T2A*-TMPRSS2. The vector was used to express both ST3GAL1 and TMPRSS2 in WT DF-1 cells, termed O/E-ST3T2. (B) Expression of ST3GAL1 and TMPRSS2 in O/E-ST3T2 and WT DF-1 cells was analyzed by qRT-PCR. Data were normalized to the expression of chicken ACTB and expressed as the mean ± standard deviation (n = 3). Significant differences (compared with WT DF-1 cells) were determined using Student's t-test (**P<0.01 and *P <0.05).

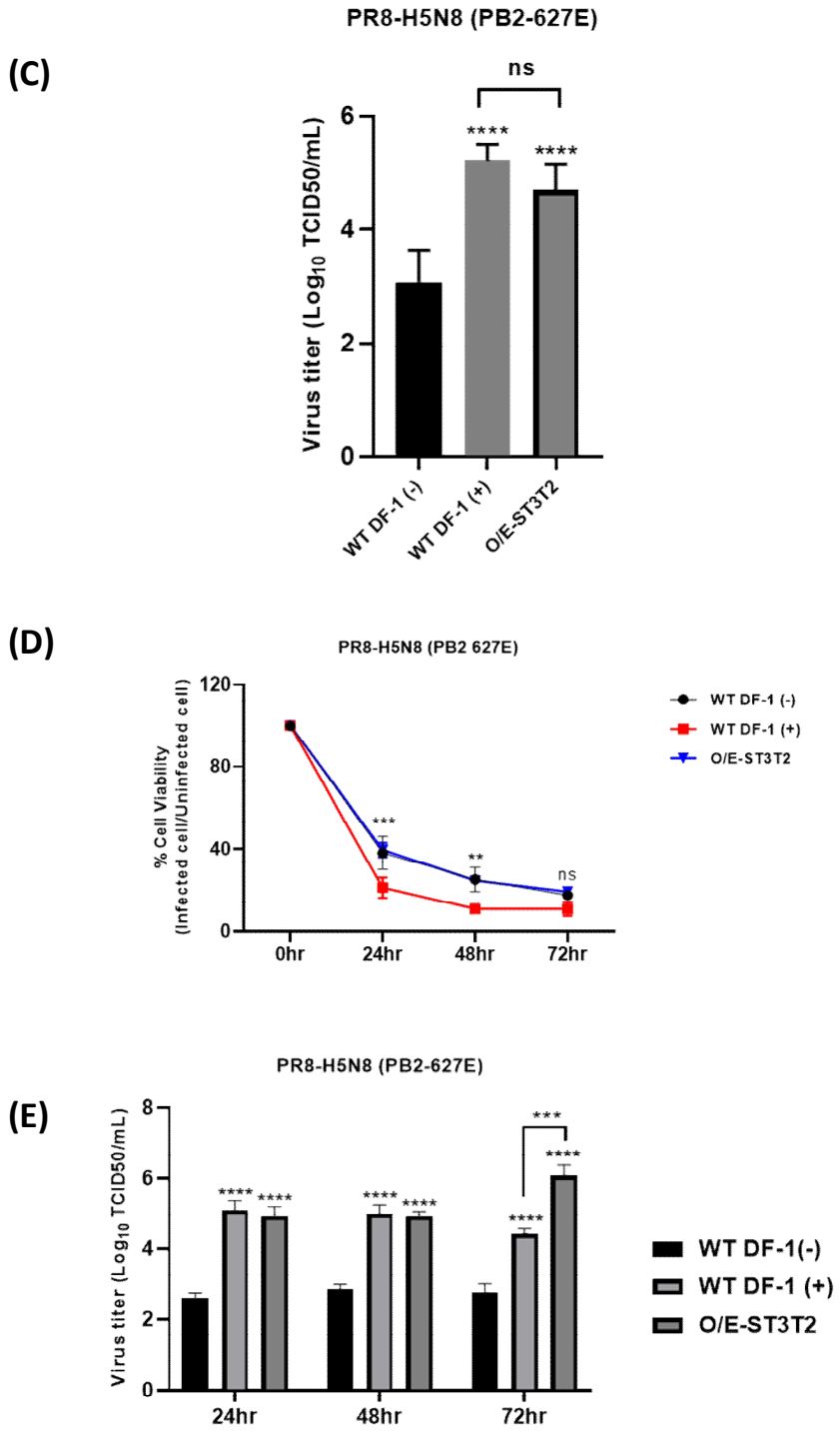

Figure 4-3. Combined overexpression of ST3GAL1 and TMPRSS2 and the resulting viral titer in cells. (C) Titer of PR8-H5N8 (PB2-

627E) in O/E-ST3T2 cells and WT DF-1 cells in the absence (WT DF-1(-)) and presence (WT DF-1(+)) of trypsin. (D) Cell proliferation at 24 h, 48 h, and 72 h post-infection. Error bars indicate the mean ± standard deviation of triplicate analyses. (E) Viral titer at 24 h, 48 h, and 72 h. Significant differences (compared with WT DF-1 cells) were determined by two-way ANOVA. A P value <0.05 was considered significant (****P<0.0001 and ***P<0.001). Error bars indicate the mean ± standard deviation of triplicate analyses.

Table 4-1. List of primers used for RT-PCR and qRT-PCR

Gene symbol	Primer sequence (5' - 3')	Product Size (bp)	Annealing Temperature
ACTB	F: AGGAGATCACAGCCCTGGCA R: CAATGGAGGGTCCGGATTCA	165	60
ST3GAL1	F: CACCCACCATTGGCTACGAA R: AGGCCTGTGGAAGGGTATCT	168	60
TMPRSS2	F: TTCTGCCAGGCCACAAGTAG R: GGAGAAATGCACACTCCCGA	297	60
TMPRSS4	F: TCCCCTCTGGATCCTCACTG R: TCCAGCTCCTCGTCGAAGTA	250	60

4. Discussion

Vaccine production in egg-based systems has many disadvantages (Wu et al. 2019; Audsley and Tannock, 2008); therefore, cell-based systems are a suitable alternative. Several cell lines have been established (Petiot et al., 2018) to meet different parameters for virus amplification. However, cell-based systems require the addition of exogenous trypsin to support viral replication. Furthermore, developing cell lines from different species is beneficial as it will obviate the need for virus adaptation.

Here, chicken DF-1 cells overexpressing chicken protease TMPRSS2 was established; this means that they do not require the addition of exogenous trypsin for virus amplification. Consistent with other reports (Böttcher et al., 2009; Baron et al., 2013; Limburg et al., 2019), it was found that cells expressing TMPRSS2 supported influenza replication without the need for trypsin. Studies show that HA is cleaved by TMPRSS2 at the plasma membrane during post-translational modification (Böttcher-Friebertshäuser et al., 2010). However, with respect to the expression of TMPRSS4, it was speculated that cells may not support viral replication in the absence of trypsin. Interestingly, overexpression of proteases was not toxic to cells, which grew as well as WT DF-1 cells, even after several serial passages.

Increased expression of sialic acid receptors on the cell surface

increases the uptake of influenza viruses by cells. Therefore, cells to overexpress ST3GAL1 were engineered. These cells generated high virus titers at 24 h post-infection, suggesting that high expression of α-2,3 linked sialic acid residues increased viral uptake. These results are consistent with those of other studies suggesting that abundant expression of sialic acid increases infection of host cells by influenza viruses (Kimble et al., 2010; van Riel et al., 2007).

Finally, it was shown that cells overexpressing both ST3GAL1 and TMPRSS2 generated greater viral titers than WT DF-1 cells treated with trypsin. Intriguingly, at 24 h and 48 h post-infection, virus titers were comparable with those in WT DF-1 cells treated with trypsin. However, the viability of overexpressing cells was superior at later time points, suggesting that cell viability had an effect on the final viral titers. As expected, viral titers were highest in O/E-ST3T2 cells at 72 h post-infection; this was most likely due to prolonged viability.

Conclusion

Here is was shown that genetically engineered chicken DF-1 cells overexpressing TMPRSS2 and ST3GAL1 support infection and replication of the influenza virus in the absence of trypsin. These cell lines could be useful for studying influenza virus replication and host responses in the absence of trypsin, which can degrade interferons

secreted by cultured cells. Furthermore, this system can be adapted to amplify influenza for vaccine production because avian-adapted influenza viruses do not require cell adaptation. Collectively, these cell lines can be added to the growing pool of cell-based systems useful for influenza virus amplification.

CHAPTER 5

GENERAL DISCUSSION

Influenza amplification is important, especially in vaccine production and in studies on various strains with host factors. The HA of influenza is critical for viral attachment to cell surface receptors and endosomal membrane fusion. To be infectious, the HA has to be cleaved by proteases. In the egg-based system, the HA is cleaved by different proteases present in the egg's allantoic fluid. Nonetheless, this system cannot amplify the desirable viral titer of some reassorted viral strains, and highly pathogenic strains also grow poorly in it. In this regard, the cell-based system has been a viable alternative for the amplification of the virus.

Nonetheless, the cell-based system requires the addition of trypsin to support the viral life cycle and thus amplification, which could raise the cost for production. Furthermore, some cell lines are dependent on trypsin thus requiring optimization. Therefore, cell engineering has been used as a viable way to eliminate the need for trypsin and virus adaptation.

In the first study, first the 250 bp region of the chicken polymerase I was identified from the origin of the rDNA transcription start site for cloning. The chicken polymerase I from the genomic DNA was cloned into a plasmid that could express both for mRNA by the CMV promoter and vRNA by the chicken polymerase I. Next, the viral genome of PB2, PB1, PA, and NP was codon optimized. The synthetic fragments were

then ligated into the plasmid under the chicken polymerase I promoter. Further, a plasmid with a luciferase reporter in the reverse direction under the chicken polymerase promoter I flanked by non-coding regions of the NS segment of the influenza virus was constructed. The plasmids were transfected into wild-type DF-1 cells, and the polymerase activity was accessed through the luciferase activity. In conclusion, in this study, a luciferase reporter assay was created that could be used to determine the polymerase activity of different strains of the influenza virus.

In the second study, the expression levels of serine proteases TMPRSS2 and TMPRSS4 in some chicken tissues compared to wild-type DF-1 cells (WT DF-1) were examined. It was found that the expression levels in WT DF-1 cells were remarkably low compared to the lung and trachea, respectively. These results suggest that WT DF-1 cells poorly support the replication of the influenza virus. Previous studies have shown that the expression of proteases in MDCK supported virus amplification even in the absence of trypsin. To understand the expression of TMPRSS2 and TMPRSS4, the protein-coding regions of TMPRSS2 and TMPRSS4 were ligated into piggyBac vectors and transfected them into the cells. After selection and recovery, TMPRSS2 was significantly higher compared to

TMPRSS4 expression in the engineered cells. Intriguingly, after examination for cell proliferation, both engineered cell lines proliferated the same as WT DF-1. Then, the cells were infected with PR8-H5N8 (PB2-627E) and noticed that only the overexpressing cells of TMPRSS2 had a significant viral titter compared to WT DF-1 and cell line overexpressing TMPRSS4. These results suggest that TMPRSS2 proteolytically activated the HA, thus supporting virus amplification even in the absence of trypsin. To understand whether the increased expression of ST3GAL1 could positively be correlated with an increased viral titer, first, the expression of ST3GAL1 in different chicken tissues was examined compared to WT DF-1. It was shown that the lung and trachea expressed significantly higher levels of ST3GAL1 compared to WT DF-1. Therefore, a piggyBac vector was constructed to overexpress ST3GAL1. It was found that ST3GAL1 was highly expressed, and the cell proliferation assay revealed that overexpression had no antagonistic impact on the cells. The impact of ST3GAL1 on the viral titer was investigated and found that overexpressing cells had a significantly higher viral titer compared to WT DF-1. Finally, if the combinatory effect of TMPRSS2 and ST3GAL1 could increase viral titer was investigated. After transfection and recovery of the cell line, the gene expression was analyzed and found both TMPRSS2 and ST3GAL1 were significantly increased.

Then, the established cell line (O/E-ST3T2) was infected with PR8-H5N8 (PB2-627E) in the absence of trypsin, and the results revealed that the combinatory expression of TMPRSS2 and ST3GAL1 had a significantly higher final virus titer. Collectively, these results showed that the genetically engineered cells of TMPRSS2 and those of the combinatory TMPRSS2-ST3GAL1 could be used for influenza virus amplification in the absence of trypsin.

REFERENCES

Ahn, Insung, and Hyeon-Seok Son. 2010. 'Comparative study of the nucleotide bias between the novel H1N1 and H5N1 subtypes of influenza A viruses using bioinformatics techniques', *J Microbiol Biotechnol*, 20: 63-70.

Ahn, Insung, and Hyeon Seok Son. 2012. 'Evolutionary analysis of human-origin influenza A virus (H3N2) genes associated with the codon usage patterns since 1993', *Virus genes*, 44: 198-206.

Audsley, Jennifer M, and Gregory A Tannock. 2008. 'Cell-based influenza vaccines', *Drugs*, 68: 1483-91.

Baron, Joanna, Carolin Tarnow, Deborah Mayoli-Nüssle, Eva Schilling, Daniela Meyer, Maya Hammami, Folker Schwalm, Torsten Steinmetzer, Yi Guan, and Wolfgang Garten. 2013. 'Matriptase, HAT, and TMPRSS2 activate the hemagglutinin of H9N2 influenza A viruses', *Journal of virology*, 87: 1811-20.

Baudin, F, C Bach, S Cusack, and RW Ruigrok. 1994. 'Structure of influenza virus RNP. I. Influenza virus nucleoprotein melts secondary structure in panhandle RNA and exposes the bases to the solvent', *The EMBO journal*, 13: 3158-65.

Bertram, Stephanie, Ilona Glowacka, Paulina Blazejewska, Elizabeth Soilleux, Paul Allen, Simon Danisch, Imke Steffen, So-Young Choi, Youngwoo Park, and Heike Schneider. 2010. 'TMPRSS2 and TMPRSS4 facilitate trypsin-independent spread of influenza virus in Caco-2 cells', *Journal of virology*, 84: 10016-25.

Bosch, FX, W Garten, H-D Klenk, and R Rott. 1981. 'Proteolytic cleavage of influenza virus hemagglutinins: primary structure of the connecting peptide between HA1 and HA2 determines proteolytic cleavability and pathogenicity of Avian influenza viruses', *Virology*, 113: 725-35.

Böttcher-Friebertshäuser, Eva, Catharina Freuer, Frank Sielaff, Sarah Schmidt, Markus Eickmann, Jennifer Uhlendorff, Torsten Steinmetzer, Hans-Dieter Klenk, and Wolfgang Garten. 2010. 'Cleavage of influenza virus hemagglutinin by airway proteases TMPRSS2 and HAT differs in subcellular localization and susceptibility to protease inhibitors', *Journal of virology*, 84:

5605-14.

Böttcher-Friebertshäuser, Eva, Hans-Dieter Klenk, and Wolfgang Garten. 2013. 'Activation of influenza viruses by proteases from host cells and bacteria in the human airway epithelium', *Pathogens and disease*, 69: 87-100.

Böttcher, Eva, Catharina Freuer, Torsten Steinmetzer, Hans-Dieter Klenk, and Wolfgang Garten. 2009. 'MDCK cells that express proteases TMPRSS2 and HAT provide a cell system to propagate influenza viruses in the absence of trypsin and to study cleavage of HA and its inhibition', *Vaccine*, 27: 6324-29.

Bouvier, N. M., and P. Palese. 2008. 'The biology of influenza viruses', *Vaccine*, 26 Suppl 4: D49-53.

Chen, Weisan, Paul A. Calvo, Daniela Malide, James Gibbs, Ulrich Schubert, Igor Bacik, Sameh Basta, Robert O'Neill, Jeanne Schickli, Peter Palese, Peter Henklein, Jack R. Bennink, and Jonathan W. Yewdell. 2001. 'A novel influenza A virus mitochondrial protein that induces cell death', *Nature Medicine*, 7: 1306-12.

Chen, Yao-Qing, Teddy John Wohlbold, Nai-Ying Zheng, Min Huang, Yunping Huang, Karlynn E Neu, Jiwon Lee, Hongquan Wan, Karla Thatcher Rojas, and Ericka Kirkpatrick. 2018. 'Influenza infection in humans induces broadly cross-reactive and protective neuraminidase-reactive antibodies', *Cell*, 173: 417-29. e10.

Chou, Yi-ying, Reza Vafabakhsh, Sultan Doğanay, Qinshan Gao, Taekjip Ha, and Peter Palese. 2012. 'One influenza virus particle packages eight unique viral RNAs as shown by FISH analysis', *Proceedings of the National Academy of Sciences*, 109: 9101-06.

Claas, Eric CJ, Albert DME Osterhaus, Ruud Van Beek, Jan C De Jong, Guus F Rimmelzwaan, Dennis A Senne, Scott Krauss, Kennedy F Shortridge, and Robert G Webster. 1998. 'Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus', *The Lancet*, 351: 472-77.

Compans, Richard W, Jean Content, and Peter H Duesberg. 1972. 'Structure of the ribonucleoprotein of influenza virus', *Journal of virology*, 10: 795-800.

Cros, Jerome F, Adolfo García□Sastre, and Peter Palese. 2005. 'An unconventional NLS is critical for the nuclear import of the influenza A virus nucleoprotein and ribonucleoprotein', *Traffic*, 6: 205-13.

De Jong, JC, ECJ Claas, Albert DME Osterhaus, Robert G Webster, and WL Lim. 1997. 'A pandemic warning?', *Nature*, 389: 554.

De Vries, Erik, Donna M Tscherne, Marleen J Wienholts, Viviana Cobos-Jiménez, Florine Scholte, Adolfo García-Sastre, Peter JM Rottier, and Cornelis AM De Haan. 2011. 'Dissection of the influenza A virus endocytic routes reveals macropinocytosis as an alternative entry pathway', *PLoS pathogens*, 7: e1001329.

de Wit, Emmie, Monique IJ Spronken, Gaby Vervaet, Guus F Rimmelzwaan, Albert DME Osterhaus, and Ron AM Fouchier. 2007. 'A reverse-genetics system for Influenza A virus using T7 RNA polymerase', *Journal of General Virology*, 88: 1281-87.

Dou, Dan, Rebecca Revol, Henrik Östbye, Hao Wang, and Robert Daniels. 2018. 'Influenza A virus cell entry, replication, virion assembly and movement', *Frontiers in immunology*, 9.

Engelhardt, Othmar G. 2013. 'Many ways to make an influenza virus–review of influenza virus reverse genetics methods', *Influenza and other respiratory viruses*, 7: 249-56.

Fodor, Ervin, Louise Devenish, Othmar G Engelhardt, Peter Palese, George G Brownlee, and Adolfo García-Sastre. 1999. 'Rescue of influenza A virus from recombinant DNA', *Journal of virology*, 73: 9679-82.

Fouchier, R. A., V. Munster, A. Wallensten, T. M. Bestebroer, S. Herfst, D. Smith, G. F. Rimmelzwaan, B. Olsen, and A. D. Osterhaus. 2005. 'Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls', *J Virol*, 79: 2814-22.

Fujii, Yutaka, Hideo Goto, Tokiko Watanabe, Tetsuya Yoshida, and Yoshihiro Kawaoka. 2003. 'Selective incorporation of influenza virus RNA segments into virions', *Proceedings of the National Academy of Sciences*, 100: 2002-07.

Fukuda, Makoto, Shiro Asano, Takahiro Nakamura, Makoto Adachi, Minoru Yoshida, Mitsuhiro Yanagida, and Eisuke Nishida. 1997.

'CRM1 is responsible for intracellular transport mediated by the nuclear export signal', *Nature*, 390: 308.

Gabriel, G., K. Klingel, A. Otte, S. Thiele, B. Hudjetz, G. Arman-Kalcek, M. Sauter, T. Shmidt, F. Rother, S. Baumgarte, B. Keiner, E. Hartmann, M. Bader, G. G. Brownlee, E. Fodor, and H. D. Klenk. 2011. 'Differential use of importin-alpha isoforms governs cell tropism and host adaptation of influenza virus', *Nat Commun*, 2: 156.

Galloway, Summer E, Mark L Reed, Charles J Russell, and David A Steinhauer. 2013. 'Influenza HA subtypes demonstrate divergent phenotypes for cleavage activation and pH of fusion: implications for host range and adaptation', *PLoS pathogens*, 9: e1003151.

Gao, Huijie, Yipeng Sun, Jiao Hu, Lu Qi, Jinliang Wang, Xin Xiong, Yu Wang, Qiming He, Yang Lin, and Weili Kong. 2015. 'The contribution of PA-X to the virulence of pandemic 2009 H1N1 and highly pathogenic H5N1 avian influenza viruses', *Scientific reports*, 5: 8262.

Gao, Rongbao, Bin Cao, Yunwen Hu, Zijian Feng, Dayan Wang, Wanfu Hu, Jian Chen, Zhijun Jie, Haibo Qiu, and Ke Xu. 2013. 'Human infection with a novel avian-origin influenza A (H7N9) virus', *New England Journal of Medicine*, 368: 1888-97.

García-Sastre, Adolfo. 2001. 'Inhibition of interferon-mediated antiviral responses by influenza A viruses and other negative-strand RNA viruses', *Virology*, 279: 375-84.

Genzel, Yvonne, Ilona Behrendt, Jana Rödig, Erdmann Rapp, Claudia Kueppers, Stefan Kochanek, Gudrun Schiedner, and Udo Reichl. 2013. 'CAP, a new human suspension cell line for influenza virus production', *Applied microbiology and biotechnology*, 97: 111-22.

Genzel, Yvonne, Christian Dietzsch, Erdmann Rapp, Jana Schwarzer, and Udo Reichl. 2010. 'MDCK and Vero cells for influenza virus vaccine production: a one-to-one comparison up to lab-scale bioreactor cultivation', *Applied microbiology and biotechnology*, 88: 461-75.

Gilles, HJ. 1974. 'Calculation of the index of acute toxicity by the method of linear regression. Comparison with the method of"

Karber and Behrens'", *European journal of toxicology and environmental hygiene. Journal europeen de toxicologie*, 7: 77.

Goñi, Natalia, Andrés Iriarte, Victoria Comas, Martín Soñora, Pilar Moreno, Gonzalo Moratorio, Héctor Musto, and Juan Cristina. 2012. 'Pandemic influenza A virus codon usage revisited: biases, adaptation and implications for vaccine strain development', *Virology journal*, 9: 263.

Hai, Rong, Florian Krammer, Gene S Tan, Natalie Pica, Dirk Eggink, Jad Maamary, Irina Margine, Randy A Albrecht, and Peter Palese. 2012. 'Influenza viruses expressing chimeric hemagglutinins: globular head and stalk domains derived from different subtypes', *Journal of virology*, 86: 5774-81.

Hamilton, Brian S, Gary R Whittaker, and Susan Daniel. 2012. 'Influenza virus-mediated membrane fusion: determinants of hemagglutinin fusogenic activity and experimental approaches for assessing virus fusion', *Viruses*, 4: 1144-68.

Hayashi, Tsuyoshi, Leslie A MacDonald, and Toru Takimoto. 2015. 'Influenza A virus protein PA-X contributes to viral growth and suppression of the host antiviral and immune responses', *Journal of virology*, 89: 6442-52.

Heix, Jutta, and Ingrid Grummt. 1995. 'Species specificity of transcription by RNA polymerase I', *Current opinion in genetics & development*, 5: 652-56.

Hempel, William M, Alice H Cavanaugh, Ross D Hannan, Laura Taylor, and Lawrence I Rothblum. 1996. 'The species-specific RNA polymerase I transcription factor SL-1 binds to upstream binding factor', *Molecular and cellular biology*, 16: 557-63.

Hershberg, Ruth, and Dmitri A Petrov. 2008. 'Selection on codon bias', *Annual review of genetics*, 42: 287-99.

Hoffmann, Erich, Gabriele Neumann, Gerd Hobom, Robert G Webster, and Yoshihiro Kawaoka. 2000. '"Ambisense" approach for the generation of influenza A virus: vRNA and mRNA synthesis from one template', *Virology*, 267: 310-17.

Hoffmann, Erich, Gabriele Neumann, Yoshihiro Kawaoka, Gerd Hobom, and Robert G Webster. 2000. 'A DNA transfection system for generation of influenza A virus from eight plasmids',

Proceedings of the National Academy of Sciences, 97: 6108-13.

Hoffmann, Erich, and Robert G Webster. 2000. 'Unidirectional RNA polymerase I–polymerase II transcription system for the generation of influenza A virus from eight plasmids', *Journal of General Virology*, 81: 2843-47.

Horimoto, Taisuke, Kazuhisa Nakayama, Steven P Smeekens, and Yoshihiro Kawaoka. 1994. 'Proprotein-processing endoproteases PC6 and furin both activate hemagglutinin of virulent avian influenza viruses', *Journal of virology*, 68: 6074-78.

Hu, Jiao, Yiqun Mo, Xiaoquan Wang, Min Gu, Zenglei Hu, Lei Zhong, Qiwen Wu, Xiaoli Hao, Shunlin Hu, and Wenbo Liu. 2015. 'PA-X decreases the pathogenicity of highly pathogenic H5N1 influenza A virus in avian species by inhibiting virus replication and host response', *Journal of virology*, 89: 4126-42.

Jiang, Yongping, Kangzhen Yu, Hongbo Zhang, Pingjing Zhang, Chenjun Li, Guobin Tian, Yanbing Li, Xijun Wang, Jinying Ge, and Zhigao Bu. 2007. 'Enhanced protective efficacy of H5 subtype avian influenza DNA vaccine with codon optimized HA gene in a pCAGGS plasmid vector', *Antiviral research*, 75: 234-41.

Kim, Eun-Ha, Hyeok-Il Kwon, Su-Jin Park, Young-Il Kim, Young-Jae Si, In-Won Lee, Se Mi Kim, Soo-In Kim, Dong-Ho Ahn, and Young-Ki Choi. 2018. 'Generation of a High-Growth Influenza Vaccine Strain in MDCK Cells for Vaccine Preparedness', *Journal of microbiology and biotechnology*, 28: 997-1006.

Kimble, Brian, Gloria Ramirez Nieto, and Daniel R Perez. 2010. 'Characterization of influenza virus sialic acid receptors in minor poultry species', *Virology journal*, 7: 365.

Klenk, Hans-Dieter, Rudolf Rott, Michaela Orlich, and Jochen Blödorn. 1975. 'Activation of influenza A viruses by trypsin treatment', *Virology*, 68: 426-39.

Kobasa, Darwyn, Shantha Kodihalli, Ming Luo, Maria R Castrucci, Isabella Donatelli, Yasuo Suzuki, Takashi Suzuki, and Yoshihiro Kawaoka. 1999. 'Amino acid residues contributing to the substrate specificity of the influenza A virus neuraminidase', *Journal of virology*, 73: 6743-51.

Koudstaal, W, L Hartgroves, M Havenga, I Legastelois, C Ophorst, M Sieuwerts, D Zuijdgeest, R Vogels, J Custers, and E de Boer-Luijtze. 2009. 'Suitability of PER. C6® cells to generate epidemic and pandemic influenza vaccine strains by reverse genetics', *Vaccine*, 27: 2588-93.

Krammer, Florian, and Peter Palese. 2013. 'Influenza virus hemagglutinin stalk-based antibodies and vaccines', *Current opinion in virology*, 3: 521-30.

———. 2015. 'Advances in the development of influenza virus vaccines', *Nature reviews Drug discovery*, 14: 167-82.

Kurland, CG. 1991. 'Codon bias and gene expression', *FEBS letters*, 285: 165-69.

Lamb, Robert A, Purnell W Choppin, Robert M Chanock, and Ching-Juh Lai. 1980. 'Mapping of the two overlapping genes for polypeptides NS1 and NS2 on RNA segment 8 of influenza virus genome', *Proceedings of the National Academy of Sciences*, 77: 1857-61.

Lamb, Robert A, Ching-Juh Lai, and Purnell W Choppin. 1981. 'Sequences of mRNAs derived from genome RNA segment 7 of influenza virus: colinear and interrupted mRNAs code for overlapping proteins', *Proceedings of the National Academy of Sciences*, 78: 4170-74.

Lazarowitz, Sondra G, and Purnell W Choppin. 1975. 'Enhancement of the infectivity of influenza A and B viruses by proteolytic cleavage of the hemagglutinin polypeptide', *Virology*, 68: 440-54.

Le Ru, Audrey, Danielle Jacob, Julia Transfiguracion, Sven Ansorge, Olivier Henry, and Amine A Kamen. 2010. 'Scalable production of influenza virus in HEK-293 cells for efficient vaccine manufacturing', *Vaccine*, 28: 3661-71.

Lee, C-W, K Jung, SJ Jadhao, and DL Suarez. 2008. 'Evaluation of chicken-origin (DF-1) and quail-origin (QT-6) fibroblast cell lines for replication of avian influenza viruses', *Journal of virological methods*, 153: 22-28.

Lee, C. W., and Y. M. Saif. 2009. 'Avian influenza virus', *Comp Immunol Microbiol Infect Dis*, 32: 301-10.

Limburg, Hannah, Anne Harbig, Dorothea Bestle, David A Stein, Hong M Moulton, Julia Jaeger, Harshavardhan Janga, Kornelia Hardes, Janine Koepke, and Leon Schulte. 2019. 'TMPRSS2 is the major activating protease of influenza A virus in primary human airway cells and influenza B virus in human type II pneumocytes', *Journal of virology*, 93: e00649-19.

Lindstrom, Stephen E, Yasuaki Hiromoto, Reiko Nerome, Katsuhiko Omoe, Shigeo Sugita, Yoshinao Yamazaki, Tomoko Takahashi, and Kuniaki Nerome. 1998. 'Phylogenetic analysis of the entire genome of influenza A (H3N2) viruses from Japan: evidence for genetic reassortment of the six internal genes', *Journal of virology*, 72: 8021-31.

Lohr, V, Y Genzel, I Behrendt, K Scharfenberg, and U Reichl. 2010. 'A new MDCK suspension line cultivated in a fully defined medium in stirred-tank and wave bioreactor', *Vaccine*, 28: 6256-64.

Luczo, J. M., M. Tachedjian, J. A. Harper, J. S. Payne, J. M. Butler, S. I. Sapats, S. L. Lowther, W. P. Michalski, J. Stambas, and J. Bingham. 2018a. 'Evolution of high pathogenicity of H5 avian influenza virus: haemagglutinin cleavage site selection of reverse-genetics mutants during passage in chickens', *Sci Rep*, 8: 11518.

Luczo, Jasmina M, Mary Tachedjian, Jennifer A Harper, Jean S Payne, Jeffrey M Butler, Sandra I Sapats, Suzanne L Lowther, Wojtek P Michalski, John Stambas, and John Bingham. 2018b. 'Evolution of high pathogenicity of H5 avian influenza virus: haemagglutinin cleavage site selection of reverse-genetics mutants during passage in chickens', *Scientific reports*, 8: 11518.

Lutz, Andrew, Julie Dyall, Paul D Olivo, and Andrew Pekosz. 2005. 'Virus-inducible reporter genes as a tool for detecting and quantifying influenza A virus replication', *Journal of virological methods*, 126: 13-20.

Luytjes, Willem, Mark Krystal, Masayoshi Enami, Jeffrey D Parvin, and Peter Palese. 1989. 'Amplification, expression, and packaging of a foreign gene by influenza virus', *Cell*, 59: 1107-13.

Mahy, John W. McCauley and Brian W. J. 1983. 'Structure and function of the influenza virus genome', *Biochem. J.*, 211: 13.

Manicassamy, Balaji, Santhakumar Manicassamy, Alan Belicha-Villanueva, Giuseppe Pisanelli, Bali Pulendran, and Adolfo García-Sastre. 2010. 'Analysis of in vivo dynamics of influenza virus infection in mice using a GFP reporter virus', *Proceedings of the National Academy of Sciences*, 107: 11531-36.

Marsh, Glenn A, Raheleh Hatami, and Peter Palese. 2007. 'Specific residues of the influenza A virus hemagglutinin viral RNA are important for efficient packaging into budding virions', *Journal of virology*, 81: 9727-36.

Massin, Pascale, Pierre Rodrigues, Monica Marasescu, Sylvie van der Werf, and Nadia Naffakh. 2005. 'Cloning of the chicken RNA polymerase I promoter and use for reverse genetics of influenza A viruses in avian cells', *Journal of virology*, 79: 13811-16.

Matrosovich, Mikhail N, Tatyana Y Matrosovich, Thomas Gray, Noel A Roberts, and Hans-Dieter Klenk. 2004. 'Human and avian influenza viruses target different cell types in cultures of human airway epithelium', *Proceedings of the National Academy of Sciences*, 101: 4620-24.

Matrosovich, Mikhail, Alexander Tuzikov, Nikolai Bovin, Alexandra Gambaryan, Alexander Klimov, Maria R Castrucci, Isabella Donatelli, and Yoshihiro Kawaoka. 2000. 'Early alterations of the receptor-binding properties of H1, H2, and H3 avian influenza virus hemagglutinins after their introduction into mammals', *Journal of virology*, 74: 8502-12.

Matrosovich, MN, AS Gambaryan, S Teneberg, VE Piskarev, SS Yamnikova, DK Lvov, JS Robertson, and K-A Karlsson. 1997. 'Avian influenza A viruses differ from human viruses by recognition of sialyloligosaccharides and gangliosides and by a higher conservation of the HA receptor-binding site', *Virology*, 233: 224-34.

Matthews, James T. 2006. 'Egg-based production of influenza vaccine: 30 years of commercial experience', *BRIDGE-WASHINGTON-NATIONAL ACADEMY OF ENGINEERING-*, 36: 17.

Mitnaul, Lyndon J, Mikhail N Matrosovich, Maria R Castrucci, Alexander B Tuzikov, Nikolai V Bovin, Darwyn Kobasa, and Yoshihiro Kawaoka. 2000. 'Balanced hemagglutinin and neuraminidase activities are critical for efficient replication of influenza A virus', *Journal of virology*, 74: 6015-20.

Moncorgé, Olivier, Jason S Long, Anna V Cauldwell, Hongbo Zhou, Samantha J Lycett, and Wendy S Barclay. 2013. 'Investigation of influenza virus polymerase activity in pig cells', *Journal of virology*, 87: 384-94.

Muster, Thomas, Andrej Egorov, and Markus Wolschek. 2014. "Method for generation of RNA virus." In.: Google Patents.

Nadler, Steven G, Douglas Tritschler, Omar K Haffar, James Blake, A Gregory Bruce, and Jeffrey S Cleaveland. 1997. 'Differential expression and sequence-specific interaction of karyopherin α with nuclear localization sequences', *Journal of Biological Chemistry*, 272: 4310-15.

Nayak, Debi P, Rilwan A Balogun, Hiroshi Yamada, Z Hong Zhou, and Subrata Barman. 2009. 'Influenza virus morphogenesis and budding', *Virus research*, 143: 147-61.

Neumann, Gabriele, Ken Fujii, Yoichiro Kino, and Yoshihiro Kawaoka. 2005. 'An improved reverse genetics system for influenza A virus generation and its implications for vaccine production', *Proceedings of the National Academy of Sciences*, 102: 16825-29.

Neumann, Gabriele, and Yoshihiro Kawaoka. 2002. 'Generation of influenza A virus from cloned cDNAs—historical perspective and outlook for the new millenium', *Reviews in medical virology*, 12: 13-30.

Neumann, Gabriele, Takeshi Noda, and Yoshihiro Kawaoka. 2009. 'Emergence and pandemic potential of swine-origin H1N1 influenza virus', *Nature*, 459: 931.

Neumann, Gabriele, Tokiko Watanabe, Hiroshi Ito, Shinji Watanabe, Hideo Goto, Peng Gao, Mark Hughes, Daniel R Perez, Ruben Donis, and Erich Hoffmann. 1999. 'Generation of influenza A viruses entirely from cloned cDNAs', *Proceedings of the National Academy of Sciences*, 96: 9345-50.

Nogales, Aitor, Steven F Baker, Emilio Ortiz-Riaño, Stephen Dewhurst, David J Topham, and Luis Martínez-Sobrido. 2014. 'Influenza A virus attenuation by codon deoptimization of the NS gene for vaccine development', *Journal of virology*, 88: 10525-40.

Ozawa, Makoto, Junko Maeda, Kiyoko Iwatsuki-Horimoto, Shinji

Watanabe, Hideo Goto, Taisuke Horimoto, and Yoshihiro Kawaoka. 2009. 'Nucleotide sequence requirements at the 5′ end of the influenza A virus M RNA segment for efficient virus replication', *Journal of virology*, 83: 3384-88.

Palese, Peter, and Jerome L Schulman. 1976. 'Mapping of the influenza virus genome: identification of the hemagglutinin and the neuraminidase genes', *Proceedings of the National Academy of Sciences*, 73: 2142-46.

Park, Young Hyun, Kelly Chungu, Su Bin Lee, Seung Je Woo, Ho Yeon Cho, Hong Jo Lee, Deivendran Rengaraj, Ji-Ho Lee, Chang-Seon Song, and Jeong Mook Lim. 2019. 'Host-specific restriction of avian influenza virus caused by differential dynamics of ANP32 family members', *The Journal of Infectious Diseases*, 221: 71 - 80.

Pau, MG, C Ophorst, Martin H Koldijk, G Schouten, M Mehtali, and Fons Uytdehaag. 2001. 'The human cell line PER. C6 provides a new manufacturing system for the production of influenza vaccines', *Vaccine*, 19: 2716-21.

Paule, Marvin R, and Robert J White. 2000. 'Survey and summary transcription by RNA polymerases I and III', *Nucleic acids research*, 28: 1283-98.

Petiot, Emma, Anaïs Proust, Aurélien Traversier, Laurent Durous, Frédéric Dappozze, Marianne Gras, Chantal Guillard, Jean-Marc Balloul, and Manuel Rosa-Calatrava. 2018. 'Influenza viruses production: Evaluation of a novel avian cell line DuckCelt®-T17', *Vaccine*, 36: 3101-11.

Pinto, Lawrence H, Leslie J Holsinger, and Robert A Lamb. 1992. 'Influenza virus M2 protein has ion channel activity', *Cell*, 69: 517-28.

Pleschka, Stephan, R Jaskunas, Othmar G Engelhardt, T Zürcher, Peter Palese, and Adolfo Garcia-Sastre. 1996. 'A plasmid-based reverse genetics system for influenza A virus', *Journal of virology*, 70: 4188-92.

Plotkin, Joshua B, and Jonathan Dushoff. 2003. 'Codon bias and frequency-dependent selection on the hemagglutinin epitopes of influenza A virus', *Proceedings of the National Academy of Sciences*, 100: 7152-57.

Quax, Tessa EF, Nico J Claassens, Dieter Söll, and John van der Oost. 2015. 'Codon bias as a means to fine-tune gene expression', *Molecular cell*, 59: 149-61.

Reuther, Peter, Kristina Göpfert, Alexandra H Dudek, Monika Heiner, Susanne Herold, and Martin Schwemmle. 2015. 'Generation of a variety of stable Influenza A reporter viruses by genetic engineering of the NS gene segment', *Scientific reports*, 5: 11346.

Robert G. Webster, William J. Bean, Owen T. Gorman, Thomas M. Chambers, and Yoshihiro Kawaoka. 1992. 'Evolution andd ecology of influenza A viruses', *Microbiological Reviews*, 56: 27.

Rogers, Gary N, Thomas J Pritchett, Jeri L Lane, and James C Paulson. 1983. 'Differential sensitivity of human, avian, and equine influenza A viruses to a glycoprotein inhibitor of infection: selection of receptor specific variants', *Virology*, 131: 394-408.

Russell, Charles J, Meng Hu, and Faten A Okda. 2018. 'Influenza hemagglutinin protein stability, activation, and pandemic risk', *Trends in microbiology*, 26: 841-53.

Rust, Michael J, Melike Lakadamyali, Feng Zhang, and Xiaowei Zhuang. 2004. 'Assembly of endocytic machinery around individual influenza viruses during viral entry', *Nature structural & molecular biology*, 11: 567.

Samji, Tasleem. 2009. 'Influenza A: understanding the viral life cycle', *The Yale journal of biology and medicine*, 82: 153.

Scheiffele, Peter, Anton Rietveld, Thomas Wilk, and Kai Simons. 1999. 'Influenza viruses select ordered lipid domains during budding from the plasma membrane', *Journal of Biological Chemistry*, 274: 2038-44.

Schroeder, Cornelia, Harald Heider, Elisabeth Möncke-Buchner, and Tse-I Lin. 2005. 'The influenza virus ion channel and maturation cofactor M2 is a cholesterol-binding protein', *European Biophysics Journal*, 34: 52-66.

Shi, Mang, Brett W Jagger, Helen M Wise, Paul Digard, Edward C Holmes, and Jeffery K Taubenberger. 2012. 'Evolutionary conservation of the PA-X open reading frame in segment 3 of

influenza A virus', *Journal of virology*, 86: 12411-13.

Sieczkarski, Sara B, and Gary R Whittaker. 2002. 'Influenza virus can enter and infect cells in the absence of clathrin-mediated endocytosis', *Journal of virology*, 76: 10455-64.

Skowronski, Danuta M, Naveed Z Janjua, Gaston De Serres, Suzana Sabaiduc, Alireza Eshaghi, James A Dickinson, Kevin Fonseca, Anne-Luise Winter, Jonathan B Gubbay, and Mel Krajden. 2014. 'Low 2012–13 influenza vaccine effectiveness associated with mutation in the egg-adapted H3N2 vaccine strain not antigenic drift in circulating viruses', *PloS one*, 9: e92153.

Soema, P. C., R. Kompier, J. P. Amorij, and G. F. Kersten. 2015a. 'Current and next generation influenza vaccines: Formulation and production strategies', *Eur J Pharm Biopharm*, 94: 251-63.

Soema, Peter C, Ronald Kompier, Jean-Pierre Amorij, and Gideon FA Kersten. 2015b. 'Current and next generation influenza vaccines: formulation and production strategies', *European Journal of Pharmaceutics and Biopharmaceutics*, 94: 251-63.

Stachyra, Anna, Patrycja Redkiewicz, Piotr Kosson, Anna Protasiuk, Anna Góra-Sochacka, Grzegorz Kudla, and Agnieszka Sirko. 2016. 'Codon optimization of antigen coding sequences improves the immune potential of DNA vaccines against avian influenza virus H5N1 in mice and chickens', *Virology journal*, 13: 143.

Stegmann, Toon. 2000. 'Membrane fusion mechanisms: the influenza hemagglutinin paradigm and its implications for intracellular fusion', *Traffic*, 1: 598-604.

Steinhauer, David A. 1999. 'Role of hemagglutinin cleavage for the pathogenicity of influenza virus', *Virology*, 258: 1-20.

Stieneke□Gröber, A, M Vey, H Angliker, E Shaw, G Thomas, Ch Roberts, HD Klenk, and W Garten. 1992. 'Influenza virus hemagglutinin with multibasic cleavage site is activated by furin, a subtilisin□like endoprotease', *The EMBO journal*, 11: 2407-14.

Suphaphiphat, Pirada, Bjoern Keiner, Heidi Trusheim, Stefania Crotta, Annunziata Barbara Tuccino, Pu Zhang, Philip R Dormitzer, Peter W Mason, and Michael Franti. 2010. 'Human RNA

polymerase I-driven reverse genetics for influenza a virus in canine cells', *Journal of virology*, 84: 3721-25.

Tenbusch, M, T Grunwald, T Niezold, M Storcksdieck genannt Bonsmann, D Hannaman, S Norley, and K Überla. 2010. 'Codon-optimization of the hemagglutinin gene from the novel swine origin H1N1 influenza virus has differential effects on CD4+ T-cell responses and immune effector mechanisms following DNA electroporation in mice', *Vaccine*, 28: 3273-77.

Tran, Vy, Lindsey A Moser, Daniel S Poole, and Andrew Mehle. 2013. 'Highly sensitive real-time in vivo imaging of an influenza reporter virus reveals dynamics of replication and spread', *Journal of virology*, 87: 13321-29.

van Riel, Debby, Vincent J Munster, Emmie de Wit, Guus F Rimmelzwaan, Ron AM Fouchier, Albert DME Osterhaus, and Thijs Kuiken. 2007. 'Human and avian influenza viruses target different cells in the lower respiratory tract of humans and other mammals', *The American journal of pathology*, 171: 1215-23.

Walker, Alexander P, and Ervin Fodor. 2019. 'Interplay between influenza virus and the host RNA polymerase II transcriptional machinery', *Trends in microbiology*.

Walker, John A, Sean S Molloy, Gary Thomas, Takemasa Sakaguchi, Tetsuya Yoshida, Thomas M Chambers, and Yoshihiro Kawaoka. 1994. 'Sequence specificity of furin, a proprotein-processing endoprotease, for the hemagglutinin of a virulent avian influenza virus', *Journal of virology*, 68: 1213-18.

Wang, Ping, Peter Palese, and Robert E O'Neill. 1997. 'The NPI-1/NPI-3 (karyopherin alpha) binding site on the influenza a virus nucleoprotein NP is a nonconventional nuclear localization signal', *Journal of virology*, 71: 1850-56.

Wang, Zhaoti, and Gregory M Duke. 2007. 'Cloning of the canine RNA polymerase I promoter and establishment of reverse genetics for influenza A and B in MDCK cells', *Virology journal*, 4: 102.

Webster, RG, and DJ Hulse. 2004. 'Microbial adaptation and change: avian influenza', *Revue Scientifique et Technique-Office International des Epizooties*, 23: 453-66.

Weis, W, JH Brown, S Cusack, JC Paulson, JJ Skehel, and DC Wiley.

1988. 'Structure of the influenza virus haemagglutinin complexed with its receptor, sialic acid', *Nature*, 333: 426.

Wen, Zhiyuan, Chao Wu, Weiye Chen, Xianying Zeng, Jianzhong Shi, Jinying Ge, Hualan Chen, and Zhigao Bu. 2015. 'Establishment of MDCK stable cell lines expressing TMPRSS2 and MSPL and their applications in propagating influenza vaccine viruses in absence of exogenous trypsin', *Biotechnology research international*, 2015.

WHO. 2019. 'World Health Organization. Influenza (seasonal) fact sheet'.https://www.who.int/en/news-room/fact-sheets/detail/influenza-(seasonal).

Wiethoelter, Anke K, Daniel Beltrán-Alcrudo, Richard Kock, and Siobhan M Mor. 2015. 'Global trends in infectious diseases at the wildlife–livestock interface', *Proceedings of the National Academy of Sciences*, 112: 9662-67.

Wilson, Ian A, John J Skehel, and DC Wiley. 1981. 'Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 Å resolution', *Nature*, 289: 366-73.

Wise, Helen M, Agnes Foeglein, Jiechao Sun, Rosa Maria Dalton, Sheetal Patel, Wendy Howard, Emma C Anderson, Wendy S Barclay, and Paul Digard. 2009. 'A complicated message: Identification of a novel PB1-related protein translated from influenza A virus segment 2 mRNA', *Journal of virology*, 83: 8021-31.

Wu, Nicholas C, Huibin Lv, Andrew J Thompson, Douglas C Wu, Wilson WS Ng, Rameshwar U Kadam, Chih-Wei Lin, Corwin M Nycholat, Ryan McBride, and Weiwen Liang. 2019. 'Preventing an antigenically disruptive mutation in egg-based H3N2 seasonal influenza vaccines by mutational incompatibility', *Cell host & microbe*, 25: 836-44.

Yamayoshi, S., and Y. Kawaoka. 2019a. 'Current and future influenza vaccines', *Nat Med*, 25: 212-20.

Yamayoshi, Seiya, and Yoshihiro Kawaoka. 2019b. 'Current and future influenza vaccines', *Nature Medicine*, 25: 212-20.

Yoon, Sun-Woo, Richard J Webby, and Robert G Webster. 2014. 'Evolution and ecology of influenza A viruses.' in, *Influenza*

Pathogenesis and Control-Volume I (Springer).

York, Ashley, and Ervin Fodor. 2013. 'Biogenesis, assembly, and export of viral messenger ribonucleoproteins in the influenza A virus infected cell', *RNA biology*, 10: 1274-82.

Yuen, KY, PKS Chan, M Peiris, DNC Tsang, TL Que, KF Shortridge, PT Cheung, WK To, ETF Ho, and R Sung. 1998. 'Clinical features and rapid viral diagnosis of human disease associated with avian influenza A H5N1 virus', *The Lancet*, 351: 467-71.

Zhang, Xiangmin, Wei Kong, Shamaila Ashraf, and Roy Curtiss. 2009. 'A one-plasmid system to generate influenza virus in cultured chicken cells for potential use in influenza vaccine', *Journal of virology*, 83: 9296-303.