Effects of PB1-F2 and PA-X on the pathogenicity of H1N1 influenza virus

by

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D.V.M., Kangwon National University, 2009
M.S., Kangwon National University, 2011

AN ABSTRACT OF A DISSERTATION

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DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine/Pathobiology
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Abstract

Influenza A virus (IAV) is a negative sense, single-stranded, segmented RNA virus with eight gene segments. It is an important respiratory pathogen which causes annual epidemics and occasional pandemics worldwide in humans and leads to considerable economic problems for the livestock industry. To control and prevent this significant disease, understanding the pathogenesis of IAVs is critical. Although some molecular mechanisms regarding virulence have been determined, IAV pathogenesis is not completely understood and is difficult to predict.

The eight viral gene segments of IAV were thought to encode for 10 viral proteins. Since 2001, eight additional viral proteins have been identified, including PB1-F2, PB1-N40, PA-X, NS3, PA-N155, PA-N182, M42, and PB2-S1. However, the functions of these novel proteins in influenza virus replication as well as pathogenesis have not been fully elucidated.

Although PB1-F2 protein is an important virulence factor of IAV, the effects of this protein on viral pathogenicity of swine influenza virus (SIV) remain unclear. In Chapter 2, we investigated the contribution of the PB1-F2 protein to viral pathogenicity of a virulent triple-reassortant (TR) H1N1 SIV in different hosts, pigs and mice. Our data indicate that PB1-F2 expression in virulent TR H1N1 SIV modulates virus replication and pathogenicity in the natural host, pigs, but not in mice. In addition, single amino acid (aa) substitution at position 66 (N/S) in the PB1-F2 has a critical role in virulence in mice but no effect was found in pigs.

A novel IAV protein, PA-X consists of the N-terminal 191aa of PA protein and a unique C-terminal 41 (truncated form) or 61 (full-length form) aa residues encoded by +1 ribosomal frameshifting. Although several studies have demonstrated the PA-X protein as an important immune modulator and virulence factor, the impact of different expressions of PA-X protein including full-length, truncated or PA-X deficient forms on viral pathogenicity and host response

remains unclear. In Chapter 3, we showed that expression of either truncated or full-length PA-X protein in 2009 human pandemic H1N1 (pH1N1) viruses suppresses host antiviral response by host shutoff activity which promotes viral growth and virulence in mice when compared to loss of PA-X expression. Furthermore, full-length PA-X expression displayed stronger impact on viral pathogenicity and host immune response compared to truncated PA-X expression.

Taken together, our results provide new insights into the impact of PB1-F2 and PA-X proteins on virus replication, pathogenicity and modulation of host immune responses. This knowledge is important for better understanding of IAV pathogenesis.

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Chapter 1 - General Introduction

1.1 Influenza

Influenza is an important acute respiratory disease caused by influenza viruses in humans and animals. Although the disease outcome is usually mild in humans with common symptoms, including fever, cough and runny nose, influenza virus infection can also cause severe symptoms such as pneumonia and death. In animals, the severity of disease varies from asymptomatic infection to severe clinical signs and high mortality, depending on the species, virus strains and host immune condition.

1.2 Influenza virus classification

Influenza viruses are single-stranded, negative-sense RNA viruses which belong to the *Orthomyxoviridae* family. There are three genera of influenza viruses, including influenza A, B and C viruses. Influenza A and B viruses have similar spherical or filamentous shapes in contrast to influenza C viruses which have distinct cordlike long structures. Influenza A and B have eight RNA segments, while influenza C contains only seven genome segments. In addition, influenza A and B have two major surface glycoproteins, the hemagglutinin (HA) which are responsible for cell attachment and entry, and the neuraminidase (NA) which are responsible for virus release. On the other hand, influenza C virus has one major surface glycoprotein, which is called the hemagglutinin-esterase-fusion (HEF) protein that has both the HA and NA functions of influenza A and B viruses (Herrler & Klenk, 1991; Vlasak *et al.*, 1989). While influenza A virus has many genetically distinct subtypes depending on HA and NA proteins, influenza B virus contains two distinguishable lineages which are termed the Victoria lineage and the Yamagata lineage (Rota *et al.*, 1990). Influenza A virus infects a wide variety of animals, ranging from wild

birds to mammals, including humans, and circulates worldwide causing severe human epidemics and pandemics (Webster, 1997). Influenza B virus is also a major pathogen causing human influenza epidemics together with influenza A but it does not cause pandemics. Although influenza B virus mainly affects humans, it has also been isolated from harbor seals and the genome was detected in domestic pigs (Osterhaus *et al.*, 2000; Ran *et al.*, 2015) . Compared to these two influenza viruses, influenza C virus causes mild and asymptomatic disease predominantly observed in humans. However, several studies have reported serological evidence of influenza C virus infections in dogs and pigs (Manuguerra & Hannoun, 1992; Ohwada *et al.*, 1987; Youzbashi *et al.*, 1996). In 2011, a new influenza virus, named influenza D virus, was identified from pigs as an influenza C-like virus and was proposed as a new genus in the *Orthomyxoviridae* family (Hause *et al.*, 2014; Hause *et al.*, 2013). Influenza D virus has been detected in cattle and pigs in the United States, France and China (Ducatez *et al.*, 2015; Ferguson *et al.*, 2015; Hause *et al.*, 2014; Jiang *et al.*, 2014).

1.3 Influenza A virus (IAV)

Influenza A virus (IAV) known as "flu" is the significant respiratory pathogen in humans worldwide and causes seasonal epidemics and periodic pandemics. Although wild waterfowl are the natural reservoir of IAVs, viruses constantly establish evolutionary changes to adapt to new hosts and consequently have been shown to infect many animal species such as domestic birds, humans, pigs, horses and dogs (Webster *et al.*, 1992). Due to the susceptibility of a variety of animal hosts and continuous adaptation of IAVs, it is difficult to control IAV infections. Furthermore, outbreaks of annual epidemics, unpredictable pandemics and zoonotic infections pose significant threat to public health.

IAVs are classified into different subtypes based on the genetic and antigenic properties of the two surface glycoproteins HA and NA. To date, 18 HA (H1-H18) and 11 NA (N1-N11) subtypes have been identified. Among them, 16 HA subtypes (H1-H16) and 9 NA subtypes (N1-N9) are circulating in wild birds (Alexander, 2007; Fouchier *et al.*, 2005), while the genome sequences of H17, H18, N10 and N11 were recently identified in bats (Tong *et al.*, 2012; Tong *et al.*, 2013).

1.4 IAV genome organization and proteins

The genome of IAV is comprised of eight segmented negative-sense RNAs (Palese & Schulman, 1976; Ritchey *et al.*, 1976). Each viral RNA (vRNA) segment consists of a 3´end non-coding region, an internal coding region, and a 5´end non-coding region. The 3´ and 5´ termini of all vRNA segments in IAVs contain highly conserved 12 and 13 terminal nucleotides, respectively (Desselberger *et al.*, 1980; Skehel & Hay, 1978). The eight IAV segments are numbered from segment 1 to 8 and encode PB2, PB1, PA, HA, NP, NA, M and NS, respectively according to the genome size. These eight gene segments can encode up to 18 identified proteins (Table 1-1) (Vasin *et al.*, 2014; Yamayoshi *et al.*, 2016).

Surface and structural proteins

IAVs have three surface proteins, including HA, NA and matrix (M1 and M2) proteins. IAVs are subtyped based on two glycoproteins, HA and NA, so far 18 different HA and 11 different NA subtypes have been identified (Tong *et al.*, 2012; Tong *et al.*, 2013). The HA glycoprotein performs a critical role during viral entry, serving as the receptor binding and membrane fusion protein. The HA protein recognizes the sialic acid (SA) which is bound to galactose (Gal) in either α2,3 or α2,6 linkages on the host cell surface. The HA protein has a

host-specific recognition based on its affinity to different SA receptors in different species. For instance, avian influenza viruses have a preference to recognize α2,3-linked SA, which are mainly present in the avian respiratory and intestinal epithelium, while human influenza viruses prefer to recognize α2,6-linked SA, which are mostly found in the human respiratory tract epithelium (Connor *et al.*, 1994; Naeve *et al.*, 1984; Rogers & Paulson, 1983). During IAV entry, the HA protein is cleaved into two subunits, HA1 and HA2 by host extracellular proteases (Klenk *et al.*, 1975; Lazarowitz & Choppin, 1975). This proteolytic cleavage is necessary for HA conformational change to enable fusion of the viral and endosomal membranes. Mammalian influenza virus and low pathogenic avian influenza viruses generally have a HA cleavage site with a Q/E-X-R monobasic motif which is recognized and cleaved by trypsin-like proteases produced by respiratory and intestinal epithelia (Kawaoka & Webster, 1988). However, H5 and H7 subtypes of avian influenza viruses can possess a polybasic cleavage site with the R-X-R/K-R motif acquired by insertional mutations and it is recognized and cleaved by the ubiquitous furin-like protease (Stieneke-Grober *et al.*, 1992). This specificity of the protease recognition determines the virus replication site and pathogenicity in hosts. The viruses possessing a monobasic motif at the HA cleavage site are confined to replicate within the respiratory or intestinal tract which produce trypsin-like protease, whereas the viruses containing a polybasic HA cleavage site can induce systemic replication, which results in the occurrence of highly pathogenic avian influenza (Chen *et al.*, 1998; Steinhauer, 1999).

The NA protein encoded by segment 6 has neuraminidase enzymatic activity which is necessary for release and spread of progeny virions from the cells. The NA protein cleaves the SAs from host cells to release the newly budded viruses and also removes the SAs from viral glycoproteins to avoid the aggregation of virus particles at the release site (Palese *et al.*, 1974).

To date, four NA inhibitors have been approved for treatment of IAVs: oseltamivir (F. Hoffmann-La Roche, Basel, Switzerland), zanamivir (GlaxoSmithKline, Brentford, UK), laninamivir (Daiichi Sankyo Company Ltd, Tokyo, Japan) and peramivir (BioCryst, Durham, NC, USA). NA inhibitors bind to the enzyme active site of NA and prevent the release and spread of new virus from infected cells (Colman, 1994; McKimm-Breschkin, 2013). The Centers for Disease Control (CDC) currently recommend the use of NA inhibitors for the treatment of influenza infections (CDC, Influenza antiviral medications: summary for clinicians, May 2016). Although NA-specific antibodies are not sufficient to prevent IAV infections, they can inhibit viral spread and reduce disease severity (Monto & Kendal, 1973; Schulman *et al.*, 1968). Recently, several studies demonstrated that NA antibodies can induce heterologous protection as well as long-term immunity against IAVs due to the slower rate of antigenic changes compared to HA (Abed *et al.*, 2002; Doyle *et al.*, 2013; Quan *et al.*, 2012; Wan *et al.*, 2015). Therefore, some studies have suggested the NA protein as a potential target for influenza vaccines along with the HA protein (Bosch *et al.*, 2010; Brett & Johansson, 2005; Kilbourne *et al.*, 2004; Wohlbold *et al.*, 2015).

These two glycoproteins, HA and NA, have complementary functions during viral replication. Thus the balance between HA binding affinity and NA sialidase enzymatic activity plays a critical role in efficient viral replication and transmission (Gen *et al.*, 2013; Mitnaul *et al.*, 2000; Wagner *et al.*, 2000). Furthermore, although HA and NA undergo constant evolutionary changes, IAVs maintain the HA/NA functional balance to have optimal viral fitness (Gaymard *et al.*, 2016; Wagner *et al.*, 2002).

The matrix 1 (M1) protein encoded in segment 7 is the most abundant protein as well as a major structure protein of IAV particles. The M1 protein forms a shell underneath the lipid layer

of viral particles and connects the viral ribonucleoprotein (vRNP) with envelope proteins: HA, NA and M2 (Elster *et al.*, 1997; Shishkov *et al.*, 1999). The M1 protein plays multiple roles during IAV replication cycles. During virus entry, the M1 protein is dissociated from the vRNP and allows the release of vRNP to enter the nucleus (Bui *et al.*, 1996; Martin & Helenius, 1991b). The newly synthesized M1 protein is transported to the nucleus and interacts with the vRNP and the nuclear export protein (NEP), forming a complex which in turn exports from the nucleus to cytoplasm (Akarsu *et al.*, 2003; Bucher *et al.*, 1989; Neumann *et al.*, 2000; O'Neill *et al.*, 1998; Yasuda *et al.*, 1993). In addition, exported M1, vRNP and NEP protein complex from the nucleus inhibit the reentry of vRNP back into the nucleus to promote viral assembly (Martin & Helenius, 1991a). During the late stage of viral replication, the M1 protein plays a critical role in the processes of viral assembly as well as budding and it influences the viral morphology (Burleigh *et al.*, 2005; Elleman & Barclay, 2004; Roberts *et al.*, 1998). In addition to encoding the M1 protein, segment 7 of IAV also encodes the matrix 2 (M2) protein via alternative splicing (Lamb & Choppin, 1981). During viral entry, the M2 protein works as a proton-selective ion channel and allows the proton influx into the virion interior (Chizhmakov *et al.*, 1996; Pinto *et al.*, 1992). The low pH of the virion by proton influx triggers the dissociation of the M1 protein with the vRNPs as well as the membrane fusion of the viral envelop with the endosomal membrane, which leads to the release of the vRNP into the cytoplasm which then migrate into the nucleus (Helenius, 1992). The adamantane derivatives, amantadine and rimantadine, are M2 ion channel blockers and the FDA-approved class of antiviral drugs for IAV infection (Davies *et al.*, 1964). They inhibit the M2 ion channel activity to prevent uncoating of IAV as well as the release of vRNPs. However, currently the adamantanes are not recommended for treatment of IAV infection due to the widespread resistance between human seasonal IAVs and no inhibitory

effect on influenza B viruses (Deyde *et al.*, 2007; Gubareva *et al.*, 2010; Hayden & Hay, 1992). Since the extracellular domain of M2 (M2e) is a highly conserved between IAVs, it has been a target for an universal IAV vaccine even if it induces low protective immunity (De Filette *et al.*, 2011; Deng *et al.*, 2015; Kim *et al.*, 2014; Neirynck *et al.*, 1999).

RNA polymerase complex

The three largest IAV genome segments 1, 2 and 3 encode three polymerase subunits, named polymerase basic protein 2 (PB2), polymerase basic protein 1 (PB1), and polymerse acidic protein (PA), respectively. The viral polymerase subunits form the polymerase heterotrimeric complex which is an essential complex in the viral replication cycle as it is reponsible for the RNA-dependent RNA polymerase activity. The influenza viral ribonucleo-protein (vRNP) complex is composed of a heterotrimeric polymerase complex and each vRNA genome segment which is surrounded by mutilple nucleoproteins (NP). The polymerase complexes bind to the short hairpin structure formed from complementary 3´ and 5´ ternimal non-coding regions of each vRNA. The PB1 subunit is the centre of the polymerase complex containing the conserved motifs for RNA-dependent RNA polymerase activity and is also involved in interacting to the the terminal 3´ and 5´ regions of vRNA (Gonzalez & Ortin, 1999; Kobayashi *et al.*, 1996; Li *et al.*, 1998). To initiate the transcription process of vRNA, IAV polymerse uses a capped primer which is derived from the 5´ end of cellular pre-mRNA by cap-snatching due to the absence of IAV inherent capping activity (Krug *et al.*, 1979; Plotch *et al.*, 1979). The PB2 subunit recognizes and binds to host mRNA 5´ cap structures and the PA protein cleaves the 5´cap structure of host pre-mRNA by endonucleolytic activity from its N-terminal domain (Blaas *et al.*, 1982; Braam *et al.*, 1983; Dias *et al.*, 2009; Plotch *et al.*, 1981). The crystal structure analysis revealed the interactions between the C-terminal domain of PA and the N-

terminal domain of PB1, as well as between the C-terminal domain of PB1 and the N-terminal domain of PB2 (He *et al.*, 2008; Sugiyama *et al.*, 2009). Moreover, the crystal structure of the vRNP complex shows that the three polymerase subunits form a compact globular structure with multiple interactions between them. They are all invovled in the interaction with the RNA promoter (Arranz *et al.*, 2012; Coloma *et al.*, 2009; Torreira *et al.*, 2007).

The NP protein plays a critical role in transcription and replication of viral genes as an important structural component of vRNPs and cRNPs. With high affinity to RNA, the NP proteins bind to viral RNA genomes to maintain the double helical structures of vRNPs through strong interactions between the NP proteins (Yamanaka *et al.*, 1990; Ye *et al.*, 2006).

Non-structural proteins

The non-structural protein 1 (NS1) and nuclear export protein (NEP or NS2) are encoded from segment 8 of IAVs. The NS1 protein is the greatly well-known viral antagonist of type I interferon (IFN) immune response and a regulator of host gene expression which supports viral protein synthesis as well as viral replication. The NS1 protein prevents the IFN response at a pre-transcriptional level by inhibiting RIG-I activation and the RIG-I signaling pathway, which initiates the antiviral response triggered by dsRNAs (Gack *et al.*, 2009; Mibayashi *et al.*, 2007; Rajsbaum *et al.*, 2012; Talon *et al.*, 2000; Wang *et al.*, 2000). In addition, the NS1 protein inhibits the activation of the interferon-stimulated gene (ISG) products such as the protein kinase PKR and the RNAse L-pathway activator OAS at a post-transcriptional level (Lu *et al.*, 1995; Min & Krug, 2006; Min *et al.*, 2007). In addition, the NS1 protein contributes to the host shutoff activity by preventing splicing of cellular pre-mRNA, cellular polyadenylation machinery as well as the export of polyadenylated host mRNAs from the nucleus (Alonso-Caplen & Krug, 1991; Alonso-Caplen *et al.*, 1992; Fortes *et al.*, 1994; Nemeroff *et al.*, 1998; Qiu & Krug, 1994). This

host shutoff activity of the NS1protein promotes viral gene expression by recruitment of host translation-related factors to viral genes while also supporting viral infection by inhibiting the IFN-related gene expressions.

The NEP protein encoded from segment 8 by alternative splicing mediates the export of vRNPs from the nucleus by interacting with chromosomal maintenance 1 (Crm1) and the viral M1 protein (Akarsu *et al.*, 2003; Elton *et al.*, 2001; Neumann *et al.*, 2000). In addition to the nuclear export function, the NEP is involved in the viral budding process by interacting with a cellular F1Fo-ATPase (Gorai *et al.*, 2012). Moreover, it has been demonstrated that NEP promotes the synthesis of the viral complementary RNP (cRNP) from vRNPs, which subsequently leads to the increased production of vRNPs for packaging into the progeny viruses (Manz *et al.*, 2012; Robb *et al.*, 2009).

Newly discovered proteins

It was thought that 8 segmented genomic RNAs of IAVs encode 10 viral proteins including PB2, PB1, PA, HA, NP, NA, M1, M2, NS1 and NS2. However, since the PB1-F2 protein was identified in 2001 as the 11^{th} viral protein of IAVs from the PB1gene, seven additional viral proteins, PB2-S1, PB1N-40, PA-X, PA-N155, PA-N182, M42 and NS3, have been discovered.

Most recently identified is the PB2-S1 protein which is encoded by following of spliced mRNA transcribed from the segment 1, PB2 genome (Yamayoshi *et al.*, 2016). The PB2-S1 protein localizes to the mitochondria and inhibits the RIG-I-dependent IFN signaling pathway. However, the PB2-S1 protein is not essential for virus replication *in vitro* or for viral pathogenicity *in vivo* (Yamayoshi et al., 2016). The PB1-F2 protein and PB1-N40 proteins are expressed from segment 2, PB1 gene of IAVs by an alternative translation initiation process

(Chen *et al.*, 2001; Wise *et al.*, 2009). The PB1-F2 protein is considered to be an important virulence factor of IAVs. This protein is mainly localized in the mitochondria and reduces mitochondrial membrane potential, which is associated with inducing apoptosis as well as inhibiting the RIG-I-dependent IFN response (Chen *et al.*, 2001; Conenello *et al.*, 2011; Lowy, 2003; Varga *et al.*, 2012; Zamarin *et al.*, 2005). The PB1-F2 protein also interacts with the PB1 protein in the nucleus and regulates polymerase activity (Mazur *et al.*, 2008; McAuley *et al.*, 2010). Although the PB1-N40 expression is not critical for viral replication and its function still remains unclear, the PB1-N40 is obviously associated with both PB1 and PB1-F2 expression, which may affect virus replication (Tauber *et al.*, 2012; Wise *et al.*, 2009).

Segment 3 of IAV genomes encodes not only the PA protein but also three additional PA related proteins, PA-X, PA-N155 and PA-N182. The PA-X protein is produced by a +1 ribosomal frameshift process and contains an identical 191aa N-terminal domain of the PA protein along with a PA-X unique 41or 61aa C-terminal domain (Jagger *et al.*, 2012). The PA-X protein has a role in host shutoff activity which inhibits host gene expression including inflammatory and immune related genes. Thus, the PA-X protein is related with the regulation of host immune responses, leading to modulation of viral pathogenicity (Hayashi *et al.*, 2015; Jagger *et al.*, 2012). The PA-N155 and PA-N182 are expressed from the 11^{th} and 13^{th} in-frame AUG codons, respectively, by alternative translation initiation (Muramoto *et al.*, 2013). Even if AUG codons for expression of PA-N155 and PA-N182 proteins are highly conserved and they displayed the universal expressions among IAVs, the functions of these proteins have yet to be determined (Muramoto *et al.*, 2013).

The M42 and NS3 proteins are encoded from spliced mRNAs of segment 7, M gene and segment 8, NS gene, respectively (Selman *et al.*, 2012; Wise *et al.*, 2012). The M42 protein is

able to functionally replace the M2 protein as a proton ion channel, in spite of the different sequence from the M2 in the ectodomain (Wise *et al.*, 2012). The NS3 protein is produced as the result of a nucleotide substitution in the NS gene, A374G, which is associated with the adaptation of avian influenza viruses to mammalian hosts (Selman *et al.*, 2012). However, it is still arguable that NS3 expression is associated with host adaptation.

Table 1-1 The proteome of influenza A viruses

Genome segment	Protein	mRNA	Mechanism of expression	Length (aa)	Function
1	PB2	Segment 1 non-spliced mRNA	Translation from AUG1	759	Subunit of the viral polymerase complex, binding to host mRNA 5' cap structures
	PB2-S1	Segment 1 alternatively spliced mRNA	Translation from AUG1	508	Inhibiting RIG-I-dependent IFN signaling pathway
2	PB1	Segment 2 non-spliced mRNA	Translation from AUG1	757	Subunit of the viral polymerase complex, RNA-dependent RNA polymerase activity
	PB1-N40	Segment 2 non-spliced mRNA	Translation from AUG5 as the result of leaky ribosomal scanning	718	Maintaining the balance between PB1 and PB1-F2 expression
	PB1-F2	Segment 2 non-spliced mRNA	Translation from AUG4 in alternative ORF as the result of leaky ribosomal scanning	90[a]	Virulence factor, Inducing apoptosis, Inhibiting RIG-I-dependent IFN response, Regulating the polymerase activity
3	PA	Segment 3 non-spliced mRNA	Translation from AUG1	716	Subunit of the viral polymerase complex, RNA endonuclease activity (cap snatching)
	PA-X	Segment 3 non-spliced mRNA	Translation from AUG1 and +1 ribosomal frameshifting at codons 190-193	252[a]	Host shutoff activity, modulation the host response and viral pathogenicity
	PA-N155	Segment 3 non-spliced mRNA	Translation from AUG11 as the result of leaky ribosomal scanning	568	ND
	PA-N182	Segment 3 non-spliced mRNA	Translation from AUG13 as the result of leaky ribosomal scanning	535	ND
4	HA	Segment 4 non-spliced mRNA	Translation from AUG1	560	Recognition and binding to receptors of target cells, fusion with endosomal membrane
5	NP	Segment 5 non-spliced mRNA	Translation from AUG1	498	Major component of the viral RNP complex, encapsidating vRNA segments
6	NA	Segment 6 non-spliced mRNA	Translation from AUG1	465	Sialidase enzymatic activity, Cleaving the sialic acids from cells to release progeny viruses
7	M1	Segment 7 non-spliced mRNA	Translation from AUG1	252	Main component of the viral membrane, multiple roles in virion assembly and infection
	M2	Segment 7 alternatively spliced mRNA	Translation from AUG1	97	Membrane protein, forming proton ion channels for the proton influx which triggers the release of vRNPs

	M42	Segment 7 alternatively spliced mRNA	Translation from AUG2	99	Functionally replacing the M2 ion channel
8	NS1	Segment 8 non-spliced mRNA	Translation from AUG1	217	Evasion of host immune response, host shutoff activity
	NS2(NEP)	Segment 8 alternatively spliced mRNA	Translation from AUG1	121	Mediating vRNP export from nucleus to the cytoplasm
	NS3	Segment 8 alternatively spliced mRNA	Translation from AUG1	174	ND

[a] : can be truncated in some viral strains.

ND : Functions have not been determined.

1.5 IAV replication cycle

The IAV replication cycle is initiated by the attachment of virus particles to cellular receptors (Figure 1-1). The HA protein of IAVs recognizes and binds to SA containing glycolipids or glycoproteins on the cell surface such as α2,3-linked SA or α2,6-linked SA, which determines the host and tissue specificity of IAVs (Baum & Paulson, 1990; Couceiro *et al.*, 1993; Rogers & Paulson, 1983). Once a virus particle binds to the cellular receptor, the virus is internalized by either clathrin-dependent or clathrin-independent endocytosis such as micropinocytosis (de Vries *et al.*, 2011; Matlin *et al.*, 1981; Sieczkarski & Whittaker, 2002) . Inside the endosome, the highly acidic conditions trigger several changes in the virion. First, the viral M2 ion channel allows an influx of proton ions into the interior of the viral particle and increased acidity within the virion causes dissociation of vRNPs from the M1 protein (Bui *et al.*, 1996; Pinto *et al.*, 1992). Next, the acidic condition of the late endosome promotes a conformational change in the HA protein that leads to the fusion of the viral envelope with the endosomal membrane and finally, vRNPs are released from the virion into the cytoplasm (Helenius, 1992; Skehel & Wiley, 2000). The vRNPs subsequently enter the cellular nucleus, where vRNA replication and transcription occur by the RNA-dependent RNA polymerase complex (Krug *et al.*, 1987). During this process, four different RNA species are produced, including viral mRNA, positive-sense complementary RNA (cRNA), negative-sense viral genomic RNA (vRNA) and negative-sense small viral RNA (svRNA) (Etkind *et al.*, 1977; Jackson *et al.*, 1982; Perez *et al.*, 2010; Plotch & Krug, 1978; Scholtissek & Rott, 1963) . The viral RNA polymerase produces cRNA through copying of the vRNA and in turn cRNA serves as a template to synthesize more vRNAs. Synthesized cRNA and vRNA associate with the heterotrimeric polymerase complex and multiple NP proteins to form cRNP and vRNP. The

svRNA consists of 22-27 nucleotides which correspond to the 5´ end of each of the vRNA genomes. During viral replication, svRNA is thought to act in concert with NEP as an important regulator to promote cRNA synthesis over mRNA transcription, which consequently results in additional production of new vRNAs (Perez *et al.*, 2010; Umbach *et al.*, 2010). Viral mRNA has a 5´ cap which is obtained from cellular mRNA through cap-snatching and has 3´ poly-A tail (Dias *et al.*, 2009; Krug *et al.*, 1979; Luo *et al.*, 1991; Plotch *et al.*, 1979; Zheng *et al.*, 1999). Viral mRNAs are transported to the cytoplasm for viral protein synthesis using the cellular translation machinery (Compans, 1973; Garfinkel & Katze, 1993). Newly synthesized PB2, PB1, PA, NP, NEP, NS1 and M1 proteins, which are necessary for vRNA transcription and replication, are imported back into the nucleus. Imported new polymerase subunits (PB2, PB1 and PA) and NP proteins bind to newly produced vRNAs or cRNAs to assemble vRNPs and cRNPs, respectively. These newly assembled vRNPs are exported to the cytoplasm through its interacting with NEP, M1 as well as cellular nuclear export machinery (Bui *et al.*, 2000; Chase *et al.*, 2011; Elton *et al.*, 2001; O'Neill *et al.*, 1998). Exported vRNPs migrate to the plasma membrane across the cytoplasm, mediated by Rab11-containing structures (Eisfeld *et al.*, 2011). At the late time points of infection, vRNPs and viral proteins enrich at the apical plasma membrane followed by the virion assembly and budding process. The RNA packaging signals which are located in the 5´ and 3´ terminal regions of each vRNA, including both non-coding and partial coding sequences, are required to bring each of the eight segments into every virion (Chou *et al.*, 2012; Fujii *et al.*, 2003; Goto *et al.*, 2013). To assemble new virus particles, HA, NA, M1, M2 and vRNPs interact with each other and these interactions promote the budding of progeny virions (Rossman & Lamb, 2011). Viral M1, M2 and NEP proteins as well as host factors such as ATP, F1Fo-ATPase, actin filaments, G-protein and kinase activity are known to

be required for virus particle budding (Demirov *et al.*, 2012; Gorai *et al.*, 2012; Hui & Nayak, 2001; 2002; Rossman *et al.*, 2010). Once virions are assembled and bud out on the cell membrane, neuraminidase activity of the NA protein cleaves the link between the HA protein and SA molecules to release progeny virus particles from the cell surface (Palese *et al.*, 1974).

Figure 1-1 Influenza A virus replication cycle

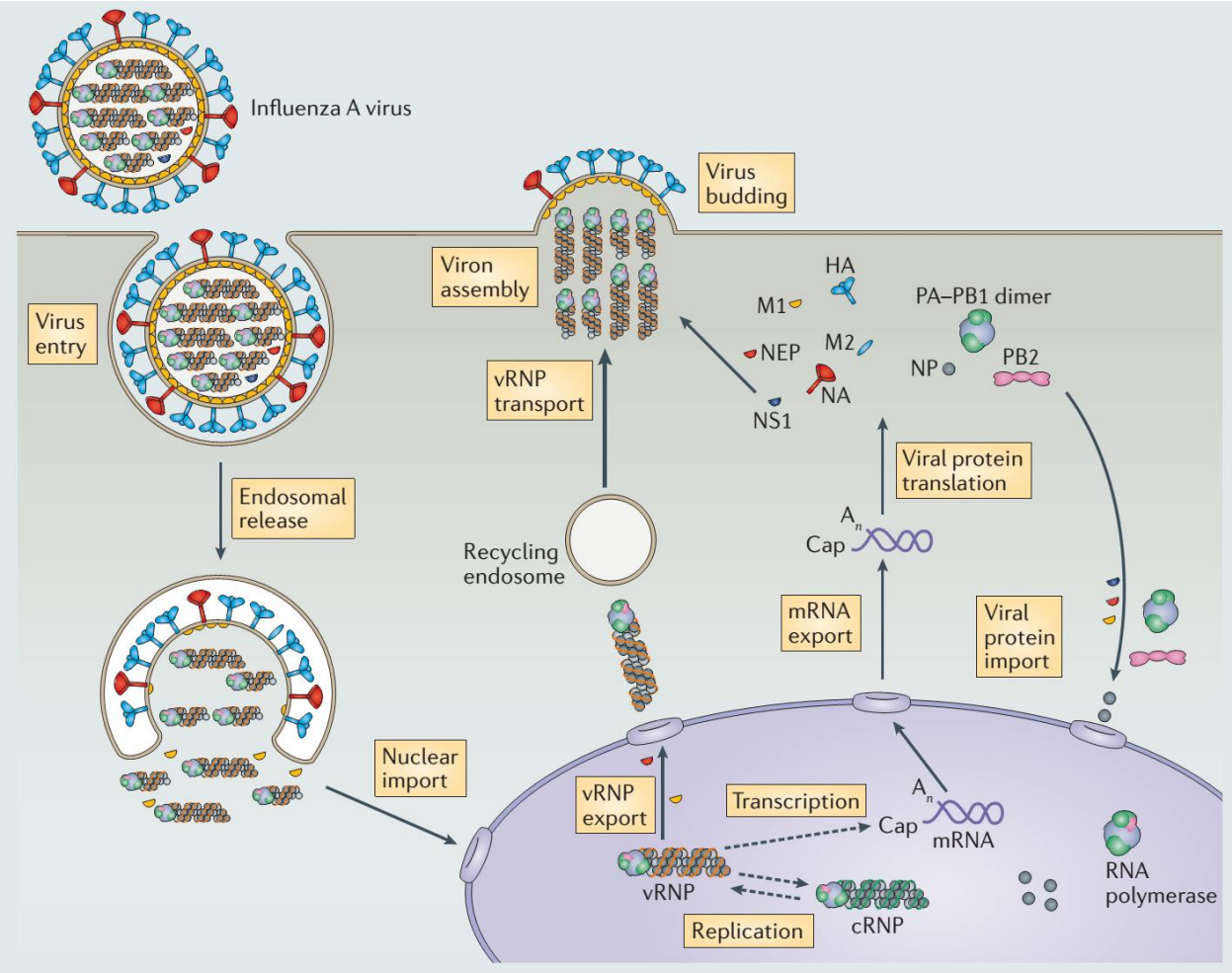


Figure 1-1 The IAV replication cycle is initiated by the attachment of viral HA protein to cellular receptors. Once virus particle binds to the cellular receptor, the virus is internalized by either endocytosis or micropinocytosis. The acidic condition of the endosome causes dissociation of vRNPs from the M1 protein and promotes a conformational change in the HA protein that leads to the fusion of the viral envelope with the endosomal membrane and finally, vRNPs are released from the virion into the cytoplasm. The vRNPs subsequently enter the nucleus, where vRNA replication and transcription occur by the RNA-dependent RNA polymerase complex. Viral mRNAs are transported to the cytoplasm for viral protein synthesis using the cellular translational machinery. Newly synthesized PB2, PB1, PA, NP, NEP, NS1 and M1 proteins which are necessary for vRNA transcription and replication are imported back into the nucleus. Newly assembled vRNPs are exported to the cytoplasm through its interacting with NEP, M1 as well as cellular nuclear export machinery. Exported vRNPs migrate to the plasma membrane across the cytoplasm. At the late time points of infection, vRNPs and viral proteins enrich at the apical plasma membrane followed by the virion assembly and budding process. (Adapted from Te Velthuis & Fodor, *Nature Reviews Microbiology*, 2016, doi: 10.1038/nrmicro.2016.87) (Te Velthuis & Fodor, 2016)

1.6 IAV hosts

It is known that the natural reservoirs of IAVs are wild waterfowl as well as shorebirds, and 16 HA and 9 NA subtypes are detected from wild aquatic birds (Webster *et al.*, 1992). Recently, there is growing evidence that bats are the possible reservoir of IAVs with finding two HA and two NA new subtypes from bats (Ma *et al.*, 2015; Tong *et al.*, 2012; Tong *et al.*, 2013). IAVs in domestic birds and mammals have evolved either indirectly or directly from avian influenza viruses (AIVs) in wild aquatic bird reservoirs (Webster *et al.*, 1992).

Avian hosts

Wild aquatic birds, especially *Anseriformes* (ducks, geese and swans) and *Charadriiformes* (shorebirds, gulls and alcids), are the primary reservoirs of IAVs. Although IAVs have been detected from over 100 wild birds, dabbling ducks and especially mallards are considered to be the major sources of IAVs in wild aquatic birds (Krauss *et al.*, 2004; Munster *et al.*, 2007; Olsen *et al.*, 2006). In aquatic birds, IAVs mainly replicate in the intestinal tract which are then shed in feces and spread by fecal-oral route through contaminated water, while some subtypes such as H3 and H4 can also replicate well in the respiratory tract (Bahl *et al.*, 2013; Runstadler *et al.*, 2013; Webster *et al.*, 1992). The migratory wild birds play a critical role in the wide geographic spread and initial introduction of IAVs. In 2014, highly pathogenic (HP) H5N8 avian influenza viruses were introduced into North America from Asia by migratory wild birds which then led to reassortment with the North American lineage resulting in a generation of new H5N1 and H5N2 genotypes (Claes *et al.*, 2016; Jhung & Nelson, 2015). IAVs in natural reservoirs display limited evolution. An interpretation is that the continued mutations do not provide additional advantages to IAVs that is already well established within natural reservoirs

(Webster *et al.*, 1992). However, to transmit to other host species from avian, IAVs show rapid mutations as well as the evolution required for adaptation to new hosts.

Since transmission of IAVs from wild birds to domestic birds occurs commonly in ponds or backyard poultry farms, domestic ducks as well as geese may act as intermediate bridge hosts to transmit IAVs to terrestrial poultry. Most AIVs cause an asymptomatic or mild disease in domestic birds as the low pathogenic avian influenza viruses (LPAIVs) and H1, H3, H5, H6, H7 and H9 subtypes are most frequently isolated from them (Alexander, 2007; Peiris & Yen, 2014). On the other hand, two subtypes of AIVs including H5 and H7 can evolve into highly pathogenic avian influenza viruses (HPAIVs), causing severe disease with up to 100% mortality in domestic poultry. The live poultry markets have been described to have a variety of IAV subtypes and provide ideal conditions for genetic mixing of IAVs as well as generation of new recombinant viruses. In addition, the live poultry market is a major source of interspecies transmission events for IAVs and range from domestic poultry to mammals, including humans through close contact (Pepin *et al.*, 2013b). Including a recent outbreak of the H7N9 subtype in China, many human cases of AIV infections with H5N1, H5N6, H9N2 and H10N8 from live poultry markets have been reported (Bi *et al.*, 2015; Liu *et al.*, 2015a; Peiris *et al.*, 1999; Pepin *et al.*, 2013a; Wu & Gao, 2013).

Mammalian hosts

Over twenty mammalian species, including humans, dogs, horses and pigs, which have established a stable infection chain, and other accidental hosts without stable infection such as cats as well as sea mammals can be infected with IAVs (Webster *et al.*, 1992). Although some AIVs can infect mammalian hosts without prior adaptation, either gene reassortment or genetic mutations are generally required to achieve the interspecies transmission and adaptation of IAVs

from avian to mammalian hosts. We will discuss the molecular determinants of mammalian adaptation further in Chapter 1.10. In humans, only three subtypes of IAVs, H1N1, H2N2 and H3N2, have been determined to be the stable lineages which have caused annual epidemics or occasional pandemics. More detailed information about human influenza virus will be discussed in Chapter 1.8.

Swine are thought to be an important host of IAVs because they can be infected with both mammalian and avian influenza viruses to generate novel reassortant IAVs which can infect other mammalian hosts and possess a potential threat to humans. This was highlighted by the swine-origin 2009 H1N1 pandemic (pH1N1) in humans as well as more recent human infections with a novel H3N2 variant virus which is a reassortant between 2009 pandemic H1N1 (pH1N1) and TR H3N2 SIV (Dawood *et al.*, 2009; Epperson *et al.*, 2013; Peiris *et al.*, 2009). Swine influenza was first reported in pigs associated with the 1918 Spanish H1N1 pandemic in humans and the virus was subsequently identified in 1930 as an H1N1 IAV (Shope, 1931). Swine influenza is an important respiratory disease and causes a continuous problem in swine populations throughout the world. Infected pigs show from asymptomatic to fatal conditions depending on the virus strain and co-infection of other pathogens. Although only limited subtypes of IAVs including H1N1, H1N2 and H3N2 are established as endemic viruses in pigs, multiple lineages and genotypes of swine influenza viruses (SIVs) are circulating in different continents and regions of the world (Vincent *et al.*, 2014). In North America, classical swine H1N1 (cH1N1) virus which evolved from the 1918 H1N1 pandemic, was circulating in swine population until late 1990s. In 1998, a novel triple-reassortant (TR) H3N2 virus emerged and rapidly adapted to the swine population. This TR H3N2 virus contains PB2 and PA genes derived from AIV, PB1, HA and NA genes from human IAV, and NP, M and NS genes from

cH1N1 swine IAV (Zhou *et al.*, 2000). After that, TR H3N2 viruses reassorted with cH1N1 SIVs as well as human seasonal H1N1 viruses, subsequently generating multiple reassortant viruses which retain a triple reassortant internal gene cassette (TRIG) consisting of human (PB1), avian (PB2 and PA) and classical swine (M, NP and NS) IAV origins (Ma *et al.*, 2009; Vincent *et al.*, 2008). Since the 2009 pH1N1 virus was introduced into pigs, reassortant viruses between the 2009 pH1N1 and the endemic SIVs have been circulating in US swine and notably, the TRIG M segment was replaced by the Eurasian swine M gene (Nelson *et al.*, 2015; Nelson *et al.*, 2012). In Europe, the Eurasian avian-like H1N1, human-like H3N2, and human-like H1N2 lineages have emerged. In the late 1970s, cH1N1 SIV was replaced by avian-like H1N1 virus which has become the dominant swine strain in many European countries (Pensaert *et al.*, 1981). After the 1968 human H3N2 pandemic, human-like H3N2 viruses were detected in swine followed by reassortment with the avian-like H1N1 SIVs in the 1980s (Castrucci *et al.*, 1993). In 1994, a new reassortant H1N2 virus containing genes from human H1N1 virus and H3N2 SIV, was detected in Great Britain and in turn became widespread in other European countries (Brown *et al.*, 1995; Kyriakis *et al.*, 2011). Following the outbreak of the 2009 pH1N1 virus, co-circulation of 2009 pH1N1 with the endemic European SIVs in swine herds led to generation of several reassortant viruses (Howard *et al.*, 2011; Moreno *et al.*, 2011). In Asia, nearly 60% of world's pork production is produced and diverse variants of IAVs are co-circulating in the swine population. The cH1N1 SIVs were first detected in China in 1974, becoming enzootic in swine in many Asian countries, including China, Japan, Thailand and Vietnam. In China, H1N2 virus containing the human origin N2 segment co-circulated with cH1N1 SIVs and H3N2 human IAVs were occasionally transmitted to swine (Peiris *et al.*, 2001; Shortridge *et al.*, 1977). In addition, the H1N1 AIVs which are independent of Eurasian avian-like H1N1 viruses were

detected in 1993 in China (Guan *et al.*, 1996). With the enhanced intercontinental movement of pigs, diverse SIVs were introduced and co-circulated in swine of Asian countries, which led to genetic diversity of SIVs in Asia. For example, the European H1N1 as well as H3N2 SIVs were found in 1999 and 2001, respectively and the North American TR SIVs were first isolated in China in 2002 (Vijaykrishna *et al.*, 2011). Recently, the 2009 pH1N1 virus and its reassortant viruses with endemic SIVs have been detected in swine in many Asian countries (Vijaykrishna *et al.*, 2010). Furthermore, several AIVs such as H9N2, H5N1, H4N8 and H6N6 have been sporadically reported in the swine of a few Asian countries (Su *et al.*, 2012; Trevennec *et al.*, 2011; Xu *et al.*, 2004a; Zhang *et al.*, 2011).

Until now, two different equine IAVs, H7N7 and H3N8 are known to cause considerable respiratory disease in the horse population (Sovinova *et al.*, 1958; Waddell *et al.*, 1963). The H7N7 subtype has not been reported in over three decades and is considered to be extinct in equine (Cullinane *et al.*, 2010) . However, equine H7N7 subtype has been studied for its potential virulence in other mammalian hosts, since the H7N7 lineage contains the HPAIV feature, a multi-basic cleavage site in its HA protein and displaying high virulence in mice without prior adaptation (Gibson *et al.*, 1992; Kawaoka, 1991; Murcia *et al.*, 2010). Since the 1970s, H3N8 equine virus has been circulating in the equine population worldwide (Daly *et al.*, 1996). This H3N8 subtype has also been reported to cross the species barrier and transmit to dogs as well as pigs (Crawford *et al.*, 2005; Tu *et al.*, 2009).

Since 2004, two stable circulating IAV strains, H3N8 and H3N2, have been described in canines. The H3N8 influenza virus which is derived from equine was first isolated in 2004 and caused respiratory disease in dogs (Crawford *et al.*, 2005). Avian-origin H3N2 canine IAV has been detected, generating reassortant viruses with other IAVs such as H5N1 and H1N1 (Lee *et*

al., 2009; Li *et al.*, 2010; Song *et al.*, 2012; Zhu *et al.*, 2015). Serological data indicated that dogs can be infected with the 2009 pH1N1 virus and transmission of pH1N1 virus can also occur between dogs (Dundon *et al.*, 2010). Although there are no known IAVs which are established and stably circulate in cats, several cat infection cases with different IAVs have occurred. These include human-origin H2N2, H3N2, H1N1 including the 2009 pH1N1 virus, avian-origin H7N3 and HP H5N1 virus (Hinshaw *et al.*, 1981; Kuiken *et al.*, 2004; McCullers *et al.*, 2011; Romvary *et al.*, 1975; Songserm *et al.*, 2006; Sponseller *et al.*, 2010) .

Sporadic IAV isolations from marine mammals have been reported. In 1980, H7N7 IAV was isolated from dead seals and later, H4N5, H4N6 and H3N3 IAVs outbreaks were also observed in seals (Callan *et al.*, 1995; Geraci *et al.*, 1982; Webster *et al.*, 1981). In 1984, H13N2 as well as H13N9 subtypes were detected from a pilot whale (Hinshaw *et al.*, 1986). Recently, the 2009 pH1N1 virus was detected from an elephant seal in 2010 and H3N8 IAV of avian origin was isolated from Atlantic harbor seals in 2011 (Anthony *et al.*, 2012; Goldstein *et al.*, 2013).

The mixing vessels

The HA proteins of IAVs bind to sialic acids (SAs) which are linked to galactose with either α2,3 or α2,6 linkage on the host cell surface. Differnet HA proteins have different specificities for recognition of these receptors and the expression of specific SAs on cell surfaces varies between different tissues and host species. Most human IAVs preferentially bind to α2,6-linked SAs while avian and equine IAVs recognize α2,3-linked SAs (Connor *et al.*, 1994). To achieve the interspecies transmission of IAVs, a switch in receptor preference of HA proteins is necessary to target new host species. Therefore, IAVs overcome host barriers by rapid evolving through either gene reassortments or genetic substitutions. The “mixing vessel” theory explains the mechanism that simultaneous infection of one host with avian- and human-like viruses can

generate the novel reassortant viruses which might be able to transmit from avian hosts to humans (Ito *et al.*, 1998; Scholtissek *et al.*, 1985). Pigs are susceptible to both avian- and human-like IAVs due to the distribution of both α2,3- and α2,6-linked SAs in the cells lining the pig respiratory tracts. Therefore, pigs are widely known as a mixing vessel of IAVs and generate the avian/human/swine reassortants which can obtain the preference to recognize human virus receptors and infect humans. The most notable example is the emergence of the swine-origin 2009 pH1N1 virus that caused a recent pandemic in humans. This virus was generated by reassortment between the North American TR H3N2 or H1N2 swine viruses and the Eurasian avian-like H1N1 SIVs (Smith *et al.*, 2009b). Other animal species also express both α2,3- and α2,6-linked SAs receptors suggesting the possibility of acting as the mixing vessel for IAVs. For example, domestic poultry species, such as quails and turkeys possess both avian- and human-type receptors in the intestinal and respiratory tract, while wild waterfowls carry predominantly α2,3-linked SAs (Costa *et al.*, 2012; Gambaryan *et al.*, 2002; Pillai *et al.*, 2010). In fact, several well established AIVs in poultry such as the H9N2, HP H5N1 and H7 subtypes have been shown to have an increased affinity to α2,6-linked SAs receptors compared to other AIVs, while also having the capability to transmit to mammals, including humans (de Wit *et al.*, 2010; Li *et al.*, 2014; Xiong *et al.*, 2014). In addition, several studies demonstrated the possibility of both quails and turkeys as being mixing vessels (Cilloni *et al.*, 2010; Giannecchini *et al.*, 2010; Pillai *et al.*, 2010; Thontiravong *et al.*, 2012). The respiratory tract of both dogs and cats express both α2,3- and α2,6-linked SAs receptors, indicating their potential as intermediate hosts for IAVs (Lin *et al.*, 2012; Wang *et al.*, 2013; Zhang *et al.*, 2013a).

1.7 IAV evolution

IAV evolution by different mechanisms is related with changing antigenicity and viral adaptation to the new species. Two major mechanisms which are involved in IAV evolution are antigenic drift and antigenic shift.

Antigenic drift

Antigenic drift refers to the genetic mutation which results in amino acid substitutions (Smith *et al.*, 1951). The RNA-dependent RNA polymerase of IAV has an error prone nature by lack of a proof reading process during viral replication. Lack of proof-reading results in very high IAV mutation rates, nearly from 1×10^{-3} to 8×10^{-3} substitutions per base pair per year (Chen & Holmes, 2006). The HA and NA surface glycoproteins are associated with inducing the host's neutralizing antibodies to block viral infection. Therefore, mutations which change the amino acid composition in the antigenic sites of HA and NA proteins may permit IAVs to escape from the preexisting host immunity with different antigenic properties and also provide selective advantages for these viruses to emerge as a novel seasonal epidemic strain (Both *et al.*, 1983). Thus, antigenic drift is the reason why the annual influenza vaccine composition needs to be reviewed and updated every year to keep up with evolving IAVs. In addition, this mutation allows IAVs to establish the antiviral drug resistances which have been observed in various IAV strains (de Jong *et al.*, 2005; Lackenby *et al.*, 2008; McKimm-Breschkin, 2000).

Antigenic shift

The eight segmented RNAs of IAVs allow shuffling of gene segments when two or more IAVs co-infect one host cell, which leads to the generation of novel reassortant viruses containing genes from both parental viruses. In particular, antigenic shift is able to produce new

subtypes of IAVs by introducing new HA and/or NA genes from other IAVs (Kilbourne, 1969; Muramoto *et al.*, 2006). These new IAV subtypes are responsible for emerging pandemics when most human populations do not have protective immunity to the novel IAVs (Garten *et al.*, 2009; Scholtissek *et al.*, 1978a).

1.8 IAV epidemics and pandemics

IAVs are the main cause of seasonal epidemics and occasional pandemics. To date, only three IAV subtypes, H1N1, H2N2 and H3N2 have caused pandemics while H1N1 and H3N2 subtypes are currently circulating to cause seasonal epidemics in humans.

Seasonal epidemics

Since 1977, two seasonal epidemic viruses (H1N1 and H3N2) have been co-circulating with frequent reassortment between these subtypes in humans (Holmes *et al.*, 2005; Nelson *et al.*, 2008). The annual epidemics generally affect 10-20% of the population and are estimated to lead to approximately 3 to 5 million cases of severe infections as well as 250,000 to 500,000 deaths worldwide (WHO, 2014) and 200,000 hospitalizations with up to 49,000 deaths in the United State (CDC, 2010). The influenza epidemics mainly occur during wintertime with dry and cold conditions which appear to support efficient IAV transmission and spread (Lowen *et al.*, 2007).

1918 Spanish H1N1 pandemic

The Spanish influenza pandemic caused by the H1N1 virus is known to be the most lethal pandemic in human history. This pandemic affected hundreds of millions and killed approximately 50 million people worldwide (Johnson & Mueller, 2002). An unusual mortality pattern was seen during the 1918 pandemic, presenting a high mortality rate among young aged

adults between 15-34 years old (Glezen, 1996). Although several studies suggested that the 1918 pandemic virus evolved from avian viruses or mammalian hosts, its origin is still undetermined (Smith *et al.*, 2009a; Taubenberger, 2006). The complete genomes of 1918 influenza virus were sequenced from fixed and frozen lung samples from infected patients (Taubenberger *et al.*, 1997). Moreover, reconstruction of this virus using reverse genetics revealed more information regarding the pathogenesis and genetic features of 1918 pandemic influenza virus (Kash *et al.*, 2006; Kobasa *et al.*, 2007; Tumpey *et al.*, 2005a; Tumpey *et al.*, 2007; Weingartl *et al.*, 2009).

1957 Asian H2N2 pandemic

In 1957, a new pandemic H2N2 influenza virus emerged in China and spread to East Asia, North America and Europe. Previously circulating H1N1 human virus which was derived from 1918 pandemic virus was replaced by the new pandemic H2N2 virus. This Asian H2N2 pandemic virus was generated by reassortment between circulating human H1N1 virus and avian H2N2 virus: HA, NA and PB1 genes from avian virus and the remaining genes from human virus (Kawaoka *et al.*, 1989; Scholtissek *et al.*, 1978a). By 1958, its circulation caused 2 million deaths worldwide including 70,000 deaths in the United States (Kilbourne, 2006).

1968 Hong Kong H3N2 pandemic

The Hong Kong H3N2 virus emerged in 1968 and replaced the circulating H2N2 virus. The pandemic H3N2 virus was also the consequence of reassortment between human and avian viruses. Circulating H2N2 virus acquired the new HA and PB1 gene from avian H3 virus and generated the new reassortant H3N2 virus. Until 1970, approximately one million people died globally and about 34,000 people were killed in the United States by the Hong Kong H3N2 pandemic influenza (Kilbourne, 2006).

1977 Russian H1N1 influenza

In 1977, H1N1 influenza virus reoccurred in the Russian-Chinese border region affecting young adults under 23 years old. The recurrent H1N1 virus had high genetic similarity to previous H1N1 viruses which circulated in the early 1950s before the H2N2 outbreak (Nakajima *et al.*, 1978; Scholtissek *et al.*, 1978b) . With regards to the lack of genetic evolution in comparison with the H1N1 virus in 1950s, it is assumed that the 1977 H1N1 virus was an unexpected release from a research lab. The reemerged H1N1 virus co-circulated with H3N2 viruses instead of replacing them and in turn these two viruses continue to circulate together in humans to date (Rambaut *et al.*, 2008).

2009 H1N1 pandemic

The first influenza pandemic of the 21st century was caused by a novel reassortant H1N1 virus of swine origin which spread to worldwide in 2009 (Dawood *et al.*, 2009; Peiris *et al.*, 2009). Since 2009 pH1N1 virus was antigenically similar to previously circulating H1N1 viruses prior to 1957, it mainly affected young or middle-aged adults who didn't have prior immunity, while the elderly in 33% of those aged over 60 years had cross-protective immunity established from previous exposure to H1N1 virus before 1957 or by H1N1 influenza vaccination in 1976 (Hancock *et al.*, 2009). This 2009 pH1N1 virus affected more than 214 countries and territories resulting in hundreds of millions infection cases including over 18,000 deaths throughout the world by August 2010 (WHO, 2010).

Genetic analysis indicated that 2009 pH1N1 virus was generated by the reassortment between triple-reassortant H3N2 swine virus harboring human, avian and swine origin gene segments, classical H1N1 swine virus and Eurasian avian-like H1N1 swine virus (Smith *et al.*, 2009b). The PB2 and PA segments were contributed by North American avian viruses, while the

PB1 gene was from the human H3N2 virus. The HA, NP and NS segments originated from classical swine viruses while the other segments, NA and M, were from the Eurasian avian-like swine viruses (Figure 1-2). It is thought that the ancestor of pH1N1 virus was circulating in swine populations without symptoms for more than 10 years before transmission to humans and the 2009 outbreak (Mena *et al.*, 2016; Smith *et al.*, 2009b). Previous studies with several animal models including mice, pigs, ferrets and non-human primates indicated that 2009 pH1N1 strain is more pathogenic in these animal models compared with seasonal H1N1 strains (Itoh *et al.*, 2009; Maines *et al.*, 2009; Munster *et al.*, 2009; Perez *et al.*, 2009). Recently, this pandemic virus replaced the previous seasonal H1N1 strains and stably co-circulates with seasonal H3N2 viruses in humans. Additionally reassortant viruses between 2009 pH1N1 and human H3N2 viruses have also been detected (Lee *et al.*, 2010; Liu *et al.*, 2010; Myers *et al.*, 2011; Rith *et al.*, 2015) .

Figure 1-2 Origin of 2009 pandemic H1N1

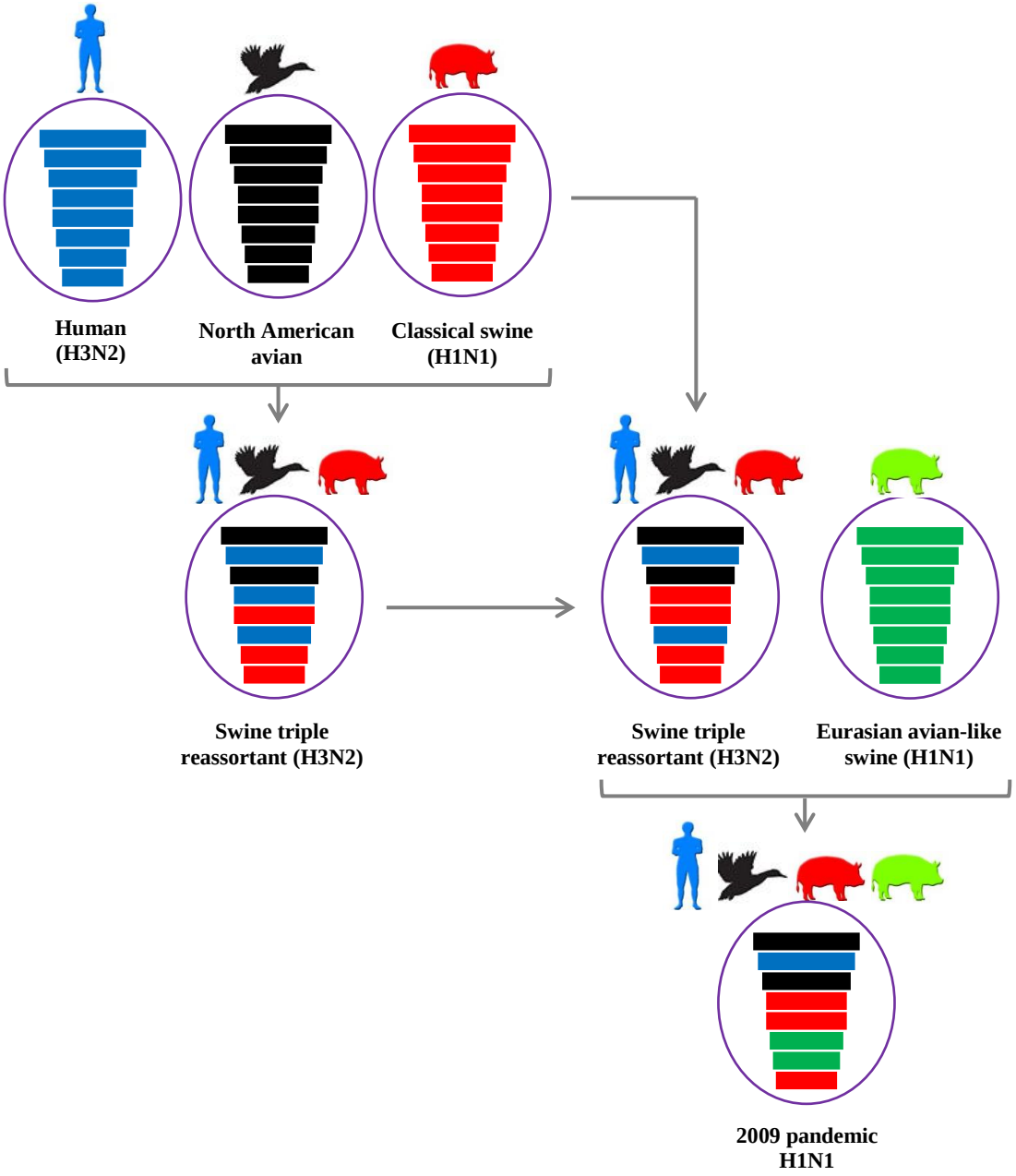


Figure 1-2 2009 pH1N1 virus was generated from the reassortment of triple-reassortant H3N2 swine virus harboring human, avian and swine origin gene segments, classical H1N1 swine virus and Eurasian avian-like H1N1 swine virus. Each gene segments of human, avian, or swine origin corresponds to a characteristic feature above the schematic viral particles.

Pandemic potential

In humans, only three HA subtypes of IAVs (H1, H2 and H3) caused pandemics and have established the ability of efficient human-to-human transmission. However, some other influenza subtypes such as H5, H6, H7, H9 and H10 have been reported to be able to cross the species barrier to infect humans which has raised concerns (Arzey *et al.*, 2012; Ostrowsky *et al.*, 2012; Peiris *et al.*, 1999; Subbarao *et al.*, 1998; Yuan *et al.*, 2013; Zhang *et al.*, 2014).

The first zoonotic case of HPAI H5N1 virus human infections through direct exposure to infected birds was reported in Hong Kong in 1997 and resulted in 6 deaths out of 18 infection cases (de Jong *et al.*, 1997). Since 2003, severe HPAI H5N1 outbreaks have been reported in Asia, Europe and Africa with occasional human infections. As of October 2016, over 850 cases of HPAI H5N1 infections in humans have been confirmed, of which 452 were fatal (WHO, 2016a). Although the HPAI H5N1 virus has limited human-to-human transmission, it is possible that evolutionary changes to the H5N1 virus and reassortment with current human IAVs may lead to better adaptation of avian H5N1 virus to humans, which give rise to significant pandemic threats.

Several H7 subtypes of avian influenza viruses have infected humans with some fatal cases (Belser *et al.*, 2009). In 2003, HPAI H7N7 virus emerged in the Netherlands, causing 89 human cases with one fatality (Fouchier *et al.*, 2004). In addition, there were three human infection cases of poultry workers with HPAI H7N7 virus in Italy in 2013 (Bonfanti *et al.*, 2014; Puzelli *et al.*, 2014). Interestingly, these H7N7 viruses usually induced conjunctivitis in humans, whereas they were highly virulent to poultry (Bonfanti *et al.*, 2014; Fouchier *et al.*, 2004; Puzelli *et al.*, 2014). In 2013, a novel LPAI H7N9 virus of avian origin emerged in China and human infection cases are still being reported (Gao *et al.*, 2013c; Husain, 2014; Liu *et al.*, 2015b). In

humans, this H7N9 virus has caused severe pneumonia as well as acute respiratory distress syndrome, while only mild or no symptoms are observed in poultry (Gao *et al.*, 2013a). As of August 2016, there have been 859 laboratory-confirmed human cases of H7N9 infection including 315 deaths according to the WHO (WHO, 2016b). The outbreaks of H7 subtypes have been reported in poultry along with occasional human infections resulting in serious disease, increasing the consideration of pandemic potential risks (Lopez-Martinez *et al.*, 2013; Ostrowsky *et al.*, 2012; Skowronski *et al.*, 2006; Tanner *et al.*, 2015; Tweed *et al.*, 2004).

LPAI H9N2 viruses have been widely circulating in domestic birds and have become endemic in many Eurasian countries (Alexander, 2003; 2007). Numerous recently emerged avian influenza viruses, such as the H5N1, H7N9, H10N8 and H5N2 subtypes are produced by reassortment between other subtype precursor viruses from wild birds and widespread H9N2 viruses (Guan *et al.*, 1999; Liu *et al.*, 2015a; Pu *et al.*, 2015; Xu *et al.*, 2015). Reassortment with H9N2 viruses gives rise to the ability of wild-bird avian influenza viruses to infect domestic birds and humans more efficiently. Importantly, H9N2 virus and its reassortants have sporadically caused infections in pigs as well as humans with several fatal cases (Gao *et al.*, 2013c; Liu *et al.*, 2014; Yu *et al.*, 2008; Yu *et al.*, 2011; Zhang *et al.*, 2014). Thus, we cannot exclude the possibility that reassortment events between H9N2 virus, human and other influenza viruses may generate new viruses with pandemic potential.

Since the outbreak of 2009 pH1N1 in humans, transmission of pH1N1 virus from humans to swine called reverse zoonosis has been observed around the world (Charoenvisal *et al.*, 2013; Harder *et al.*, 2013; Howden *et al.*, 2009; Keenliside, 2013; Nagarajan *et al.*, 2010; Zhao *et al.*, 2012). Since 2009, the pH1N1 virus has been maintained in the swine population and subsequently reassorted with circulating triple-reassortant H1N1 and H3N2 swine viruses to

generate novel genotype of viruses (Ali *et al.*, 2012; Ducatez *et al.*, 2011; Liu *et al.*, 2012; Vijaykrishna *et al.*, 2010). Notably, the novel generated reassortant H1N2 and H3N2 variant viruses, known as H1N2v and H3N2v, have been reported to infect humans (Epperson *et al.*, 2013; Komadina *et al.*, 2014). In particular, H3N2v which contains the M gene from 2009 pH1N1 virus was first isolated in human in 2011 and there have been over 350 confirmed cases of human infection with H3N2v viruses including one death reported in USA according to CDC report, as of September 2016 (CDC, 2016; Jhung *et al.*, 2013; Nelson *et al.*, 2012). Although the H3N2v viruses have displayed limited human-to-human infection, this virus is antigenically distinct from recently circulating H3N2 human seasonal viruses and has raised the potential risk of a large number of human infections due to the lack of cross-protective immunity. Moreover, with increasing reverse zoonosis events, novel reassortant viruses may increase adaptation and transmissibility among humans.

1.9 Host response to IAV

The host responses play critical roles to protect the host from IAV infection. The innate and adaptive immune responses are the major host defense mechanism against virus infections. However, unbalanced and excessive immune responses to IAV infection cause immunopathology by inducing severe lung damage, respiratory failure and fatal pneumonia. Therefore, an appropriate host response is important to protect the host efficiently.

Innate and adaptive immunity

The first line of defense against IAV infection is the mucus and collectin barriers covering respiratory epithelial cells to prevent viral binding and invading to cells. If IAVs successfully overcome these barriers and start replication in cells, rapid innate cellular defenses

are induced to control virus replication. The IAV infection is recognized by pattern-recognition receptors (PRRs), such as Toll-like receptor (TLR) 3/7 and retinoic acid-inducible gene-I (RIG-I) through detection of the viral double-strand or single-strand RNAs (Diebold *et al.*, 2004; Le Goffic *et al.*, 2007; Pichlmair *et al.*, 2006). The initiated signaling pathways of PRRs induce the production of type I as well as type III IFNs and activate antiviral immune responses (Garcia-Sastre, 2011). During IAV infections, IFNs are mainly produced by respiratory epithelial cells, macrophages and plasmacytoid dendritic cells (pDCs) (Cheung *et al.*, 2002; Hogner *et al.*, 2013; Jewell *et al.*, 2007). The secretion of IFNs leads to the expression of hundreds of different interferon-stimulated genes (ISGs) which establish the antiviral activity to inhibit virus replication efficiently (Randall & Goodbourn, 2008). Several ISGs, including Mx family, viperin and IFN-induced transmembrane (IFITM) family have been shown to have the direct anti-influenza activity (Brass *et al.*, 2009; Pavlovic *et al.*, 1992; Wang *et al.*, 2007; Zimmermann *et al.*, 2011). In addition to the IFN responses, PRRs induce the release of pro-inflammatory cytokines and chemokines by cells of the innate immune system. These pro-inflammatory cytokines and chemokines are responsible for the recruitment of immune cells, such as neutrophils, natural killer (NK) cells and monocytes, to the virus infected site and in turn, activate the adaptive immune response by development and modulation of virus specific T and B cell responses (Braciale *et al.*, 2012; Kawai & Akira, 2008). IAV infection upregulates several inflammatory cytokines and chemokines, including CCL2 (MCP-1), CCL3 (MIP-1α), CCL5 (RANTES), CXCL10 (IP-10) , IL-1β, IL-6, IL-8 and TNFα (Dawson *et al.*, 2000; Herold *et al.*, 2008; Lin *et al.*, 2008; Perrone *et al.*, 2008). The adaptive immune responses are critical for virus clearance and host protection through a virus-specific response as well as the development of immunologic memory (Chiu & Openshaw, 2015). IAV infections elicit virus-specific antibodies

(Mancini *et al.*, 2011; Potter & Oxford, 1979). Particularly antibodies targeting for the two surface glycoproteins, HA and NA proteins, are important for their role in inducing the protective immunity (Gerhard, 2001). HA-specific antibodies are able to neutralize the virus infection by inhibiting virus attachment and entry into the cells. Antibodies binding to NA proteins also have protective potential by inhibiting the release and spread of progeny virus particles. These antibodies which are elicited through either past infection or vaccination mainly protect against the matching strains causing the infections. Therefore, the surface proteins of IAVs undergo the rapid evolutionary changes in response to the selective pressure of the host immune system and as a result, new IAV variants are generated to escape the host protective immunity (Smith *et al.*, 2004). This is why the seasonal influenza vaccine needs to be updated annually to avoid the antigenic mismatch between epidemic strains and vaccine strains. However, some of the antibodies binding to the HA stem region, M2 or NP proteins which are highly conserved among different IAV subtypes can provide a broad immunity not only against the same IAV subtypes but also different IAV subtypes (Carragher *et al.*, 2008; Ekiert *et al.*, 2009; Epstein *et al.*, 2005; Frace *et al.*, 1999; Sui *et al.*, 2009). During IAV infections, cytotoxic T lymphocytes (CTLs) contribute to the viral clearance and heterosubtypic immunity. The CTLs are activated in lymphoid tissues and are recruited into influenza virus-infected sites. Subsequently, they recognize and eliminate the cells infected with IAVs and as a result, inhibit spread of progeny viruses (McMichael *et al.*, 1983). Upon IAV infection, the activated CTLs mostly target the conserved internal proteins of IAVs, such as PA, NP and M1 proteins (Boon *et al.*, 2002; Gotch *et al.*, 1987a; Gotch *et al.*, 1987b; Jameson *et al.*, 1998; Yewdell *et al.*, 1985). Therefore, CTL-mediated immunity provides a cross-reactive protection during heterosubtypic IAV infections (Jameson *et al.*, 1999; Kreijtz *et al.*, 2008; Lee *et al.*, 2008; Tu *et al.*, 2010).

Immunopathology

The host immune responses are critical for the protection of the host from virus infections. However, an excessive immune response can be detrimental. Following viral infection, the initial innate immune response activates pro-inflammatory cytokines and chemokines which induce the recruitment of inflammatory cells at the viral infected site. In some cases, the hyperinduction of cytokines and chemokine also known as a cytokine storm, induces severe inflammation with excessive immune cell infiltration and causes serious pathological tissue damage (Tisoncik *et al.*, 2012). Furthermore, the severe inflammatory responses associated with cytokine storms can cause systemic cytokine storms to occur, which may subsequently lead to multi-organ dysfunction (Tisoncik *et al.*, 2012). In most cases of IAV infection, innate and adaptive immune systems induce appropriate protective immunity for the host. However, several fatal cases, particularly 1918 H1N1, HPAI H5N1, 2009 pandemic H1N1 as well as the recent H7N9 human infections have displayed the influenza-induced cytokine storm (de Jong *et al.*, 2006; Gao *et al.*, 2013b; Shen *et al.*, 2014; Tiwari *et al.*, 2014; Tumpey *et al.*, 2005b; Yu *et al.*, 2013). This excessive immune response damages lung tissues with serious immunopathological changes, reducing the respiratory capacity and ultimately leads to death. In addition to the virus strains, the differential expressions of IAV proteins, such as NS1, PB1-F2 and PA-X proteins which are correlated with modulation of host innate immune response can affect the induction of immunopathological changes in the lower respiratory tract (Hayashi *et al.*, 2015; Jagger *et al.*, 2012; Jiao *et al.*, 2008; McAuley *et al.*, 2007). There is accumulating evidence that CTL-meditated immunity is also responsible for immunopathology during IAV infections. Excessive infiltration of CTLs and overproduction of inflammatory cytokines, such as TNF-α and IFN-γ

from CTLs can cause severe lung immunopathology (DeBerge *et al.*, 2014; Ramana *et al.*, 2015; Small *et al.*, 2001; Xu *et al.*, 2004b).

1.10 IAV virulence factors

To achieve interspecies transmission and infect other hosts, IAVs need to overcome the species barriers and interact with host-specific factors. Hence, IAVs change the genetic traits which are related with virulence and host range to increase viral virulence and to achieve the successful transmission and adaptation in new hosts.

HA

Since the HA protein initiates IAV infection by attachment to cellular receptors and by inducing membrane fusion, the HA protein is an important determinant of the host range and tissue tropism of IAVs, which influences viral pathogenicity. It is known that different IAVs have different receptor specificities. Human IAVs have the preference to recognize α2,6-linked SA receptors which are abundantly expressed on epithelial cells along the human upper respiratory tract (URT), while avian IAVs preferentially bind to α2,3-linked SA receptors which are predominantly found in the human lower respiratory tract (LRT) and in the avian intestinal tract (Connor *et al.*, 1994; Matrosovich *et al.*, 1997; Rogers & Paulson, 1983; Shinya *et al.*, 2006). Thus, avian IAVs can more efficiently replicate deep in the lungs of humans (van Riel *et al.*, 2006). However, to achieve transmission to humans, AIVs need to gain binding affinity to α2,6-linked SA receptors in the URT of humans. Specific amino acid substitutions, particularly in the receptor-binding domain (RBD) of the HA protein can change the receptor specificities from α2,3-linked SAs to α2,6-linked SAs. For H2 and H3 IAV subtypes, amino acid substitutions at position 226 and 228 from the H3 numbering system (Q226L and G228S) in the

RBD can alter the receptor preference from α2,3-linked SAs to α2,6-linked SAs (Matrosovich *et al.*, 2000; Naeve *et al.*, 1984; Rogers & Paulson, 1983). For 1918 H1N1 and 2009 pandemic H1N1 viruses, substitutions E190D and D225G from the H3 numbering system are critical in altering receptor preference from avian to human receptors (Chutinimitkul *et al.*, 2010a; Gamblin *et al.*, 2004; Glaser *et al.*, 2005; Kilander *et al.*, 2010; Tumpey *et al.*, 2007). Several studies have reported amino acid substitutions which are associated with altering the receptor preference from α2,3-linked SAs to α2,6-linked SAs in the HA protein of H5N1 HPAIVs(Chutinimitkul *et al.*, 2010b; Watanabe *et al.*, 2011; Yamada *et al.*, 2006). The novel H7N9 virus which emerged in China in 2013 is able to bind to both avian and human receptors with the mammalian adaptation mutation Q226L (van Riel *et al.*, 2013). These AIVs which acquired the human receptor preference can infect epithelial cells in both the URT and LRT and increase the severity of disease in humans. In addition to amino acid substitutions in the RBD, changes to the glycosylation site can influence the HA affinity for the two different receptors as well as the virulence of IAVs. For the 1918 H1N1 and 2009 pandemic H1N1 viruses, glycosylation patterns are important to determine antigenicity as well as the HA receptor affinity and additional glycosylation sites increase the viral virulence (Sun *et al.*, 2013; Zhang *et al.*, 2013b). In H5N1 HPAIVs, loss of glycosylation sites near the RBD of HA was shown to alter the binding preference to the receptors and increase the virulence (Chen *et al.*, 2007; Wang *et al.*, 2010).

The HA protein is initially synthesized as the precursor protein HA0 and is cleaved into two subunits, HA1 and HA2 (Klenk *et al.*, 1975; Lazarowitz & Choppin, 1975). HPAIVs containing a multibasic cleavage site in the HA protein have different protease sensitivities, in contrast to both LPAIVs and mammalian IAVs containing a monobasic cleavage site in the HA protein. Therefore, the amino acid sequences in the cleavage site of the HA protein determine

cleavage activity by specific proteases from different tissues, which is the main determinant of pathogenicity of AIVs. The HA proteins of LPAIVs and mammalian IAVs are cleaved by trypsin-like proteases which are only expressed in the respiratory and intestinal tract of avian species and in the respiratory tract of humans (Kawaoka & Webster, 1988). Thus, LPAIV infections are restricted to these organs. However, the HA protein of HPAIVs are activated by the ubiquitous furin-like proteases which provide the proteolytic activation of HA proteins in multiple organs, and consequently cause systemic viral replication with severe disease (Stieneke-Grober *et al.*, 1992). HPAIVs emerge from LPAIVs, specifically H5 and H7 subtypes by acquisition of a polybasic cleavage motif. Recent studies showed that introduction of a multibasic cleavage site into non-H5 and -H7 subtypes including H2, H4, H6, H8 and H14 subtypes can induce a highly pathogenic phenotype in chickens (Munster *et al.*, 2010; Veits *et al.*, 2012). In mammals, the effect of a multibasic cleavage site on viral pathogenicity is more diverse than in avian species, depending on hosts and virus strains. Deletion of a multibasic cleavage site from HPAI H5N1 virus only induced respiratory tract infection in mice (Hatta *et al.*, 2001). In ferrets, the multibasic cleavage site in HPAI H5N1 virus was shown to be critical for systemic spread of viruses (Schrauwen *et al.*, 2012; Suguitan *et al.*, 2012). On the other hand, insertion of a mutibasic cleavage site in the HA protein of human H3N2 IAV did not show increased virulence in ferrets (Schrauwen *et al.*, 2011). In the non-human primates, cynomolgus macaques, infection with HPAI H5N1 viruses only induced respiratory tract disease (Rimmelzwaan *et al.*, 2001).

NA

Considering the functions of HA and NA proteins, the optimal balance between HA and NA activities is important for efficient viral transmission and replication (Wagner *et al.*, 2002).

The NA proteins of AIVs preferentially cleave α2,3-linked SAs, while the NA proteins from mammalian influenza viruses can cleave both α2,3-linked SAs and α2,6-linked SAs, suggesting the host-specific adaptation of NA proteins (de Graaf & Fouchier, 2014). In AIVs, amino acid deletions in the stalk regions of the NA protein have been frequently occurred during transmission of AIVs from wild waterfowls to domestic poultry. Although it has yet to be determined why the shortened NA stalk domain is selected in poultry, it could be correlated with sustaining functional HA and NA balance since it supports virus transmission from waterfowl to poultry with enhanced virulence (Baigent & McCauley, 2001; Hoffmann *et al.*, 2012; Munier *et al.*, 2010; Sorrell & Perez, 2007; Sorrell *et al.*, 2010). In mammals, the short stalk region of the NA protein in H5N1 virus contributed to virulence in mice whereas the shortened NA stalk domain limited transmission of 2009 pandemic H1N1 virus between ferrets (Blumenkrantz *et al.*, 2013; Matsuoka *et al.*, 2009). In 2003, HPAI H7N7 emerged in poultry in the Netherlands and caused one fatal case out of 89 human cases of conjunctivitis. Four unique amino acid substitutions in the NA gene were found in the HPAI H7N7 virus isolated from the fatal case compared to the viruses obtained from other patients (Fouchier *et al.*, 2004). It was revealed that these substitutions promote NA enzymatic activity, supporting the efficient virus replication (de Wit *et al.*, 2010).

NS1

The NS1 protein has multiple functions as an important virulence determinant during IAV infection. The key role of the NS1 protein is IFN antagonism which is achieved through several different mechanisms. The NS1 protein prevents the activation of RIG-I by interacting with tripartite motif-containing protein 25 (TRIM25) which inhibits RIG-I ubiquitination, and by binding to viral RNAs to escape the detection by PRRs (Gack *et al.*, 2009; Min & Krug, 2006;

Rajsbaum *et al.*, 2012). The NS1 protein also inhibits the function of the RNA-dependent protein kinase (PKR) by forming a complex with the PKR (Lu *et al.*, 1995; Min *et al.*, 2007). In addition, the NS1 protein prevents the nuclear migration of transcription factors, such as NF-κB and IFN-regulatory factor 3 (IRF3) which induce type1 IFN expression (Talon *et al.*, 2000; Wang *et al.*, 2000). The NS1 protein downregulates the expression of host genes, including ISGs and IFNs by preventing the processing as well as nuclear export of cellular mRNAs and by interacting with cellular factor CPSF30 (cleavage and polyadenylation specific factor 4, 30kDa subunit) (Alonso-Caplen & Krug, 1991; Fortes *et al.*, 1994; Nemeroff *et al.*, 1998; Qiu & Krug, 1994).

Specific residues in the NS1 protein are closely associated with virulence of IAVs. One amino acid substitution at position 92 (D92E) of the NS1 protein is responsible for enhanced pathogenicity of HPAI H5N1 virus in mice and increased virulence of recombinant H1N1 IAVs which carried the NS gene of HPAI H5N1 in pigs by escaping the IFN antiviral response (Seo *et al.*, 2002). The substitution at position 42 (P42S) of the NS1 protein in HPAI H5N1 virus increased virulence in mice and reduced type I IFN production in vitro (Jiao *et al.*, 2008). In addition, L103F and I106M substitutions in the NS1 protein promoted NS1 binding to the CPSF30, which supports viral replication by suppressing the expression of type I IFNs (Twu *et al.*, 2007). The C-terminus of the NS1 protein in both 1918 H1N1 and H5N1 HPAI viruses contains a PDZ ligand domain (X-S/T-X-V), which increased the virus virulence when this domain was introduced into a mouse adapted IAV strain (Jackson *et al.*, 2008). Although the PDZ ligand domain is linked to viral virulence, the role of the PDZ ligand motif in IAV pathogenesis remains unclear. The 2009 pandemic H1N1virus lacks the CPSF30 binding ability and loses the PDZ ligand domain due to the expression of a truncated NS1 protein (Hale *et al.*, 2010b; Neumann *et al.*, 2009). Interestingly, even if these functions are restored, they did not

show obvious effects on virus virulence and transmission in different animal models (Hale *et al.*, 2010a; Hale *et al.*, 2010b).

Polymerase complex

The polymerase complex of IAVs has been shown to be an important contributor to viral virulence. Particularly, the PB2 protein has been recognized to be a key determinant for virus adaptation to mammalian hosts and virulence. The E627K substitution in the PB2 protein is a remarkable genetic signature for the mammalian adaptation of AIVs. This mutation is present in previous pandemic viruses, including the 1918 H1N1, 1957 H2N2 and 1968 H3N2 viruses (Taubenberger & Morens, 2006; Taubenberger *et al.*, 2005). This 627K adaptation was found in over 30% of human HPAI H5N1 isolates since 1997 and in most of the recent human H7N9 isolates (Liu *et al.*, 2013; Long *et al.*, 2013). Since the polymerase activity of AIVs with the PB2 627K residue was more active than with the PB2 627E residue at a lower temperature (33ºC), it was suggested that 627K in the PB2 protein could overcome the cold sensitivity of AIVs and support efficient viral replication at 33ºC in the human URT. Viruses with the PB2 627E can replicate efficiently at a higher temperature (41ºC) which is nearly same temperature of avian intestinal tract (Hatta *et al.*, 2007; Massin *et al.*, 2001). In the absence of the E627K mutation, the substitution D701N in the PB2 protein can compensate for the mammalian adaptation function of 627K in both human HPAI H5N1 and HPAI H7N7 viruses (de Wit *et al.*, 2010; Li *et al.*, 2005; Steel *et al.*, 2009). The D701N substitution increases binding of the PB2 protein to importin-α which is a component of the nuclear import machinery for vRNPs and enables efficient viral replication in mammalian cells (Gabriel *et al.*, 2008; Resa-Infante *et al.*, 2008). The 2009 pandemic H1N1 virus possesses the typical avian virus residues in the PB2 protein, such as 627E and/or 701D while the introduction of substitutions E627K or D701N does not

increase the viral virulence in mice (Herfst *et al.*, 2010). Instead, the substitutions G590S and Q591R in the PB2 protein of 2009 pandemic H1N1 virus compensate for the absence of 627K and support the virus replication in mammalian cells (Mehle & Doudna, 2009).

In the PB1 protein, the substitution N375S is the genetic signature related with host adaptation from avian species to mammalians. The previous 1918, 1957 and 1968 human pandemic viruses contain this N375S substitution in the PB1 protein, while some H3N2 human strains have the 375N (Naffakh *et al.*, 2008; Taubenberger *et al.*, 2005). This indicates that the N375S substitution is not a strict host-range determinant.

Several residues in the PA protein have been reported with the ability to increase the polymerase activity but they have no or only minimal influence on viral virulence in mice (Bussey *et al.*, 2011; Gabriel *et al.*, 2005).

The NP protein determines the sensitivity of IAVs to host myxovirus resistance A (MxA) protein which is induced by IFNs as an important antiviral factor. The HPAI H5N1 virus with a reverse engineered NP protein from human H1N1 virus acquired the MxA resistance in mammalian cells (Dittmann *et al.*, 2008). Adaptive mutations in the NP protein of 1918 H1N1 and 2009 pandemic H1N1 viruses were identified to restrict the MxA sensitivity in human cells and these mutations in the NP protein increased viral virulence of HPAI H5N1 virus in mice (Manz *et al.*, 2013). The N319K substitution in the NP protein has been reported to increase AIV replication in mammalian cells through promoting the interaction with imiportin-α (Gabriel *et al.*, 2005; Gabriel *et al.*, 2008; Gabriel *et al.*, 2011).

PB1-F2

The PB1-F2 protein is an important virulence factor with diverse functions. The PB1-F2 protein has pro-apoptotic activity in immune cells. It is localized to the mitochondria and

interacts with components of the permeability transition pore complex (PTPC), such as the voltage-dependent anion channel 1 (VDAC1) and the adenine nucleotide translocator 3 (ANT3), which results in alteration of the mitochondria permeability. This change in mitochondria induces the formation of membrane pores followed by cytochrome C release and subsequently leads to apoptosis (Chanturiya *et al.*, 2004; Chen *et al.*, 2001; Lowy, 2003; Zamarin *et al.*, 2005). The PB1-F2 protein regulates polymerase activity by interacting with PB1 protein in the nucleus (Mazur *et al.*, 2008; McAuley *et al.*, 2010). In addition, the PB1-F2 protein enhances viral pathogenicity by modulating the innate immune response which promotes lung inflammation and also by increasing the frequency as well as severity of secondary bacterial infection (Alymova *et al.*, 2014; Le Goffic *et al.*, 2007; McAuley *et al.*, 2007; Zamarin *et al.*, 2006). Several amino acid residues in the PB1-F2 protein have been identified as inflammatory residues (L62, R75, R79 and L82) or cytotoxic residues (I68, L69 and V70), which are related with the enhancement of viral pathogenicity (Alymova *et al.*, 2011; Alymova *et al.*, 2014). Moreover, the single substitution N66S in PB1-F2 has been shown to increase the viral virulence of 1918 H1N1 and HPAI H5N1 viruses in mice due to the delayed IFN response (Conenello *et al.*, 2007; Schmolke *et al.*, 2011). The PB1-F2 protein with 66S can interact with the mitochondrial antiviral signaling protein (MAVS) more efficiently than the PB1-F2 protein with 66N and this increased interaction suppresses the early induction of type I IFNs as well as ISGs (Conenello *et al.*, 2011; Varga *et al.*, 2012). Most avian IAVs express the full-length PB1-F2 protein with over 78 amino acid residues, whereas swine and human IAVs, including 2009 pandemic H1N1 virus commonly have truncated PB1-F2 proteins (Pasricha *et al.*, 2013; Zell *et al.*, 2007). This suggests that there could be selective benefits for truncated PB1-F2 protein in IAVs to adapt to the mammalian hosts. However, several recent studies found that these PB1-F2 functions act in a virus strain and

host dependent manner. In a previous study, the PB1-F2 expression in H5N1 HPAIV had a minor effect on pathogenicity in a mouse model, while PB1-F2 protein contributed to the virulence in ducks (Schmolke *et al.*, 2011). In contrast, one recent study demonstrated that the PB1-F2 protein attenuated pathogenicity of H5N1 HPAIV in chickens (Leymarie *et al.*, 2014). In a swine model, restored PB1-F2 protein had minimal effect on viral pathogenicity of the 2009 pH1N1 virus (Pena *et al.*, 2012b). In addition, the PB1-F2 protein of TR H3N2 SIV modulated the viral virulence in a strain-dependent fashion in pigs and also altered viral pathogenicity and transmission in turkeys (Deventhiran *et al.*, 2015; Pena *et al.*, 2012a).

PA-X

It has been demonstrated that the PA-X protein is involved in the modulation of host response as well as viral virulence. The functional role of the PA-X protein is clearly related with the host-shutoff activity. The PA-X protein contains the N-terminal endonuclease domain which mediates the degradation of the cellular mRNAs (Jagger *et al.*, 2012). The PA-X protein targets polymerase II-transcribed RNAs in nucleus and selectively degrades the host mRNAs, while IAV mRNAs resist the mRNA degradation mediated by the PA-X during the IAV infections (Khaperskyy *et al.*, 2016). However, it is still unclear how the PA-X protein discriminates between the host mRNAs and IAV mRNAs, and targets the host mRNAs to degrade them. The host-shutoff activity of the PA-X protein mainly targets the expression of apoptosis, inflammatory and immune response-related genes (Jagger *et al.*, 2012). Therefore, the PA-X deficient virus showed increased apoptosis and upregulated inflammatory as well as immune responses. However, the effect of the PA-X protein on viral pathogenicity is depending on virus strains and hosts. The PA-X expression in 1918 H1N1 and HPAI H5N1 viruses attenuated the viral virulence with reduced pathological lung damages by suppressed immune response in mice

or chickens (Hu *et al.*, 2015; Jagger *et al.*, 2012). On the other hand, 2009 pandemic H1N1 virus with the PA-X expression showed the increased viral virulence with enhanced viral replication in mouse lungs by suppressed immune response (Hayashi *et al.*, 2015). Although the PA-X protein contains the identical N-terminal endonuclease domain with the PA protein, the PA-X protein showed a stronger host shutoff activity compared to the PA protein (Desmet *et al.*, 2013). This indicates that the PA-X unique C-terminal domain plays an important role in suppression of the host gene expression and in agreement with this, recent two studies reported that the first 15 amino acids in the C-terminal region of PA-X protein are critical for maximum shutoff activity of PA-X protein (Hayashi *et al.*, 2016; Oishi *et al.*, 2015).

Chapter 2 - Effects of PB1-F2 on the pathogenicity of H1N1 swine influenza virus in mice and pigs

Abstract:

Although several studies have exploited effects of PB2-F2 in swine influenza viruses, its contribution to the pathogenicity of swine influenza viruses remains unclear. Herein, we investigated effects of PB1-F2 on the pathogenicity of influenza virus using a virulent H1N1 A/swine/Kansas/77778/2007 (KS07) virus, which expresses a full-length PB1-F2, in mice and pigs. Using reverse genetics, we generated the wild type KS07 (KS07_WT), a PB1-F2 knockout mutant (KS07_K/O) and its N66S variant (KS07_N66S). KS07_K/O showed similar pathogenicity in mice as the KS07_WT, whereas KS07_N66S displayed enhanced virulence when compared to the other two viruses. KS07_WT exhibited more efficient replication in lungs and nasal shedding in infected pigs than the other two viruses. Pigs infected with the KS07_WT had higher pulmonary levels of GM-CSF, IFN-γ, IL-6 and IL-8 at 3 and 5 days post-infection, as well as lower levels of IL-2, IL-4 and IL-12 at 1 day post infection compared to those infected with the KS07_K/O. These results indicate that PB1-F2 modulates KS07 H1N1 virus replication, pathogenicity and innate immune responses in pigs, and the single substitution at position 66 (N/S) in the PB1-F2 plays a critical role in virulence in mice. Taken together, our results provide new insights into the effects of PB1-F2 on the virulence of influenza virus in swine, and support PB1-F2 as a virulence factor of influenza A virus in a strain- and host-dependent manner.

2.1 Introduction

Influenza A virus (IAV) is a single-stranded negative sense RNA virus of the *Orthomyxoviridae* family that infects a wide variety of host species, from wild and domestic birds to several mammalian species including humans. It causes annual epidemics and occasional pandemics in humans, which leads to significant public health and economic burdens worldwide. Swine influenza is caused by IAV and is an important acute respiratory disease to the swine industry and has also become a considerable zoonotic agent that threatens public health (Dowdle & Hattwick, 1977; Myers *et al.*, 2007). This fact is exemplified by the 2009 pandemic H1N1 influenza virus that is considered to be a swine-origin virus (Peiris *et al.*, 2009).

The first swine influenza virus (SIV) isolated from pigs was the H1N1 subtype in 1930 (Shope, 1931), also known as the classical H1N1 virus (cH1N1), which circulated predominantly in North American swine herds until 1998. Then, a novel triple-reassortant (TR) H3N2 SIV emerged in 1998 and successfully became established and widespread in North American pig populations (Webby *et al.*, 2000; Zhou *et al.*, 2000). The TR H3N2 virus contains the hemagluttinin (HA), neuraminidase (NA) and PB1 genes from human IAVs; the PB2 and PA genes from avian IAVs; and the M, NP and NS genes from the cH1N1 virus (Webby *et al.*, 2000; Zhou *et al.*, 2000). Subsequently, TR H3N2 viruses reassorted with cH1N1 SIVs and human seasonal H1N1 viruses, resulting in novel reassortants such as H1N2, reassortant H1N1 (rH1N1) and H3N2 SIVs which have become endemic in North American swine herds (Karasin *et al.*, 2000; Lekcharoensuk *et al.*, 2006; Vincent *et al.*, 2008; Webby *et al.*, 2004). These reassortant viruses have a similar composition of six internal genes of human origin (PB1), avian origin (PB2 and PA) and classical swine origin (M, NS and NP), which has been called the triple-reassortant internal gene cassette (TRIG) (Ma *et al.*, 2009; Vincent *et al.*, 2008). The TRIG

cassette is readily able to accept different HA and NA combinations (Vincent *et al.*, 2008). With the introduction of 2009 pandemic H1N1 virus into pigs, the TRIG M gene was replaced by the Eurasian swine M gene (Nelson *et al.*, 2015; Nelson *et al.*, 2012).

IAV contains eight viral RNA segments which encode 10 to 17 viral proteins depending on the strain. The PB1-F2 protein discovered in 2001 is a small non-structural protein which is encoded by alternative translation from the PB1 genome (Chen *et al.*, 2001). Although it can have various lengths from 8 to 101 amino acids, it generally consists of 90 amino acids. The PB1-F2 protein is known to be an important virulence factor with diverse functions. It is localized into the mitochondria and induces apoptosis by interacting with the mitochondrial-dependent apoptotic pathway (Chen *et al.*, 2001; Lowy, 2003). In most avian IAVs, PB1-F2 proteins are expressed in their full-length form (over 78 amino acid residues), while human and swine influenza viruses commonly express truncated PB1-F2 proteins. This suggests that truncated PB1-F2 proteins could be beneficial for adaptation of the virus to the mammalian host (Pasricha *et al.*, 2013; Zell *et al.*, 2007). Overall, the PB1-F2 protein is associated with the survival advantage of IAV in different hosts and modulates virus pathogenicity in a strain- and host-dependent manner. A previous study has shown that restoring the PB1-F2 open reading frame (ORF) in the context of the 2009 pandemic H1N1 virus has minimal effects in swine (Pena *et al.*, 2012b). Although numerous studies that investigate the effects of PB1-F2 on virus pathogenicity and transmissibility of different IAVs in different animal models have been published (Deventhiran *et al.*, 2015; Leymarie *et al.*, 2014; Pena *et al.*, 2012a; Schmolke *et al.*, 2011; Zamarin *et al.*, 2006), the contribution of the PB1-F2 protein to the viral pathogenicity of IAVs in natural hosts such as birds and pigs remains unclear.

In this study, we used a virulent TR H1N1 SIV, A/swine/Kansas/77778/2007 (KS07) which has a human-origin PB1gene and expresses a full-length PB1-F2 protein, to investigate the effects of PB1-F2 on viral pathogenicity in a mouse model and in a natural host, pigs.

2.2 Materials and Methods

Cells and virus strain

Human embryonic kidney (293T) cells were maintained in Opti- modified Minimal Essential Medium (MEM) containing 10% fetal bovine serum (FBS) (Hyclone) and 1% antibiotic-antimycotic (Gibco). Madin-Darby canine kidney (MDCK) cells were cultured in Minimal Essential Medium (MEM) with 5% FBS, 1% antibiotic-antimycotic, 2mM L-glutamine (Gibco) and 1x MEM vitamin solution (Gibco). Porcine kidney (PK-15) cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% FBS, 2mM L-glutamine, 1x MEM vitamin solution and 1% antibiotic-antimycotic. MEM infecting media containing 0.3% bovine serum albumins (BSA) (Sigma-Aldrich), 1% antibiotic-antimycotic and 1μg/ml TPCK-treated trypsin (Sigma-Aldrich) were used for virus infection of cells. The TR H1N1 SIV A/swine/Kansas/77778/2007 (KS07) (GenBank accession number: GQ484355-GQ484362) was isolated from diseased pigs as previously described (Ma *et al.*, 2010) and was used for this study.

Plasmid constructions and rescue of recombinant viruses

Eight-gene segments of KS07 were amplified and cloned into a pHW2000 vector as described previously (Hoffmann *et al.*, 2000; Hoffmann *et al.*, 2001). To generate the KS07 PB1-F2 knockout gene, the PB1-F2 expression initiation start codon and three possible downstream start codons were mutated from ATG to ACG (T120C, T234C, T255C and T270C) while one stop codon was mutated from TAA to TTA (A390T) using a GeneArt® site-directed

mutagenesis kit (Invitrogen) according to the manufacturer's instructions. A single amino acid substitution at position 66 of the PB1-F2 (N66S) was introduced, which has been shown to be associated with increased virulence in mice (Conenello *et al.*, 2007). None of the mutations altered the PB1 open reading frame (ORF) as silent mutations. The sequences of the constructed plasmids and introduced mutations were confirmed by sequencing (Sanger DNA Sequencing service provided by GENEWIZ).

Wild type (KS07_WT), PB1-F2 knocked out (KS07_K/O) and N66S single mutant (KS07_N66S) viruses were generated by reverse genetics as described previously (Hoffmann *et al.*, 2000). Briefly, co-cultured MDCK and 293T cells were transfected with eight constructed pHW2000 plasmids encoding viral genomic RNA segments using Lipofectamine 2000 (Invitrogen). After 48hr of transfection, supernatants were collected and passaged three times on MDCK cells. The rescued viruses were confirmed by sequencing (Sanger DNA Sequencing service provided by GENEWIZ).

Growth kinetics

To evaluate the replication kinetics of recombinant viruses, MDCK and PK-15 cells were grown in 12-well plates and infected with each virus at a multiplicity of infection (MOI) of 0.001 in triplicate. Cell culture supernatants were collected at 12, 24, 36 and 48 hours post-infection (hpi). Virus titers were determined by calculating the 50% tissue culture infective dose ($TCID_{50}$)/ml in MDCK cells using the Reed and Muench method. The plaque assay was performed to compare the size of plaques formed by each recombinant virus on MDCK cells.

Pathogenicity study in mice

Six-week-old female BALB/c mice were used for the pathogenicity study. A total of 56 mice were randomly divided into four groups (14 mice/group). Mice from each group were inoculated intranasally (IN) with 1.5 x 10^6 $TCID_{50}$ of each virus in a volume of 50 µl under slight anesthesia with isoflurane. For the control group, mice were mock-infected with 50 µl of virus-free MEM. Mice were monitored daily for clinical signs and weighed daily until 14 days post infection (dpi). If mice lost more than 25% of their original body weight, they were humanly euthanized. Three mice from each group were euthanized at 3 and 7 dpi and lungs were collected from each mouse to assess virus replication and to perform histopathological analysis. For virus titration, 10% lung homogenates were made using fresh MEM with 1 % antibiotic-antimycotic, and virus titers in the lung homogenates were determined on MDCK cells.

Pathogenicity study in pigs

Thirty-five 3 to 4-week-old pigs, which were confirmed to be seronegative for porcine reproductive and respiratory syndrome virus and SIVs, were used in this study. Each infection group contained nine pigs while the control group contained eight pigs and each group was housed in separate isolation rooms. Pigs were intratracheally infected with 1 x 10^3 $TCID_{50}$ of each recombinant virus or with virus-free MEM as controls. Body temperature and clinical symptoms were monitored daily. At 0, 1, 3 and 5 dpi, nasal swabs were collected from each pig and processed for virus titration on MDCK cells. Three pigs from each group were necropsied at 1 and 3 dpi; three pigs from each infection group and two pigs of the control group were necropsied at 5 dpi. At necropsy, lungs were removed *in toto*, and the percentage of gross lesions on each lobe (each lung lobe is considered as 100%) was assessed by a single experienced veterinarian. The mean of gross lung lesions of seven lung lobes were calculated and presented

for the average lung lesions of each pig. Bronchoalveolar lavage fluid (BALF) samples were collected by flushing each lung with 50ml fresh MEM and viral titers were determined on MDCK cells by calculating the $TCID_{50}$/ml.

Histopathology and immunohistochemistry

Tissue samples (right lungs from mice and nasal turbinates, trachea, as well as right cardiac lung lobes from pigs) were fixed in 10% neutral buffered formalin and processed by the histopathology and immunohistochemistry laboratory of the Kansas State Veterinary Diagnostic Laboratory (KSVDL). A board-certified veterinary pathologist evaluated histopathological lesions of hematoxylin and eosin (H&E) stained tissues. Immunohistochemistry (IHC) staining for mouse lungs was conducted to detect influenza virus antigen in tissues using an anti-influenza A NP monoclonal antibody (mAb). The primary antibody was detected with the Leica Bond Polymer Refine Detection Kit on the Leica Bond-Max. For the lung microscopic lesions in mice, lungs were graded on the following seven criteria: Subjective percentage of lung involved in the histological section examined (4 point scale of 1-4); airway epithelial necrosis, neutrophilic airway inflammation, peribronchiolar lymphocyte cuffing, interstitial pneumonia, airway epithelial hyperplasia (all on a 3 point scale of 1-3); and lastly the presence and absence of hyaline membranes (2 point scale, 0=absent and 1=present). In pigs, microscopic lesions were graded on a 0-3 or 0-4 scale for percentage of airways with epithelial necrosis or inflammation (0-4), peribronchiolar lymphocyte cuffing (0-3), interstitial pneumonia (0-4), airway epithelial hyperplasia (0-4), respiratory epithelial degeneration and necrosis in nasal turbinates and trachea (0-4), and degree of inflammation in trachea and nasal turbinates (0-3) similar to that described previously.

Cytokine and chemokine levels in BALF

Expressions of 12 porcine cytokines/chemokines (GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-18 and TNF-α) in BALF were quantified by MILLIPLEX MAP Porcine Cytokine/Chemokine Magnetic Bead Panel using the Luminex technology following the manufacturer's instructions (Millipore). The data was read and analyzed by a Bio-Plex 200 Suspension Array Luminex System (Bio-Rad).

Statistical analysis

For statistical analysis among groups, analysis of variance (ANOVA) test was used in GraphPad Prism version 5.0 (GraphPad Software). For comparisons of survival curves, a log-rank test was performed. A *p* value of 0.05 or less was considered statistically significant.

2.3 Results

Generation and characterization of wild type KS07 and its mutated viruses in vitro

KS07_WT and its mutated viruses including both KS07_K/O and KS07_N66S were generated by reverse genetics and confirmed by sequencing. Since the N66S substitution in PB1-F2 protein is associated with enhanced virulence in mice as shown previously (Conenello *et al.*, 2007), we generated the mutant KS07_N66S virus to determine whether the effect of this mutation can be applied to the swine-adapted KS07 virus either in mice or in pigs. The rescued three viruses replicated efficiently in MDCK cells and amplified viruses were used for further characterization. The plaque assay showed that KS07_WT as well as both mutated KS07_K/O and KS07_N66S viruses formed similar small pinpoint plaques in MDCK cells (Figure 2-1A). KS07_WT and its two mutated viruses grew efficiently in both MDCK and PK-15 cells, and their titers reached approximately 10^8 $TCID_{50}$/ml; however, no significant difference was

observed between the three viruses in their growth kinetics (Figure 2-1B). In MDCK cells, all three viruses reached their peak virus titer at 24 hpi while in PK-15 cells, they showed their peak virus titer at 48hpi (Figure 2-1B). These results indicate that both PB1-F2 knock-out and single substitution N66S in the PB1-F2 do not affect virus replication and phenotype in tested cells.

PB1-F2 in KS07 does not affect virus replication and pathogenicity in mice while KS07_N66S enhances virulence in mice

As the KS07 H1N1 SIV is not a mouse adapted virus, we infected BALB/c mice with a high dose (1.5×10^6 $TCID_{50}$/mouse) of each virus to evaluate the effect of PB1-F2 on viral pathogenicity. All infected mice showed clinical symptoms such as depression, decreased activities, ruffled fur and obvious weight loss starting at 2 dpi when compared to the control mice. In contrast, KS07_N66S induced more severe body weight loss than both KS07_WT and KS07_K/O viruses, resulting in 100% mortality by 8 dpi (Figure 2-2). Both KS07_WT and KS07_K/O caused 75% mortality in infected mice (Figure 2-2B); however, surviving mice infected with KS07_K/O started to recover two days earlier (6 dpi) compared to those infected with KS07_WT (8 dpi) (Figure 2-2A). Interestingly, KS07_K/O replicated to a significantly higher virus titer in mouse lungs than the KS07_N66S at 3 dpi, whereas virus titers in the mouse lungs were similar among three infection groups at 7 dpi (Figure 2-3A). Histopathological analysis showed that all three infection groups exhibited more severe lung damages at 7 dpi when compared to those at 3 dpi despite no significant differences of histopathological scores observed between different groups at each time point (Figure 2-3B). In mice, most had varying degrees of neutrophilic airway inflammation, airway epithelial degeneration and necrosis, peribronchiolar lymphocytic cuffing, interstitial pneumonia, and epithelial hyperplasia (Figure 2-4). A small number of mice (n=6) also had hyaline membranes lining alveoli (Figure 2-4). NP

antigen deposition of IAV was detected in lung tissues from each mouse in all three infection groups at 3 and 7 dpi by IHC staining (Figure 2-4).

PB1-F2 has moderate effects on viral pathogenicity in pigs

A previous study showed that minimal effects were observed in pigs inoculated with a high dose (10^5 $TCID_{50}$/pig) of 2009 pandemic H1N1 virus expressing either full-length or nonfunctional PB1-F2 protein (Pena *et al.*, 2012b) and our former studies also demonstrated that the 2009 pandemic H1N1 is virulent and causes disease in pigs (Ma *et al.*, 2011). As the KS07 virus is virulent in pigs, three to four-week-old pigs were infected with a low dose (10^3 $TCID_{50}$/pig) of each virus to investigate the effect of PB1-F2 and single substitution N66S in PB1-F2 on viral pathogenicity. None of the infected pigs presented obvious respiratory symptoms such as nasal secretion, coughing or sneezing. Several pigs in each infection group (5/9 pigs in KS_WT group, 6/9 pigs in KS07_K/O or in KS07_N66S group) had mild fever starting at 1 dpi and lasted 1-2 days; only one pig in the KS07_N66S group had a fever lasting for five days. During necropsy, no gross lung lesions were observed in any of the three pigs infected with either the KS07_WT or KS07_K/O virus at 1 dpi, whereas two out of three KS07_N66S infected pigs exhibited minimal gross lung lesions (averaged less than 1 %) (Figure 2-5A). Interestingly, KS07_K/O induced more macroscopic lesions in infected pigs that spread to 5-6 lung lobes when compared to the other two viruses at 3 dpi. At 5 dpi, lung lesions were present in each lung lobe of each infected pig of three groups, but lower lesions were observed in the pigs infected with the KS07_N66S virus (Figure 2-5A).

All three recombinant viruses replicated in the lungs of infected pigs. Virus was detected from bronchoalvelolar lavage fluid (BALF) samples collected at 1, 3 and 5 dpi from all infected pigs with the KS07_WT (Figure 2-5B). In contrast, virus was detected in bronchoalveolar lavage

fluid (BALF) from only two of three pigs at 1 and 3 dpi in the KS07_K/O infected group, and in all three pigs at 5 dpi. On the other hand, virus was detected in BALF from two of three pigs in the KS07_N66S infected group at 1 and 5 dpi, and in all three infected pigs at 3 dpi (Figure 2-5B). Although virus titers were variable between different groups, there were no significant differences of virus titers between three infected groups. No virus was detected in nasal swab samples collected from infected pigs of each group at 1 dpi (Figure 2-5C). Virus nasal shedding was detected from five of six pigs infected with the KS07_WT virus and from four out of six pigs infected with either the KS07_K/O or the KS07_N66S virus at 3 dpi. Virus was found in nasal swabs collected from all three infected pigs of each group at 5 dpi (Figure 2-5C). Virus titer of nasal shedding of the KS07_WT group was higher than those of the other two infection groups despite no significant differences in virus titers observed among infected groups.

Nasal turbinate, trachea and lungs collected from pigs necropsied at 1, 3 and 5 dpi were examined for histopathological analysis (Table 2-1and Figure 2-6). Microscopic lesions were not found in these three tissues of control pigs at the three time points. All influenza infected pigs showed pulmonary lesions characterized by varying degrees of interstitial pneumonia, bronchiolar epithelial necrosis and bronchiolitis with peribronchiolar lymphocytic cuffing, airway epithelial hyperplasia and had different degrees of tracheal (Figure 2-6) and nasal cavity inflammation and necrosis. Microscopic lesions were found in the lung tissues of all three pigs infected with the KS07_WT virus at 1 dpi; however, none of the three pigs infected with the KS07_N66S has lung lesions and two of three pigs infected with the KS07_K/O displayed lesions in lung tissues (Table 2-1). Microscopic lesions were observed in trachea tissues of all three pigs infected with the KS07_WT virus at 1 dpi, but only one of three pigs infected with either the KS07_N66S or the KS07_K/O had lesions in the trachea (Table 2-1). Lung lesions

were found in three or two pigs infected with the KS07_K/O at 3 and 5 dpi respectively; one or none of pigs in the other two infection groups showed microscopic lung lesions at 3 and 5 dpi. One or two pigs from each infected group showed lesions in nasal turbinate tissues at 1 and 3 dpi, while no pigs had lesions in this tissue at 5 dpi (Table 2-1). In contrast to both KS07_WT and KS07_K/O viruses, the KS07_N66S virus induced less histopathological lung and trachea damages in pigs (Table 2-1).

PB1-F2 modulates host immune responses in pigs

To investigate the effects of PB1-F2 expression in KS07 on the host immune response, we next assessed the pulmonary levels of cytokines and chemokines in BALF collected from each pig at 1, 3 and 5 dpi (Figure 2-7). All measured chemokine and cytokine levels in BALF from KS07_N66S infected pigs were similar to those of control pigs except for IL-1β and IL-6 at 5 dpi. All tested cytokine/chemokines except TNF-α in KS07_WT infected pigs and six cytokine/chemokines including IL-1β, IL-2, IL-4, IL-6, IL-10 and IL-12 in KS07_K/O infected pigs were significantly higher compared to those detected in control pigs at 3 or 5 dpi. The KS07_WT infected group showed lower levels of all tested cytokine/chemokines including significantly lower expression of IL-2, IL-4 and IL-12 than the KS07_K/O infected group at 1 dpi. In contrast, KS07_WT infected pigs had higher levels of all tested cytokine/chemokines including significantly higher levels of GM-CSF, IFN-γ, IL-6 and IL-8 than those in the KS07_K/O infected pigs at 3 or 5 dpi. Collectively, N66S substitution in PB1-F2 protein of the KS07 virus has minor effect on innate immune responses in pigs. On the other hand, PB1-F2 protein expression suppresses innate immune responses at early time points while PB1-F2 protein upregulates cytokine/chemokine expression at later time points in pigs. This data

indicates that PB1-F2 modulates immune responses in pigs during viral infection with the KS07 virus.

2.4 Discussion

The PB1-F2 protein is an accessary protein encoded from the +1 ORF in PB1 gene through leaky ribosomal scanning. It has been shown to play important roles in IAV replication that includes cell type- and virus strain-specific proapoptotic functions (Chen *et al.*, 2010; Chen *et al.*, 2001; Lowy, 2003; Zamarin *et al.*, 2005), and regulation of polymerase activity by co-localization as well as interacting with PB1 (Mazur *et al.*, 2008; McAuley *et al.*, 2010). Furthermore, the PB1-F2 protein modulates the innate immune response and pro-inflammatory reactions which exacerbate or attenuate IAV pathogenicity (Le Goffic *et al.*, 2010; Leymarie *et al.*, 2014). It also facilitates secondary bacterial infection in a mouse model (Alymova *et al.*, 2014; McAuley *et al.*, 2007). It has been shown that the PB1-F2 protein has effects on viral pathogenicity in a strain- and host-dependent manner. In H5N1 highly pathogenic avian IAVs (HPAIVs), the PB1-F2 protein has shown different contributions to viral pathogenicity in different hosts such as mice, ducks and chickens (Leymarie *et al.*, 2014; Schmolke *et al.*, 2011). In addition, restoring PB1-F2 in the 2009 pandemic H1N1 influenza virus has minimal effects in pigs (Pena *et al.*, 2012b). In TR H3N2 SIVs, PB1-F2 expression has strain-dependent effects in swine and altered viral pathogenicity and transmission in turkeys (Deventhiran *et al.*, 2015; Pena *et al.*, 2012a).

Although previous studies have shown effects of PB1-F2 in low virulent SIVs in pigs (Deventhiran *et al.*, 2015; Pena *et al.*, 2012a) and a former study has suggested a very low level of PB1-F2 expression in SIV infected cells (Buehler et al., 2013), the effect of PB1-F2 protein in a highly virulent SIV on viral pathogenicity in different animal models remains unclear.

Therefore, in this study we selected a highly virulent H1N1 TR SIV that caused approximately 10% mortality in finishing pigs (Ma *et al.*, 2010) and expresses a full-length PB1-F2 protein. Our results showed that the expression of PB1-F2 and the presence of the N66S mutation in the KS07 virus had no effect on virus replication in both MDCK and PK-15 cells as well as on the formation of plaques in MDCK cells (Figure 2-1). These results are in agreement with a previous study that showed PB1-F2 expression in TR H3N2 SIVs did not impact virus replication in A549 and 3D4/31 cells (Deventhiran *et al.*, 2015). In contrast, Pena *et al*. described that PB1-F2 expression in two different TR H3N2 SIV backgrounds had a different impact on virus replication kinetics in porcine alveolar macrophages and porcine respiratory explants (Pena *et al.*, 2012a). These findings support the hypothesis that the effects of PB1-F2 are cell type- and virus strain-specific *in vitro*.

In mice, PB1-F2 expression in 1918 H1N1 virus enhanced the viral pathogenicity and the N66S mutation in PB1-F2 protein of this virus contributed to increase virulence (Conenello *et al.*, 2007; McAuley *et al.*, 2007). Another previous study found that expression of full-length or truncated PB1-F2 protein or its N66S substitution in 2009 pandemic H1N1 virus does not have a significant impact on virulence in both DBA/2 and BALB/c mice (Hai *et al.*, 2010). In addition, the N66S substitution in the PB1-F2 protein of H5N1 HPAIV showed increased replication and virulence, while deletion of the PB1-F2 protein had no effect on viral pathogenicity in mice (Schmolke *et al.*, 2011). In our mouse study, the PB1-F2 knockout mutant did not alter viral virulence when compare to the KS07_WT virus; however, the single substitution N66S in PB1-F2 resulted in enhanced virulence in mice, evidenced by inducing severe disease and 100% mortality in infected mice when compared to either KS07_WT or KS07_K/O virus (Figure 2-2). In full-length PB1-F2, the N66S mutation has been shown to contribute to the viral pathogenicity

and delaying type I interferon (IFN) response (Conenello *et al.*, 2007; Schmolke *et al.*, 2011). The type I IFN suppression activity of PB1-F2 is due to inhibition of the early interferon-stimulated genes (ISGs) as well as decreasing mitochondrial membrane potential via interacting with the mitochondrial antiviral signaling protein (MAVS) (Conenello *et al.*, 2011; Conenello *et al.*, 2007; Varga *et al.*, 2012). Our results confirm the findings of former studies that PB1-F2 66S is a virulence maker of IAV in mice. Consistent with previous studies, it could be possible that the N66S substitution in KS07 virus leads to enhanced IFN antagonist activity of the PB1-F2, thereby increasing the viral virulence.

In pigs, previous studies using low virulent TR H3N2 SIVs have revealed that deletion of the PB1-F2 ORF or incorporation of the N66S mutation in the PB1-F2 ORF does not influence virus replication, shedding or pathogenicity in pigs (Deventhiran *et al.*, 2015; Pena *et al.*, 2012a). In contrast, the deletion of PB1-F2 expression or N66S substitution in virulent KS07 H1N1 virus has some degree of influence on pathogenicity in swine, i.e., both mutated viruses showed less efficient virus replication in lungs at 1 dpi and nasal shedding at 3 and 5 dpi (Figure 2-5B and C), and induced histopathologic lesions in both lung and trachea tissues from less infected pigs at 1 dpi when compared to the KS07_WT virus (Table 2-1). One recent study demonstrated that swine IAVs express a very low level of PB1-F2 protein relative to human isolates (Buehler *et al.*, 2013). In particular, Buehler *et al.* showed that A/swine/Ohio/511445/2007 (H1N1), which has the highest nucleotide sequence identity (99.7% -99.9%) to the virus genome of KS07 virus (Ma *et al.*, 2010), expressed PB1-F2 protein from less than 1% of the infected cells and PB1-F2 could not be detected in virus infected cells, suggesting that carrying a full-length PB1-F2 ORF does not serve as a predictor for sufficient PB1-F2 protein expression. This could explain why no significantly different phenotypes were observed between wild type and PB1-F2 knockout

viruses in pigs in this study or in former studies. The effect of PB1-F2 protein on virus replication and host immune response needs to be investigated in future studies when it is sufficiently expressed in SIV infected cells.

The pro-inflammatory cytokine response is crucial for recruiting effector cells to the site of infection to clear virus. The PB1-F2 protein has been shown to influence viral pathogenicity by modulating the host innate immune response, which helps to promote lung inflammation (Alymova *et al.*, 2014; Le Goffic *et al.*, 2010). Our pig study showed that decreased levels of all cytokine/chemokines as well as significantly lower levels of IL-2, IL-4 and IL-12 at early time points (1 dpi) and significantly increased levels of GM-CSF, IFN-γ, IL-6 and IL-8 at later time points (3 or 5 dpi) were found in lungs of pigs infected with the KS07_WT compared to what were observed for the KS07_K/O (Figure 2-7). Downregulated and upregulated cytokine/chemokines at early and later time points seem to correlate with observed severity of microscopic lesions in the lung and trachea tissues in both KS07_WT and KS07_K/O groups (Figure 2-6 and Table 2-1). Our results suggested that PB1-F2 modulates host immune response in swine throughout the course of infection. These results are in disagreement with the findings of a former study that showed PB1-F2 modulation of host immune responses only occurs shortly after infection (24 hpi) (Pena *et al.*, 2012b). This discrepancy might be due to the difference in the virus strain used in the studies. In our study we used a virulent North American TR H1N1 virus, rather than the 2009 pandemic H1N1 virus that contains Eurasian swine-origin NA and M genes.

The presence of specific amino acid residues, known as inflammatory residues or cytotoxic residues, at the C-terminal region of the PB1-F2 protein are linked to increased pathogenicity and secondary bacterial infection (Alymova *et al.*, 2011; Alymova *et al.*, 2014;

Conenello *et al.*, 2011; Conenello *et al.*, 2007). The PB1-F2 sequence of KS07 contains only two pro-inflammatory residues (L62 and L82) and none of the cytotoxic motifs, whereas H5N1 HPAIVs and all three pandemic IAVs from the last century (H1N1 1918, H2N2 1957 and H3N2 1968) include three or more of the virulence genetic markers (Figure 2-8). In addition, the C-terminal part of the PB1-F2 protein contains a mitochondrial targeting sequence (MTS) which allows the PB1-F2 protein to localize within the mitochondria (Gibbs *et al.*, 2003; Yamada *et al.*, 2004). This localization allows PB1-F2 to interact with mitochondrial membrane-related proteins followed by mitochondrial permeability alteration resulting in the induction of apoptosis (Chanturiya *et al.*, 2004; Zamarin *et al.*, 2005). One recent report revealed that a translocated full-length PB1-F2 protein in the mitochondria attenuated the mitochondrial membrane potential which suppressed mitochondrial-mediated innate immunity, such as retinoic acid-inducible gene 1 (RIG-I) signaling pathway, and also inhibited the activation of NLRP3 inflammasomes, while a truncated PB1-F2 protein which lacks a C-terminal region was diffused in cytoplasm and did not impact mitochondrial functions (Yoshizumi *et al.*, 2014). Consistent with these findings, all of cytokine/chemokines at 1 dpi in lungs of pigs infected with the KS07_WT in our studies showed lower levels than those of pigs infected with the KS07_K/O (Figure 2-7). It is possible that full-length PB1-F2 expression in KS07_WT negatively regulated mitochondrial-mediated innate immunity and that may affect the secretion of cytokine/chemokines in virus infected pigs at early post infection time points.

Another previous study demonstrated that different secondary structural forms were observed at the C-terminal region of the PB1-F2 protein based on amino acids sequences under membranous solution conditions. One continuous C-terminal α-helix was seen in less virulent strains, while a divided C-terminal α-helix was located in the PB1-F2 proteins of HPAIVs

(Solbak *et al.*, 2013). These structural differences at the C-terminal region of PB1-F2 may allow it to serve as different danger signals and induce different immune reactions in the host. It remains to be determined whether the KS07 PB1-F2 has the C-terminal α-helix structure and how that structure affects immune responses in different hosts.

2.5 Conclusions

In summary, our data indicates that PB1-F2 expression in virulent H1N1 KS07 SIV has effects on virus replication and pathogenicity in the natural host, pigs, but not in mice. In addition, PB1-F2 expression modulates host immune responses in pigs and the substitution N66S in PB1-F2 plays a critical role in virulence in mice, while no effect was found in pigs. Our results provide new insights into the impact of PB1-F2 on virulence of IAVs in swine, and support PB1-F2 as a virulence factor of IAV in a strain- and host-dependent manner.

Figure 2-1 Plaque morphology and growth kinetics of the recombinant influenza viruses

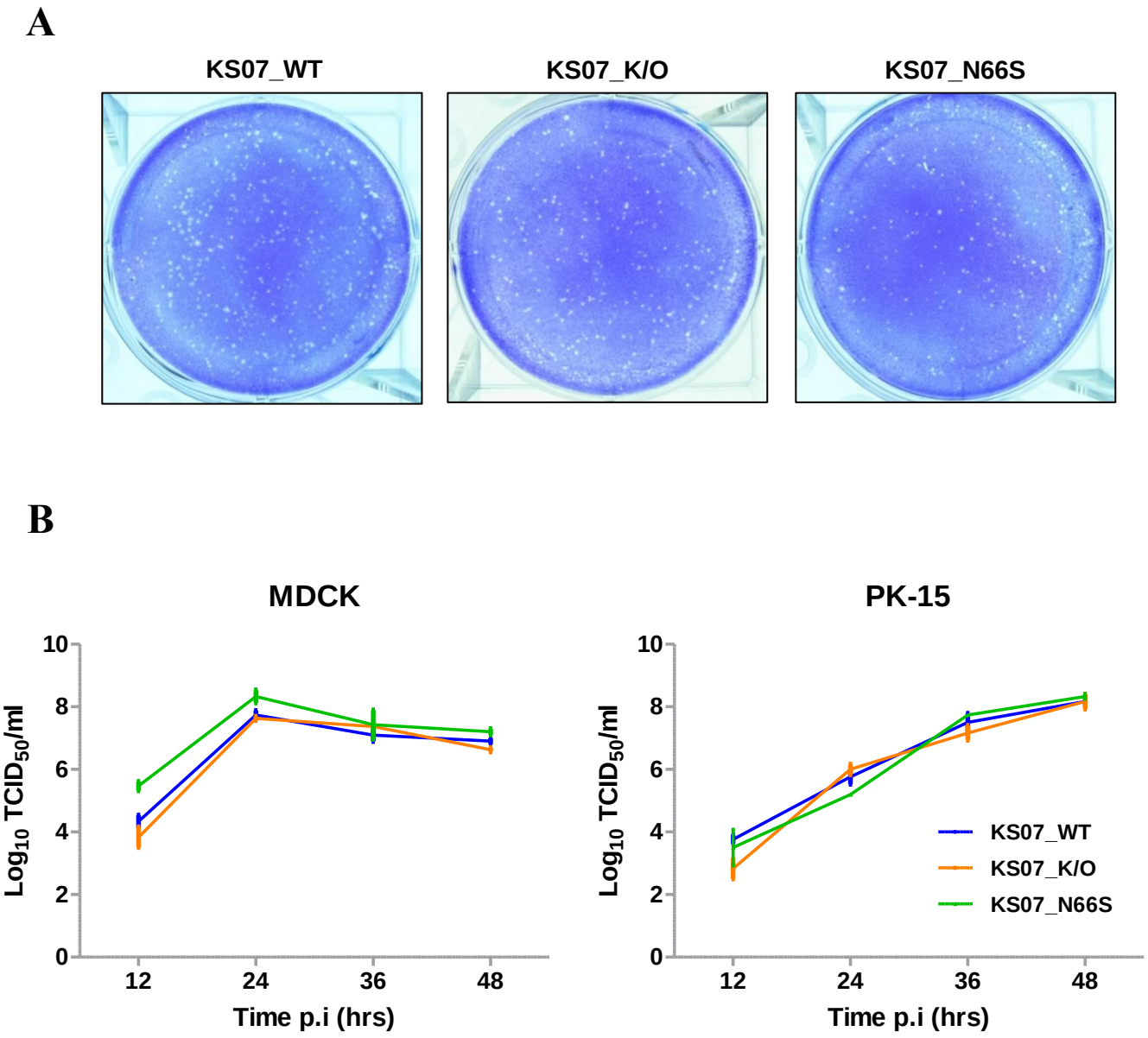


Figure 2-1 (A) Plaque sizes formed by recombinant viruses in MDCK cells at 3 days post-infection (dpi). (B) Growth kinetics of indicated recombinant viruses in MDCK or PK-15 cells infected at an MOI of 0.001. Each data point on the curve indicates the means of the results in triplicate, and the error bars indicate standard errors of the mean (SEM).

Figure 2-2 Contribution of PB1-F2 to pathogenicity of recombinant KS07 in BALB/c mice

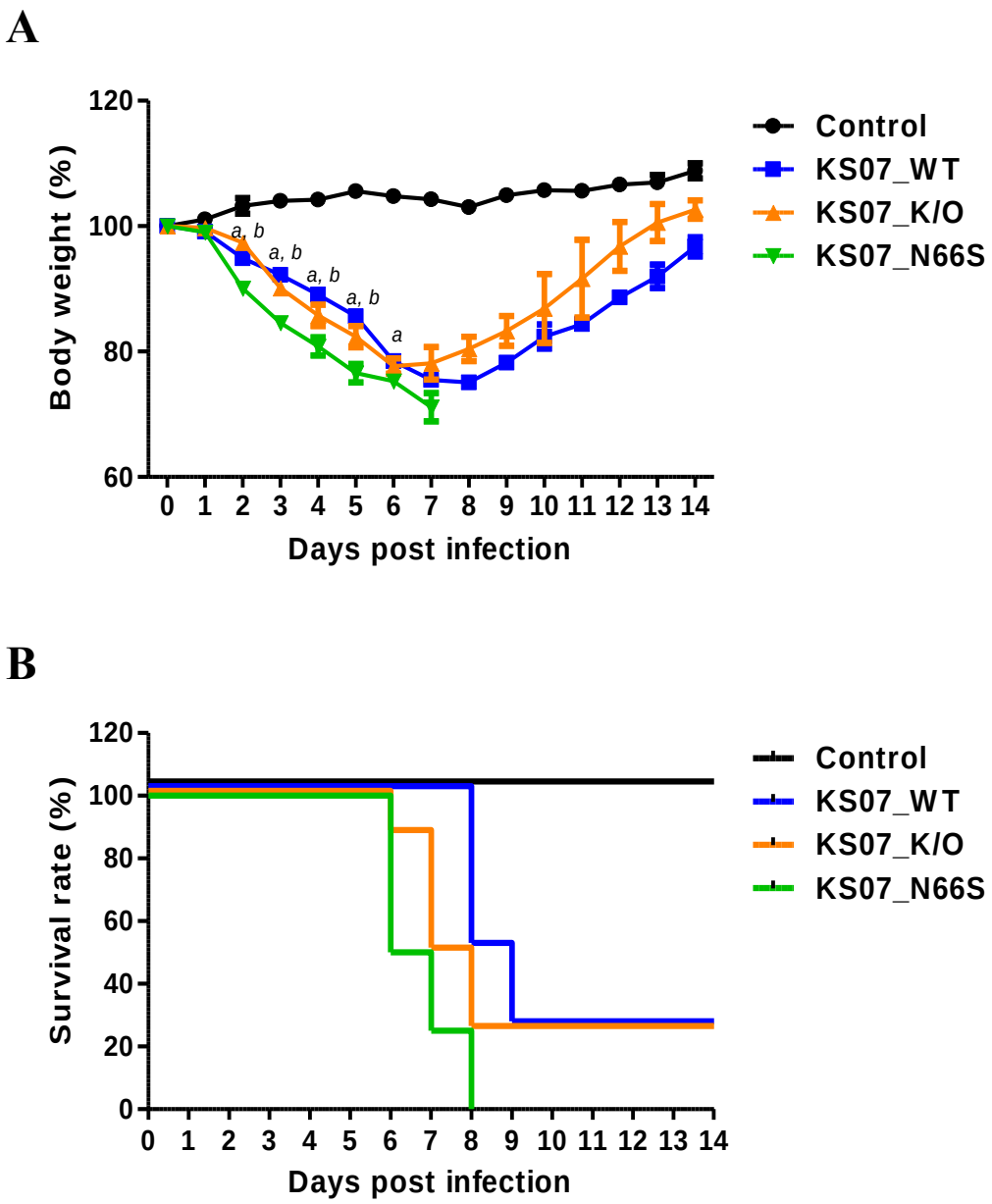


Figure 2-2 (A) Average body weights of surviving mice in each group up to 14 dpi are represented as percentages of the original weight on day 0. a and b on the value point of each day address significant differences (p<0.05) between infected groups (a: KS07_WT and KS07_N66S, b: KS07_K/O and KS07_N66S). The error bars indicate standard errors of the mean (SEM). (B) Survival rate of mice infected with indicated viruses. KS07_N66S infected group showed significantly higher mortality rate compared to KS07_WT infected group (p<0.005 by log-rank test).

Figure 2-3 Lung virus replication and histopathological scores of mouse study

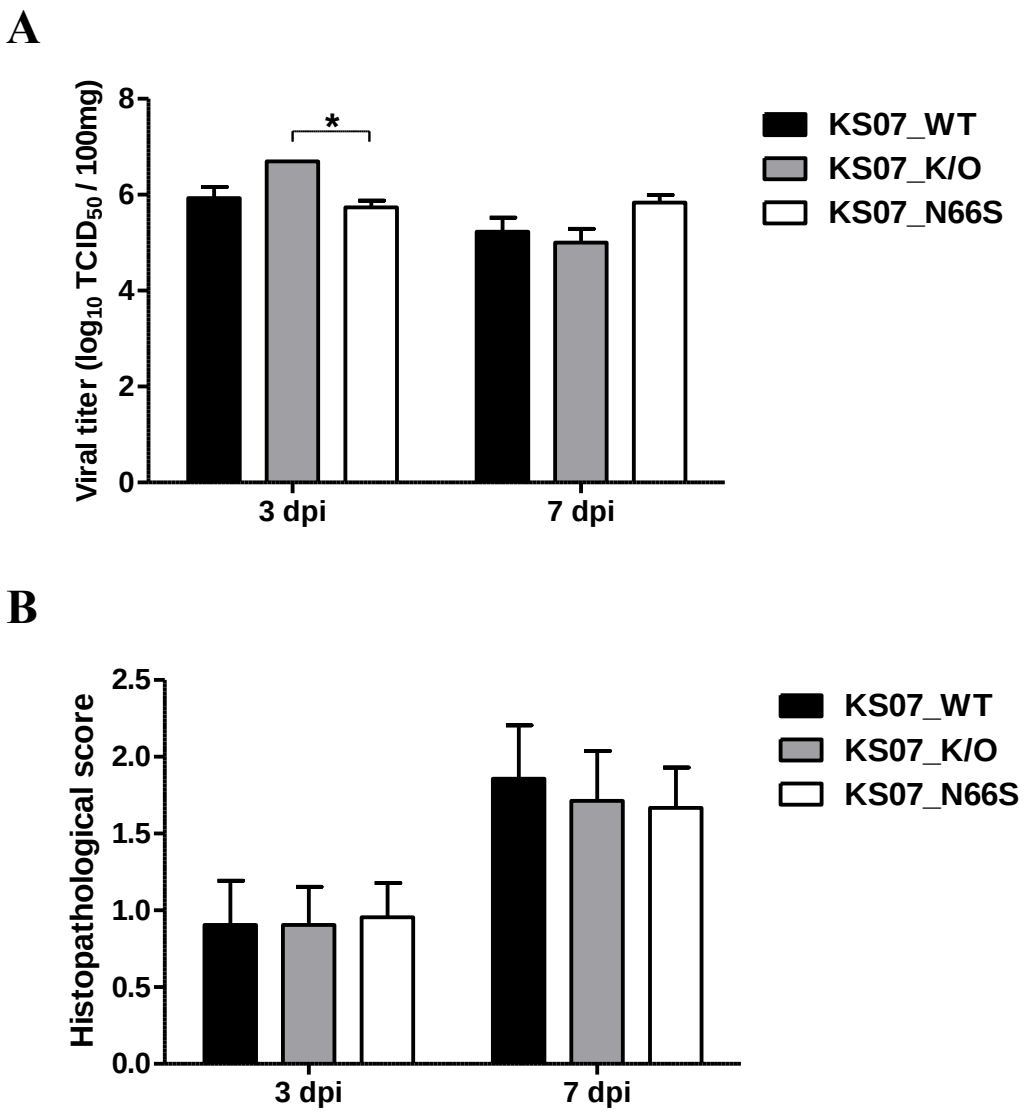


Figure 2-3 (A) Lung virus titers of infected mice were determined at 3 and 7 dpi by calculating the 50% tissue culture infective dose ($TCID_{50}$)/ml in MDCK cells. (B) Microscopic lung scores are presented as average scores ± SEM of three mice in each group at 3 and 7 dpi. The asterisks (*) indicate a statistically significant difference between groups (p<0.05). The error bars represent standard errors of the mean (SEM).

Figure 2-4 H&E and IHC staining of mouse lung sections at 7 dpi

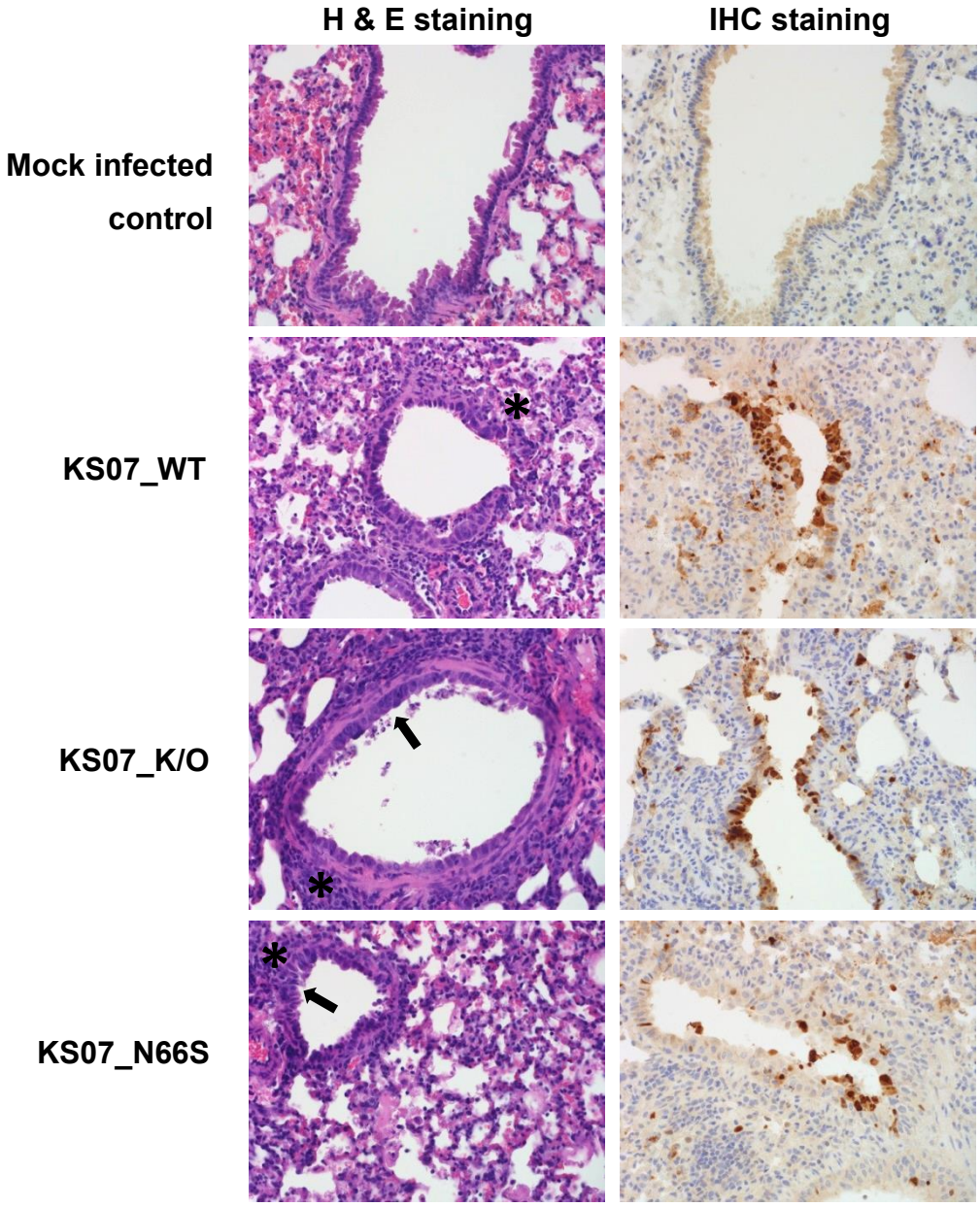


Figure 2-4 H&E stained sections of mouse lungs infected with indicated viruses at 7 dpi showed typical influenza pneumonia. Pictures are representative sections at 40× magnification. In the mock infected control, no lesions are present and there is no antigen deposition in the airway epithelium. In KS07_WT, there are areas of multifocal mild interstitial pneumonia (asterisk) and there is cuffing of bronchioles and vessels by lymphocytes and plasma cells. In KS07_K/O and KS07_N66S, there is mild epithelial hyperplasia (arrow), mild lymphocytic peribronchiolar cuffing (asterisk), and small amounts of fibrin and increased alveolar macrophages in alveolar lumina. IHC staining of lung sections were also conducted to detect influenza virus antigen using an anti-influenza A NP mAb (Brown staining).

Figure 2-5 Contribution of PB1-F2 to pathogenicity of recombinant KS07 in pigs

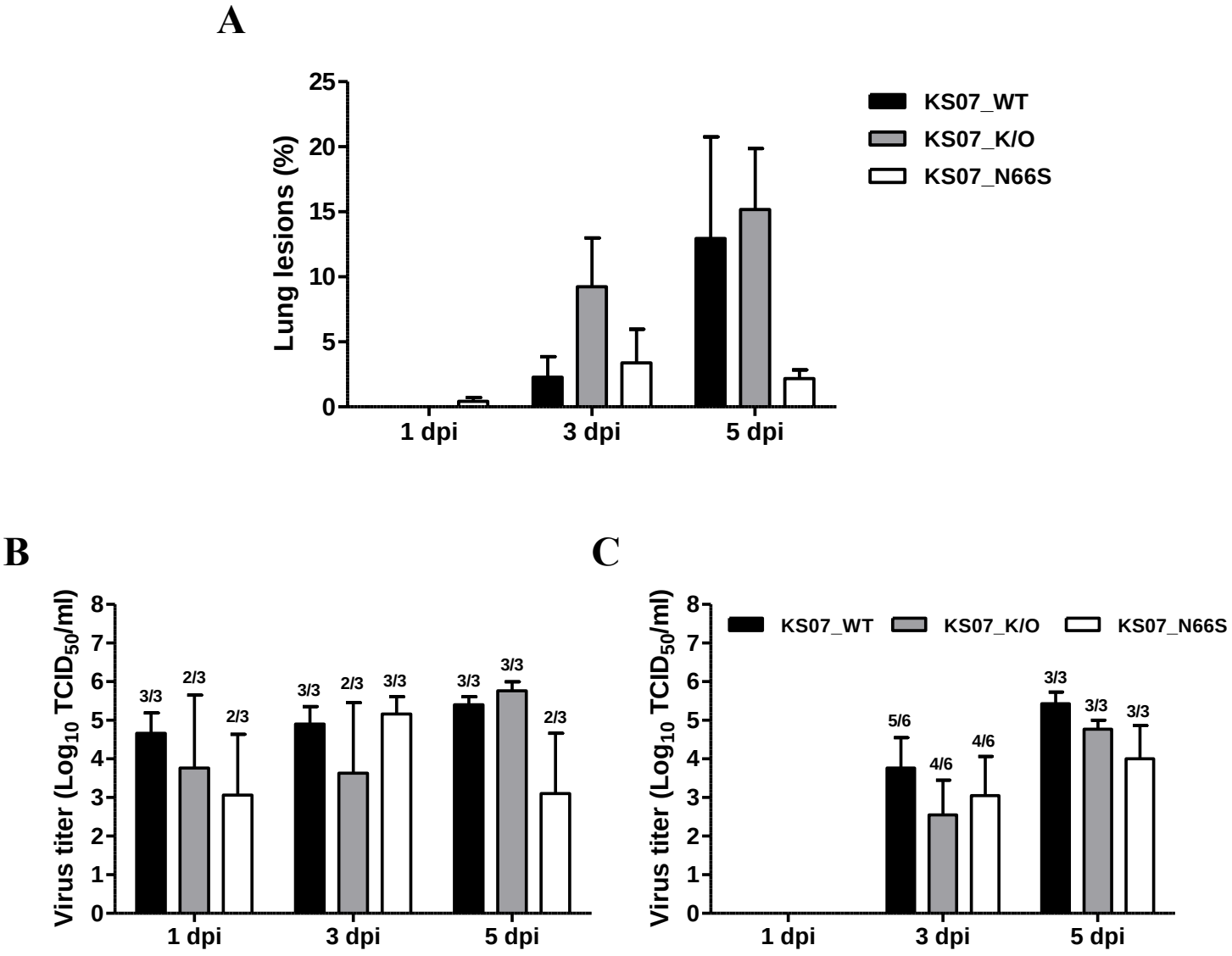


Figure 2-5 (A) Macroscopic lung lesions of infected pigs are shown as the mean percentage ± SEM of gross lesions of three pigs in each group at 1, 3 and 5 dpi. Mean of virus titers in bronchoalveolar lavage fluid (BALF) (B) and in nasal swabs (C) of infected pigs on the days indicated. The number of pigs with positive virus isolation out of the total number of tested pigs is shown above of each bar (B, C).

Figure 2-6 H&E staining of pig lung and trachea sections at 3 dpi

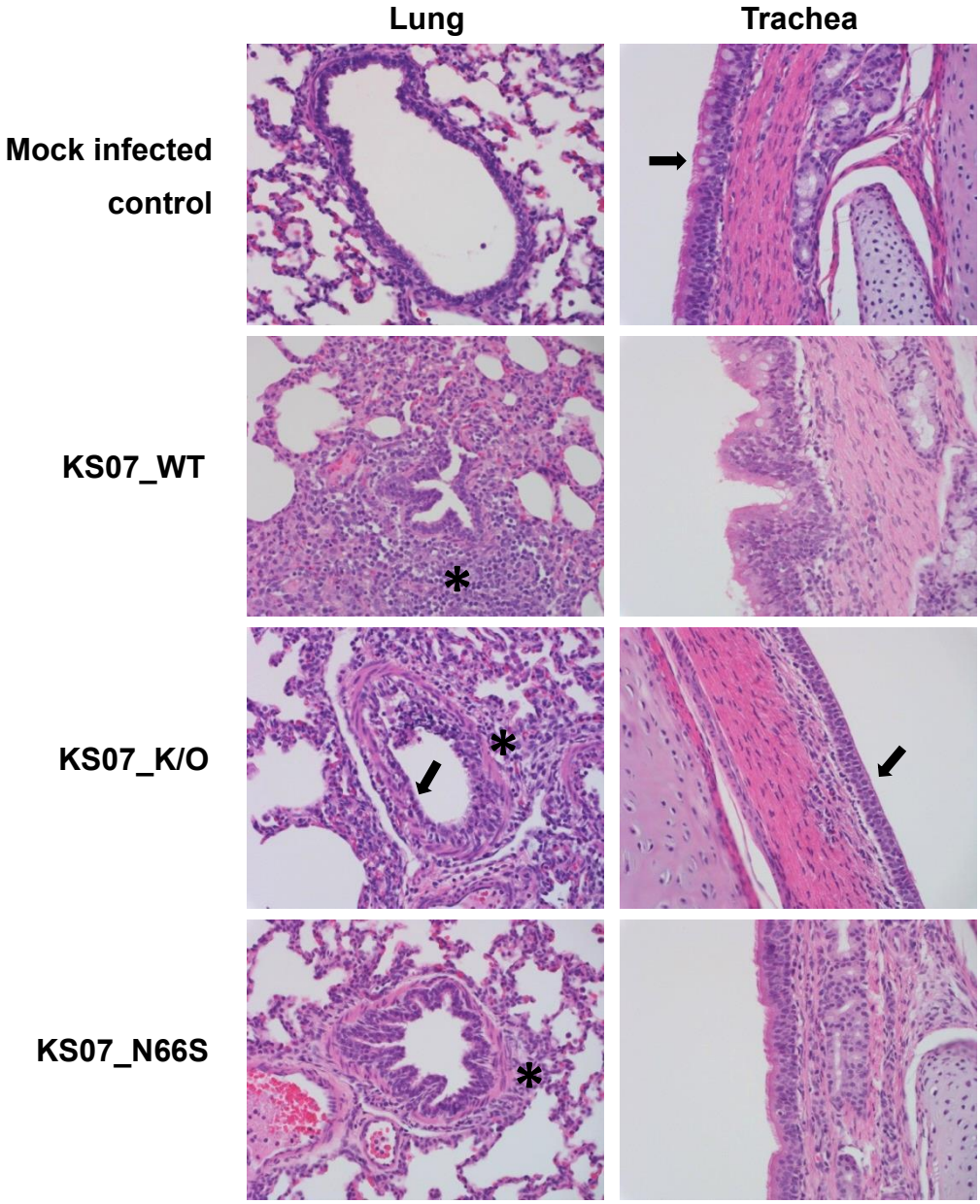


Figure 2-6 Lung and trachea sections of pigs infected with indicated viruses at 3 dpi were stained with H&E. Pictures are representative sections at 40× magnification. In the mock infected control, the lung is normal which is demonstrated by normal airway epithelium and alveoli, and tracheal epithelium has goblet cells and cilia (arrow). In KS07_WT, the bronchiole is surrounded by lymphocytes and plasma cells which extend into the alveolar septa (interstitial pneumonia) (asterisk). In KS07_K/O, there is flattening of airway epithelium (arrow) and cuffing by peribronchiolar cuffing by lymphocytes (asterisk). The trachea in this pig has diffuse loss of cilia and goblet cells consistent with early degeneration due to influenza (arrow). In KS07_N66S, there is cuffing of bronchioles and vessels by lymphocytes and plasma cells (asterisk) and the trachea lacks goblet cells but has a normal layer of cilia.

Figure 2-7 Cytokine/chemokine levels in BALF of infected and control pigs

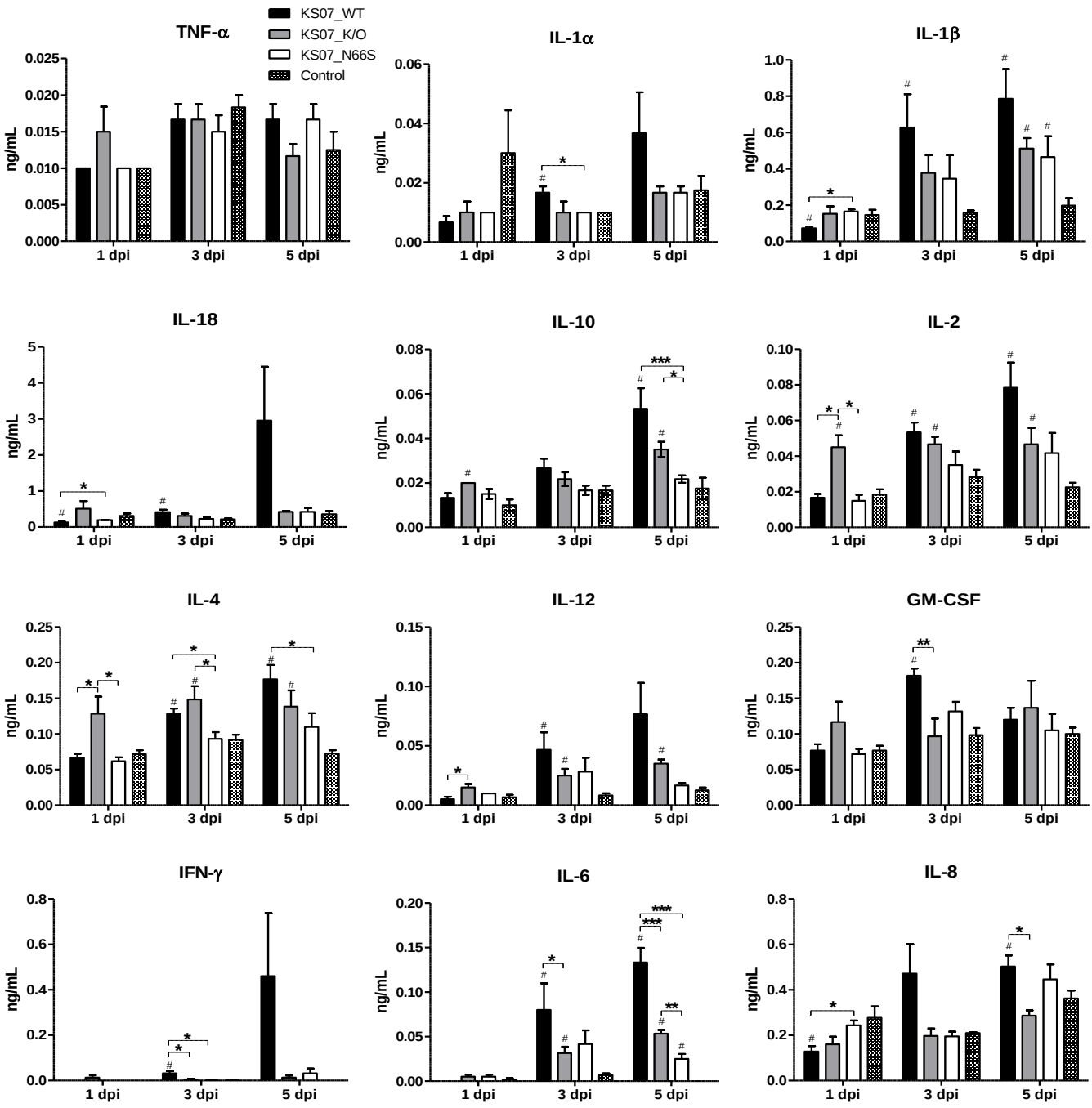


Figure 2-7 Expressions of the indicated 12 porcine cytokines/chemokines in BALF were quantified using the Luminex technology. Data represent the average values ± SEM of three pigs in each group on the days indicated. Significant differences (p<0.05) between two groups are indicated with dashed brackets. The # represents a statistically significant difference with the mock infected control group. The asterisks (*) indicate a statistically significant difference between virus infected groups (*: p<0.05. **: p<0.01 and ***: p<0.001).

Figure 2-8 Alignment of C-terminal sequences of PB1-F2 from selected IAVs

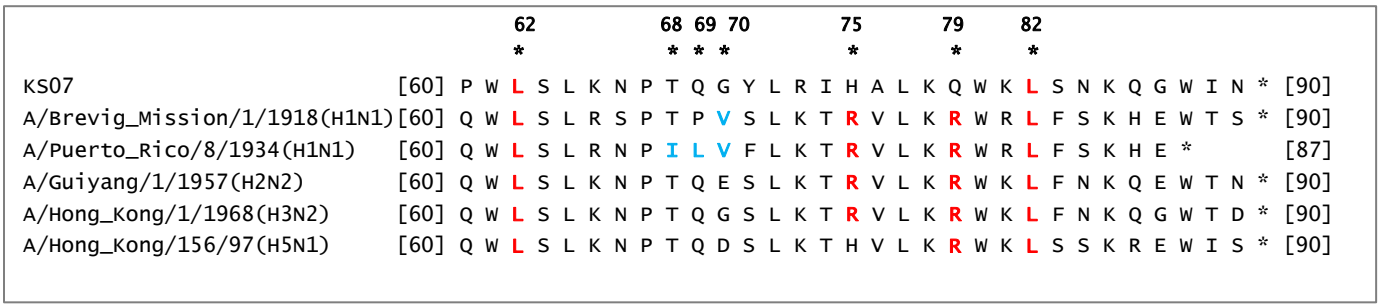


Figure 2-8 Amino acid sequences of the C-terminal region in KS07 PB1-F2 protein was aligned to those of the last century pandemic strains (H1N1 1918, H2N2 1957 and H3N2 1968), a previously characterized strain (PR8 H1N1), and one H5N1 HPIAV. Amino acid residues in red or blue letters have been identified as inflammatory residues (L62, R75, R79 and L82) or cytotoxic residues (I68, L69 and V70), respectively. Numbers with asterisks indicate amino acid positions according to PB1-F2 numbering.

Table 2-1 Microscopic lesions of respiratory tract and lungs in infected and control pigs on days 1, 3, and 5 dpi

	No. of pigs with microscopic lesions/ No. of the total euthanized pigs on the indicated days (microscopic lesion score)								
	1 dpi			3 dpi			5 dpi		
Virus	Lung	Trachea	NT	Lung	Trachea	NT	Lung	Trachea	NT
KS07_WT	3/3 (0.80±0.23)	3/3 (0.50±0.00)	1/3 (0.50)	1/3 (1.60)	2/3 (1.25±0.20)	1/3 (0.50)	1/3 (1.60)	0/3	0/3
KS07_K/O	2/3 (1.70±0.90)	1/3 (1.00)	2/3 (0.50±0.00)	3/3 (1.33±0.29)	1/3 (0.50)	1/3 (0.50)	2/3 (0.7±0.41)	1/3 (1.00)	0/3
KS07_N66S	0/3	1/3 (0.50)	2/3 (0.50±0.00)	1/3 (1.60)	1/3 (0.50)	1/3 (0.50)	0/3	0/3	0/3
Control	0/3	0/3	0/3	0/3	0/3	0/3	0/2	0/2	0/2

Results are presented as the number of pigs with microscopic lesions out of the total number of pigs on the indicated days. Three different tissues in the respiratory tract were evaluated. Numbers in parentheses indicate the mean score of microscopic lesions ± standard errors of the mean (SEM). NT, nasal turbinate.

Chapter 3 - Impacts of different expressions of PA-X protein on 2009 pandemic H1N1 virus replication, pathogenicity and host immune responses

Abstract:

A new influenza virus protein, PA-X, is expressed by ribosomal frameshifting from PA gene. Although several studies have described the function of PA-X, the impact of different expressions of PA-X protein including full-length, truncated or PA-X deficient forms on viral pathogenicity and host response remains unclear. Herein, two mutated viruses were generated using reverse genetics in the genetic background of H1N1 A/California/04/2009 (CA09) which expresses a truncated PA-X protein: one is CA09_PA-X_Full which encodes a full-length PA-X, and the other is CA09_PA-X_K/O in which the PA-X is knocked out. The CA09_PA-X_Full virus grew more efficiently in MDCK and A549 cells than both CA09_WT and CA09_PA-X_K/O viruses. In addition, expression of either full-length or truncated PA-X protein significantly increased viral polymerase activity and suppressed co-transfected gene expression in 293T cells when compared to deficient PA-X expression. The CA09_PA-X_K/O showed attenuated virulence in mice compared to the other two viruses. However, CA09_PA-X_K/O induced more severe lung damage with higher expression of pro-inflammatory cytokines in mouse lungs than the other two viruses. The results indicate that expression of either full-length or truncated PA-X protein enhanced viral replication and pathogenicity as well as reduced the host innate immune response by host shutoff activity when compared to CA09_PA-X_K/O and that full-length PA-X expression exhibited a greater effect than the truncated PA-X form. Our results provide novel insights of PA-X on viral replication and host immune responses.

3.1 Introduction

Influenza A virus (IAV) is a single-stranded negative sense RNA virus that belongs to the *Orthomyxoviridae* family. It contains eight segmented viral genomes and was thought to encode 10 viral proteins (PB2, PB1, PA, HA, NP, NA, M1, M2, NS1 and NS2). Recently, several new influenza viral proteins have been discovered, such as PB1-F2 (Chen *et al.*, 2001), PB1-N40 (Wise *et al.*, 2009), PA-X (Jagger *et al.*, 2012), NS3 (Selman *et al.*, 2012), PA-N155, PA-N182 (Muramoto *et al.*, 2013), M42 (Wise *et al.*, 2012), and PB2-S1(Yamayoshi *et al.*, 2016). Among these, the PA-X protein is translated through a +1 ribosomal frameshifting event in segment 3, the PA gene. This PA-X protein has the identical N-terminal 191 amino acids (aa) to PA protein and a PA-X unique C-terminal domain of 41 or 61 aa by alternative translation (Jagger *et al.*, 2012). Expression of PA-X protein in most IAVs is the full-length form with 61 aa in the C-terminal domain, while both the 2009 pandemic H1N1 (pH1N1) virus and triple-reassortant swine influenza viruses circulating in North American swine herds express a truncated PA-X protein with only 41 aa due to a stop codon at position 42 in the C-terminal domain (Jagger *et al.*, 2012; Shi *et al.*, 2012). Since PA-X shares the N-terminal endonuclease domain with PA, it provides the host shutoff activity for PA-X to destroy the host mRNA and suppress host protein synthesis (Desmet *et al.*, 2013; Jagger *et al.*, 2012). Although PA and PA-X have the same N-terminal domain, PA-X has been shown to have a stronger endonucleolytic activity than PA, indicating that the unique C-terminal part of PA-X is also responsible for shutoff activity (Bavagnoli *et al.*, 2015; Desmet *et al.*, 2013; Hayashi *et al.*, 2016; Oishi *et al.*, 2015).

Recently, many studies have described the effects of PA-X on viral replication and pathogenicity. Jagger et al. reported that PA-X expression reduced the viral pathogenicity of the1918 pandemic H1N1 virus, even if PA-X had no effect on viral replication (Jagger *et al.*,

2012). Two other reports also showed that loss of PA-X expression increased viral replication and pathogenicity of 2009 pH1N1 virus and H5N1 highly pathogenic avian influenza virus (HPAIV) *in vitro* and *in vivo* when compared to the wild type viruses that express PA-X (Gao *et al.*, 2015b; Hu *et al.*, 2015). In contrast to previous reports, recent studies showed that PA-X deficient 2009 pH1N1 and H9N2 viruses attenuated viral pathogenicity in mice compared to the wild type virus (Gao *et al.*, 2015c; Hayashi *et al.*, 2015). Other studies also investigated the contribution of 20aa at the C-terminal end of PA-X to viral replication and virulence by comparing PA-X full-length form with the truncated form. Gao et al. demonstrated that three different IAVs (2009 pH1N1, H5N1 HPAIV and H9N2 AIV) increased viral replication and pathogenicity when the viruses express the full-length PA-X protein with 61aa in the C-terminal domain compared to those with a truncated PA-X expression (Gao *et al.*, 2015a), whereas triple-reassortant H1N2 swine influenza virus with truncated PA-X enhanced viral pathogenicity and replication in pigs (Xu *et al.*, 2016).

The host shutoff activity of PA-X protein is expected to weaken the host antiviral response by inhibiting host protein synthesis including immune related gene expression. Previous studies reported that PA-X deficient virus markedly upregulated expression levels of apoptosis, inflammation and immune response related genes in mice or chicken lungs, using global gene expression profiling (Hu *et al.*, 2015; Jagger *et al.*, 2012). Recently, Hayashi et al. found that PA-X deficient virus induced a significantly greater amount of IFN-β mRNA as well as more anti-hemagglutinin neutralizing antibodies in mice when compared to the wild type virus (Hayashi *et al.*, 2015). Therefore, it suggests that PA-X is an important accessory protein of IAV as an immune modulator and virulence factor. However, the detailed underlying mechanisms of

PA-X function and the effects of different expressions of PA-X protein on viral pathogenicity as well as host immune response remain unclear.

In this study, we used 2009 pH1N1 A/California/04/2009 (CA09) virus which expresses a truncated PA-X protein and its two mutated viruses generated by reverse genetics to investigate the impacts of three different expressions of PA-X protein (full-length, truncated and PA-X deficient forms) on viral replication, pathogenicity and host innate immune response.

3.2 Materials and Methods

Cells

Human embryonic kidney (293T) cells were cultured in Opti-modified Minimal Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS) (Hyclone) and 1% antibiotic-antimycotic (Gibco). Madin-Darby canine kidney (MDCK) cells were grown in MEM containing 5% FBS, 1% antibiotic-antimycotic, 2mM L-glutamine (Gibco) and 1X MEM vitamin solution (Gibco). Human lung adenocarcinoma epithelial (A549) cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% FBS, 2mM L-glutamine, 1X MEM vitamin solution and 1% antibiotic-antimycotic. MEM infecting media containing 0.3% bovine serum albumins (BSA) (Sigma-Aldrich), 1% antibiotic-antimycotic and 1μg/ml TPCK-treated trypsin (Sigma-Aldrich) were used for virus infection of cells.

Plasmid constructions

Eight-plasmid reverse genetics for the 2009 pH1N1 A/California/04/2009 (CA09) virus has been established and described in our previous study (Qiao *et al.*, 2012). Eight-gene segments of CA09 virus were cloned into a pHW2000 vector (Hoffmann *et al.*, 2000; Hoffmann *et al.*, 2001). To generate the CA09_PA-X_Full virus that expresses a full-length PA-X, a single

amino acid substitution from UAG (stop) to UGG (tryptophan) at position 42 in the C-terminal domain of PA-X protein was introduced based on pHW2000-PA_WT using a GeneArt® site-directed mutagenesis kit (Invitrogen) according to the manufacturer's instructions (Figure 3-1). The resulting plasmid was named pHW2000-PA_PA-X_Full. To construct the PA-X deficient virus that does not express truncated or full-length PA-X, three nucleotide mutations in frameshifting motif were introduced from UCC UUU CGU to UCC UUC AGA based on pHW2000-PA_WT by site-directed mutagenesis to prevent PA-X expression as described previously (Figure 3-1) (Jagger *et al.*, 2012). The resulting plasmid was named pHW2000-PA_PA-X_K/O. None of the mutations altered the PA open reading frame (ORF) as silent mutations which were confirmed by sequencing. To investigate expression of different PA-X forms, the coding regions of proposed different PA-X forms were cloned into pCAGGS vector (kindly provided by Dr. Adolfo García-Sastre, Mount Sinai School of Medicine, NY, USA) and the resulting three plasmids were named pCAGGS_PA-X_WT, pCAGGS_PA-X_Full and pCAGGS_PA-X_K/O. To investigate effects of different expressions of PA-X protein on the polymerase activity, ORFs of wild type and two mutated PA genes as well as PB1, PB2 and NP genes of the CA09 virus were cloned into pCAGGS vector (. The resulting plasmids were pCAGGS_PA_PA-X_WT, pCAGGS_PA_PA-X_Full, pCAGGS_PA_PA-X_K/O, pCAGGS_PB1, pCAGGS_PB2, and pCAGGS_NP that were confirmed by sequencing**.**

Rescue of wild type and mutated CA09 H1N1 recombinant viruses

Wild type (CA09_WT), PA-X knock-out (CA09_PA-X_K/O) and full-length PA-X expression (CA09_PA-X_Full) CA09 viruses were generated by reverse genetics using the eight-plasmid system. To rescue CA09_PA-X_K/O and CA09_PA-X_Full viruses, the pHW2000-PA_WT was replaced with pHW2000-PA_PA-X_K/O or pHW2000-PA_PA-X_Full,

respectively. Briefly, MDCK and 293T cells were co-cultured in 6-well plates and transfected with eight constructed pHW2000 plasmids encoding viral genomic RNA segments using Lipofectamine 2000 (Invitrogen). After 48 hours post transfection, supernatants were collected and passaged three times on MDCK cells. The rescued viruses were confirmed by sequencing (Sanger DNA Sequencing service provided by GENEWIZ).

Replication kinetics

To evaluate the growth kinetics of recombinant viruses, MDCK and A549 cells were cultured in 12-well plates and infected with each virus at a multiplicity of infection (MOI) of 0.01 in triplicate. Supernatants were collected at 12, 24, 36 and 48 hours post-infection (hpi). Virus titers were determined by calculating the 50% tissue culture infective dose ($TCID_{50}$)/ml in MDCK cells. The plaque assay was performed to compare the size of plaques formed by each recombinant virus on MDCK cells.

Western blot analysis

Confluent 293T cells were transfected with pCAGGS_PA-X_WT, pCAGGS_PA-X_K/O or pCAGGS_PA-X_Full using Lipofectamine 2000 (Invitrogen). Mock control was transfected with empty pCAGGS plasmid. At 18 hours post-transfection, cells were washed twice with phosphate-buffered saline (PBS) and total cell lysates were extracted using CelLytic M cell lysis reagent according to the manufacturer's instructions (Sigma-Aldrich). The extracted cell lysates were loaded to 4-12% Bis-Tris polyacrylamide gel (Invitrogen) and transferred onto a polyvinylidene difluoride (PVDF) membrane using an electrophoresis system (Invitrogen). Non-specific binding on the blot was blocked using 5% skim milk dissolved in Tris-buffered saline with 0.05% Tween-20 (TBST) buffer for one hour at room temperature and then incubated with

a primary monoclonal antibody against PA-X (diluted 1:1000, kindly provided by Dr. Toru Takimoto, University of Rochester Medical Center, NY, USA) or β-actin (1:500 dilution, Santa Cruz) overnight at 4°C. The membrane was washed three times for five minutes each in TBST buffer and was incubated with horseradish peroxidase (HRP)-conjugated polyclonal rabbit anti-mouse immunoglobulins (diluted 1:1000, Dako). Target proteins were detected using SuperSignal West Femto Maximum Sensitivity Substrate according to the manufacturer's protocol (Thermo Scientific).

Viral RNP polymerase assay

The effects of different expressions of PA-X protein on polymerase activity were determined using ribonucleoprotein (RNP) minigenome assay as described previously (Bortz *et al.*, 2011). Briefly, a reporter minigenome plasmid (pPolI-NS-Luc) that contains the firefly luciferase gene flanked by the noncoding regions of influenza A virus NS gene with a polymerase I (Pol I) promoter was used. Confluent 293T cells were co-transfected with pPolI-NS-Luc (100ng) and four pCAGGS vectors encoding PB2, PB1, NP and PA (PA_PA-X_WT, PA_PA-X_Full or PA_PA-X_K/O) (the volume of the PB2, PB1, PA and NP plasmids used were 50ng, 100ng, 100ng and 500ng respectively) along with pSV-*Renilla* (50 ng) (pRL-SV40, Promega) carrying the *Renilla* luciferase gene as an internal control. As a negative control, cells were transfected with the same aforementioned plasmids mixture except the plasmid expressing PA. After 24 hours post-transfection, cells were collected using passive cell lysis buffer and luciferase activity was determined using a dual-luciferase reporter assay system according to the manufacturer's instructions (Promega). The relative polymerase activity was quantified by comparing to negative control cells which were transfected with pCAGGS blank plasmid instead

of PA expression plasmid, after normalizing it to the *Renilla* luciferase internal control. Each co-transfection experiment was performed in triplicate.

GFP expression assay

Confluent 293T cells were co-transfected with 500ng of pEGFP-N1 (Clontech) and 500ng of each PA plasmid that expresses different PA-X forms (pCAGGS_PA-WT, pCAGGS_PA_PA-X_K/O and pCAGGS_PA_PA-X_Full). After 24 hours post-transfection, cells were lysed with passive lysis buffer and the fluorescent intensity was measured using FLUOstar Omega (BMG LABTECH). The fluorescent intensity was standardized to the pCAGGS empty vector transfected control cells. The expressed GFP proteins and β-actin were detected from total cell lysates by western blotting to determine the relative expression of GFP protein using anti-GFP antibody (1:200 dilution, Santa Cruz) or anti-β-actin antibody (1:500 dilution, Santa Cruz). The detected protein bands were quantified using densitometry with AlphaEase FC Software (Alpha Innotech). The GFP protein expressions were normalized to β-actin expression.

Pathogenicity study in mice

A total of 56 six-week-old female BALB/c mice were randomly divided into four groups (14 mice/group). Mice from each group were inoculated intranasally (IN) with 3.75×10^5 $TCID_{50}$ of each virus in a volume of 50 μl under slight anesthesia with isoflurane. For the control group, mice were mock-infected with 50 μl of virus-free MEM. Mice were monitored daily for clinical signs and weighed daily until 14 days post infection (dpi). Mice that lost more than 25% of their original body weight were humanly euthanized. Three mice from each group were euthanized at 3 and 7 dpi. Lungs were collected from each mouse to assess virus replication, cytokine gene

expressions and histopathological analysis. For virus titration, 10% lung homogenates were made using fresh MEM with 1% antibiotic-antimycotic, and virus titers in the lung homogenates were determined on MDCK cells.

qRT-PCR

Total RNAs from lung homogenates were extracted using the RNeasy Plus Mini kit according to the manufacturer's protocol (Qiagen). The cDNA was synthesized from 1 μg of total RNA of each sample using the SuperScript III Reverse Transcriptase (Invitrogen) with Oligo$(dT)_{20}$ by reverse transcription reaction. The gene specific primers and probes were obtained from the TaqMan Gene Expression Assays (Applied Biosystems). The quantitative real-time PCR assays (qRT-PCR) were performed with equal volumes of cDNA from each sample and gene specific primers and probes using the TaqMan Fast Universal PCR Master Mix (Applied Biosystems). Amplification was conducted using the CXF96 Touch real-time PCR system (Bio-Rad) with the following cycle program: 1 cycle at 95ºC for 10 min, followed by 45 cycles at 95ºC for 15 s and 60ºC for 1 min. The expression fold change of each group was calculated relative to control group after normalization to 18S ribosomal RNA internal control using $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). Each experiment was performed in triplicate.

Histopathology and immunohistochemistry

Lung samples from mice were fixed in 10% neutral buffered formalin and processed by the histopathology and immunohistochemistry laboratory of the Kansas State Veterinary Diagnostic Laboratory (KSVDL). A board-certified veterinary pathologist evaluated histopathological lesions of routinely processed hematoxylin and eosin (H&E) stained tissues. For the lung microscopic lesions in mice, lungs were graded on the following seven criteria: Subjective percentage of lung involved in the histological section examined (4 point scale of 1-

4); airway epithelial necrosis, neutrophilic airway inflammation, peribronchiolar lymphocyte cuffing, interstitial pneumonia, airway epithelial hyperplasia (all on a 3 point scale of 1-3); and lastly the presence and absence of hyaline membranes (2 point scale, 0=absent and 1=present). Immunohistochemistry (IHC) staining for mouse lungs was conducted to detect influenza virus antigen in tissues using an anti-influenza A H1N1 rabbit polyclonal antibody. Antigen retrieval was performed using EDTA at pH 9.0 for 10 minutes at 100 ºC. The antibody was diluted 1:4000 and detected with the Leica Bond Polymer Refine Detection Kit on the Leica Bond-Max.

Statistical analysis

For statistical analysis among groups, analysis of variance (ANOVA) test was used in GraphPad Prism version 5.0 (GraphPad Software). For comparisons of survival curves, a log-rank test was performed. A *p* value of 0.05 or less was considered statistically significant.

3.3 Results

Generation of wild type pH1N1 and mutated viruses expressing different PA-X forms

To investigate the effects of three different expressions of PA-X protein on viral pathogenicity and host innate immune response, we rescued a recombinant wild type A/California/04/2009 (CA09_WT) virus that expresses truncated PA-X protein with 232aa and its two mutant viruses (CA09_PA-X_K/O and CA09_PA-X_Full) using reverse genetics. CA09_PA-X_Full virus expressing a full-length PA-X with 252aa was generated by substituting stop codon (UAG) with tryptophan (UGG) at the 42aa position in the C-terminal domain of PA-X (Figure 3-1A). CA09_PA-X_K/O virus, which does not express the PA-X, was generated through mutations in the frameshift motif from UUU CGU to UUC AGA according to the previous study (Jagger *et al.*, 2012) to prevent the PA-X expression (Figure 3-1A). Due to the

very low frameshifting efficiency (~1.3%) of PA-X gene (Jagger *et al.*, 2012), it is very difficult to detect PA-X expression in virus infected cells. To confirm that the generated viruses were able to express the expected PA-X, we transfected 293T cells with different PA-X ORF encoding plasmids (pCAGGS_PA-X_WT, pCAGGS_PA-X_Full or pCAGGS_PA-X_K/O) to detect expressed PA-X proteins. Both truncated PA-X (232aa) and full-length PA-X (252aa) were detected by western blotting analysis in each plasmid transfected cells, while no PA-X was detected in transfected cells with pCAGGS_PA-X_K/O plasmid that the PA-X is proposed to be knocked out (Figure 3-1B).

PA-X expression increases virus replication and polymerase activity in vitro

To further characterize wild type and its mutated viruses *in vitro*, we first performed a plaque assay in MDCK cells. Both CA09_PA-X_K/O and CA09_PA-X_Full mutated viruses as well as the CA09_WT formed similar small size of plaques in MDCK cells (Figure 3-2A). Next, we examined virus replication kinetics of three viruses in MDCK and A549 cells. CA09_WT and its two mutated viruses replicated efficiently in both MDCK and A549 cells. In contrast, CA09_PA-X_Full virus grew to a higher titer than the other two viruses (CA09_PA-X_WT and CA09_PA-X_K/O) in both cell lines and a significant higher titer was observed at 24 and 36 hpi in A549 cells (Figure 3-2B). The viral yields of PA-X deficient virus (CA09_PA-X_K/O) was significantly lower than both viruses expressing either a full-length or truncated PA-X (CA09_PA-X_Full and CA09_PA-X_WT) in MDCK cells (Figure 3-2B). The data indicates that PA-X expression enhances virus replication, particularly when the CA09 virus expresses the full-length PA-X.

To determine whether PA-X expression enhances virus replication through effects on the viral RNA polymerase activity, a minigenome assay was performed by measuring the reporter

gene expression levels which indirectly indicates the viral polymerase activity. Transfection of combination with the plasmid pCAGGS-PA_PA-X_K/O showed significantly lower polymerase activity when compared to those with either pCAGGS-PA_PA-X_WT or pCAGGS-PA_PA-X_Full, with 2.0 and 3.7 fold differences, respectively (Figure 3-2C). In addition, the polymerase complex with the pCAGGS-PA_PA-X_Full combination displayed the strongest polymerase activity (Figure 3-2C). In summary, PA-X expression increases viral RNA polymerase activity, thereby resulting in enhanced viral replication.

PA-X expression inhibits co-transfected gene expression

Recent studies revealed that PA-X protein is involved in the shutoff of host protein synthesis (Desmet *et al.*, 2013; Jagger *et al.*, 2012). To compare the contribution of different expressions of PA-X protein to shutoff activity, 293T cells were co-transfected with eGFP expression plasmid and individual PA expression plasmids (pCAGGS_PA-WT, pCAGGS_PA_PA-X_Full, or pCAGGS_PA_PA-X_K/O) for 24h. Relative GFP expression levels were determined by measuring relative fluorescence intensity and western blotting analysis. The reductions of GFP fluorescence intensity were observed from the cells co-transfected with PA expression plasmids (pCAGGS_PA-WT, pCAGGS_PA_PA-X_Full, or pCAGGS_PA_PA-X_K/O); approximately 46%, 57% or 28% of decreased fluorescence signals were found when compared to the control, respectively (Figure 3-3A and B). The GFP protein expression levels were also reduced by 21%, 52% and 7%, respectively, in the presence of pCAGGS_PA-WT, pCAGGS_PA_PA-X_Full and pCAGGS_PA_PA-X_K/O, when compared to transfection with an empty pCAGGS vector (Figure 3-3C). In the presence of both full-length and truncated PA-X, the GFP expression levels were significantly suppressed in contrast to the PA-X deficient condition (Figure 3-3B and C). Furthermore, the GFP expression was markedly

reduced in cells co-transfected with pCAGGS_PA_PA-X_Full compared to co-transfection with either pCAGGS_PA-WT or pCAGGS_PA_PA-X_K/O (Figure 3-3B and C). These results indicate that full-length PA-X in CA09 virus has the most significant effect on shutoff activity of co-expressed protein synthesis, while loss of PA-X results in decreasing the shutoff ability.

PA-X expression increases virus replication and pathogenicity in mice

To investigate the effect of different expressions of PA-X protein on virus pathogenicity *in vivo*, BALB/c mice were intranasally inoculated with 3.75×10^5 $TCID_{50}$ of each virus and monitored daily. Control animals did not show any clinical signs during the study. All infected mice in each group displayed clinical signs such as depression, less activities and obvious weight loss starting at 3 dpi when compared to the control animals. However, both PA-X expression viruses (CA09_PA-X_WT and CA09_PA-X_Full) induced more severe body weight loss (80.5% and 78.4% by 7 dpi and 8 dpi, respectively) than CA09_PA-X_K/O virus (body weight loss to 85.5% by 7 dpi) (Figure 3-4A). All mice infected with the CA09_PA-X_K/O virus survived, whereas the CA09_PA-X_Full virus caused 25% mortality and the CA09_PA-X_WT virus resulted in 12.5% mortality in infected animals (Figure 3-4B). Virus titers in mouse lungs infected with either CA09_PA-X_WT or CA09_PA-X_Full were higher than those infected with the CA09_PA-X_K/O at 3 dpi (Figure 3-5A), and a significant difference in virus titers was observed between the CA09_PA-X_Full and CA09_PA-X_K/O infected groups at 3 dpi. In contrast, a similar virus titer in mouse lungs was found in three infection groups at late time point (7 dpi) (Figure 3-5A).

Histopathological analysis showed that no lung lesions were found in control mice, while animals from three infection groups exhibited lung damages at both 3 and 7 dpi (Figure 3-5B). Lesions consisted of varying degrees and percentage of lung affected with neutrophilic

bronchiolitis, segmental loss of bronchiole epithelium, cuffing of bronchioles by lymphocytes and interstitial pneumonia (expansion of alveolar septa by histocytes) (Figure 3-6). Histologically, lung lesions varied in severity depending on dpi and the infected virus. At 3 dpi, both CA09_PA-X_WT and CA09_PA-X_Full viruses induced less histopathological lung lesions than the CA09_PA-X_K/O virus (Figure 3-5B and Figure 3-6). Furthermore, a significant difference in lung lesions was found between CA09_PA-X_K/O and CA09_PA-X_Full virus infected groups (Figure 3-5B). More severe lung damage was observed in both CA09_PA-X_WT and CA09_PA-X_Full infected mice at 7 dpi than at 3 dpi, whereas a similar lung lesion score was seen in CA09_PA-X_K/O infected mice at both days. Influenza antigens were detected in bronchiole epithelium and pneumocytes in infected mice using the H1N1 polyclonal antibody by immunohistochemistry analysis (Figure 3-6). Control mice had no staining in the lung by immunohistochemistry analysis (Figure 3-6).

PA-X expression suppresses the early inflammatory response in lungs of infected mice

To investigate the impact of different expressions of PA-X protein on the host antiviral immune response, expression of representative cytokine genes related with the inflammatory response in lungs of infected mice was determined. Overall, CA09_PA-X_K/O virus induced stronger cytokine responses than both CA09_WT and CA09_PA-X_Full virus in mice at 3 dpi (Figure 3-7). In particular, CA09_PA-X_Full virus significantly reduced the gene expression levels of TNF-α, IL-1β, IL-6 and IL-12 when compared to CA09_PA-X_K/O virus at 3 dpi (Figure 3-7). However, all three viruses induced comparable levels of gene expressions of IL-1β, IL-6, IL-12 and IFN-β at the late time point (7 dpi). At 7 dpi, IFN-α, IFN-β and IFN-γ were highly expressed, while lower levels of gene expressions of TNF-α, IL-1β, IL-6 and IL-12 were observed in infected mice with each virus compared to the early infection time point (3 dpi).

Taken together, the PA-X deficient virus elicited stronger cytokine response in lungs of infected mice at the early time point (3 dpi) than both PA-X expression viruses. Moreover, all tested cytokines and IFNs were more diminished in animals infected with CA09_PA-X_Full virus in contrast to those infected with CA09_WT expressing a truncated PA-X at the early time point (3 dpi).

3.4 Discussion

Since the PA-X protein was identified, multiple functions including effects on the viral virulence and modulation of the host immune response (Gao *et al.*, 2015b; Gao *et al.*, 2015c; Hayashi *et al.*, 2015; Hu *et al.*, 2015; Jagger *et al.*, 2012) and suppression of host protein synthesis by strong host shutoff activity (Desmet *et al.*, 2013; Hayashi *et al.*, 2015; Jagger *et al.*, 2012) have been described. Furthermore, several studies compared the roles of full-length and truncated PA-X proteins in viral pathogenicity and endonuclease activity (Bavagnoli *et al.*, 2015; Gao *et al.*, 2015a; Xu *et al.*, 2016). In contrast to previous studies, we investigated the contribution of three different expressions of PA-X protein (full-length, truncated and PA-X deficient forms) in 2009 pH1N1 virus which expresses truncated PA-X protein to viral replication, pathogenicity and host immune response. Our results showed that the expression of either full-length or truncated PA-X enhanced viral replication and pathogenicity of CA09 virus in cells as well as in mice and polymerase activity *in vitro* in contrast to the PA-X deficient expression (Figure 3-2 and Figure 3-4). Furthermore, the full-length PA-X expression resulted in more efficient replication in cells, higher virulence in mice and higher polymerase activity when compared to the truncated PA-X expression (Figure 3-2 and Figure 3-4). Our results are consistent with former findings that full-length PA-X in pH1N1, H5N1 and H9N2 viruses enhanced virus replication in cells and pathogenicity in mice compared to corresponding viruses

with truncated PA-X (Gao *et al.*, 2015a). In addition, wild type 2009 pH1N1 virus with truncated PA-X expression showed increased viral growth in cells and virulence in mice compared to PA-X deficient virus (Hayashi *et al.*, 2015). These results indicate that the enhanced viral replication seen with PA-X expression is most likely due to promotion of the polymerase activity. In addition, the longer C-terminal domain (61aa) of PA-X has stronger effect on the polymerase activity. In contrast, a previous study revealed that PA-X expression had no significant effect on viral growth of 1918 H1N1 virus in MDCK cells and reduced viral pathogenicity in mice (Jagger *et al.*, 2012). Additionally, other previous studies showed that PA-X deficient pH1N1 and H5N1 viruses increase viral replication in A549 cells or Vero cells along with higher polymerase activity and enhanced viral virulence in mice compared to corresponding wild type viruses (Gao *et al.*, 2015b; Hu *et al.*, 2015). These results indicate that the effect of PA-X expression on viral replication and pathogenicity is virus strain-dependent. Interestingly, we showed that PA-X deficiency in pH1N1 virus resulted in decreased viral replication and polymerase activity *in vitro*, whereas Gao et al. showed that loss of PA-X expression in pH1N1 virus enhanced viral replication with a higher polymerase activity than the wild type virus (Gao *et al.*, 2015b). One reason for the discrepant results on the PA-X deficiency in pH1N1 virus between two studies could be due to the different mutation approaches used in the frameshifting site to inhibit the PA-X expression. Whether different mutation approaches such as UCCUUUCGU to UCCUUCAGA used in our study, and UCCUUUCGC to AGCUUCAGA in the previous study induce different expression levels of PA-X protein leading to this discrepancy remains unknown and will be investigated in future studies.

The virus-induced host shutoff activity is expected to help the virus avoid the host antiviral responses by inhibiting antiviral factor expression (Narayanan *et al.*, 2008; Vreede &

Fodor, 2010). Influenza virus infection can cause shutoff of host protein synthesis to inhibit host antiviral defense and allow viral replication (Nemeroff *et al.*, 1998; Noah *et al.*, 2003; Plotch *et al.*, 1981). Recent studies revealed that PA-X protein in influenza virus infection has a major role in host shutoff which modulates host immune response by preventing antiviral related gene expressions (Hayashi *et al.*, 2015; Hu *et al.*, 2015; Jagger *et al.*, 2012). When we confirmed expression of expected PA-X proteins in transfected 293T cells with different PA-X ORF encoding plasmids, we could barely detect PA-X proteins because of PA-X self-suppression to PA-X expression plasmids (Figure 3-1B). In addition, our *in vitro* study showed that both full-length and truncated PA-X can shutoff co-transfected eGFP expression (Figure 3-3). Furthermore, we found that PA-X expressions in CA09 virus resulted in reduced levels of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6 and IL-12), type I IFNs (IFN-α and IFN-β) and type II IFN (IFN-γ) at 3 dpi (Figure 3-7). This result may account for the reduced inflammatory reactions and histopathological damages in lungs of mice infected with CA09_PA-X_WT and CA09_PA-X_Full viruses compared to CA09_PA-X_K/O infected mice, especially at early time point of infection (Figure 3-5B and Figure 3-6). In addition, it is possible that inhibition of host antiviral response through host shutoff activity of PA-X promoted viral replication in lungs of infected mice, which may contribute to enhance viral virulence of both PA-X expression viruses in mice in contrast to the PA-X deficient virus (Figure 3-4 and Figure 3-5A). However, previous studies showed that virulence of both 1918 H1N1 and H5N1 HPAIV viruses was attenuated due to PA-X expressions when compared to their respective PA-X deficient viruses, even though PA-X expressions in those viruses suppressed host genes associated with immune response *in vivo* (Hu *et al.*, 2015; Jagger *et al.*, 2012). One possible explanation of this discrepancy between this study and previous studies is that both influenza viruses, unlike 2009 pH1N1 virus, induce a

massive cytokine response, known as a cytokine storm which results in serious immunopathology and acute lung injury (Peiris *et al.,* 2010; Perrone *et al.,* 2008). This excessive immune response inhibits viral replication but causes serious pathological damage in infected lungs, which contribute to increased viral pathogenicity in these virulent influenza viruses. In contrast, absence of PA-X expression in 2009 pH1N1 virus elicits a more appropriate host immune response which is critical for virus clearance and reduces viral virulence. Furthermore, this indicates that the effects of PA-X protein on viral pathogenicity and host immune response are in a virus strain-dependent manner**.**

Although PA-X protein is highly conserved among different influenza viruses, the length of its C-terminal domain varies among influenza viruses (Jagger *et al.*, 2012; Shi *et al.*, 2012). Most human and avian influenza viruses express a full-length PA-X protein with 61aa in the C-terminal domain, while truncated PA-X protein possessing 41aa in the C-terminal region is common in canine, swine and 2009 pH1N1 influenza viruses (Jagger *et al.*, 2012; Shi *et al.*, 2012). We have shown that the full-length PA-X protein displays stronger host shutoff activity than the truncated PA-X protein *in vitro* and *in vivo* (Figure 3-3 and Figure 3-7), which is consistent with previous findings (Gao *et al.*, 2015a). However, two recent studies described that the first 15aa in the C-terminal region of PA-X are sufficient for maximum shutoff activity, while the addition of 20 aa of C-terminal end is not critical to shutoff activity of PA-X (Hayashi *et al.*, 2016; Oishi *et al.*, 2015). The reason for the different observations in shutoff activities of truncated PA-X between these studies could be due to differences of PA-X expression systems used, resulting in different protein expression levels, thereby showing a difference of the host shutoff activity. Because the whole PA expression plasmid was used in our study and the former studies, which could generate full-length or truncated PA-X by ribosomal frameshifting (Gao *et*

al., 2015a), while PA-X expression plasmids were used in latter studies (Gao *et al.*, 2015a; Hayashi *et al.*, 2016; Oishi *et al.*, 2015). Comprehensive phylogenetic and evolutionary analysis revealed that truncated PA-X appears in particular hosts such as dogs and swine which indicates species specificity of PA-X protein (Shi *et al.*, 2012). Additionally, truncated PA-X protein is considered to be associated with the adaptation to these new hosts (Shi *et al.*, 2012; Xu *et al.*, 2016). However, the role of the additional 20aa to the C-terminal end of PA-X protein remains unclear and further investigations are needed.

3.5 Conclusion

In summary, our data indicate that expression of either full-length or truncated PA-X protein in 2009 pH1N1 virus helps viral replication *in vitro*, likely through promoting RNA polymerase activity, and enhances viral virulence *in vivo* by inhibiting host innate immune response through host shutoff activity. Moreover, full-length PA-X protein displays a stronger effect on viral replication and pathogenicity compared to truncated PA-X. Thus, our study provides a better understanding on the contribution of different expressions of PA-X protein to viral pathogenicity and the host immune response. Additional studies are needed to understand the role of PA-X protein in influenza virus evolution and host adaptation.

Figure 3-1 Generation of PA-X deficient or full-length PA-X expression recombinant CA09 viruses

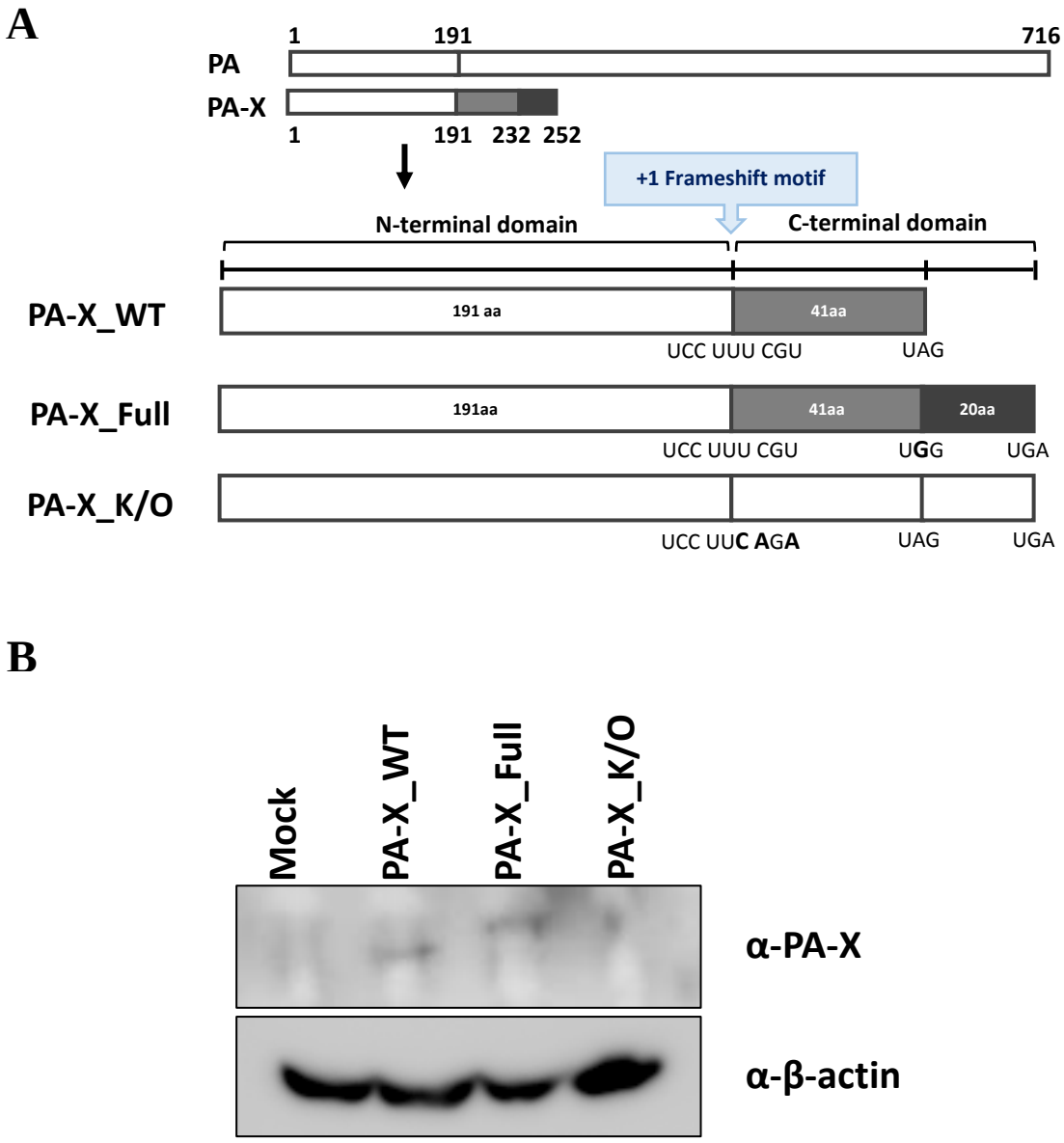
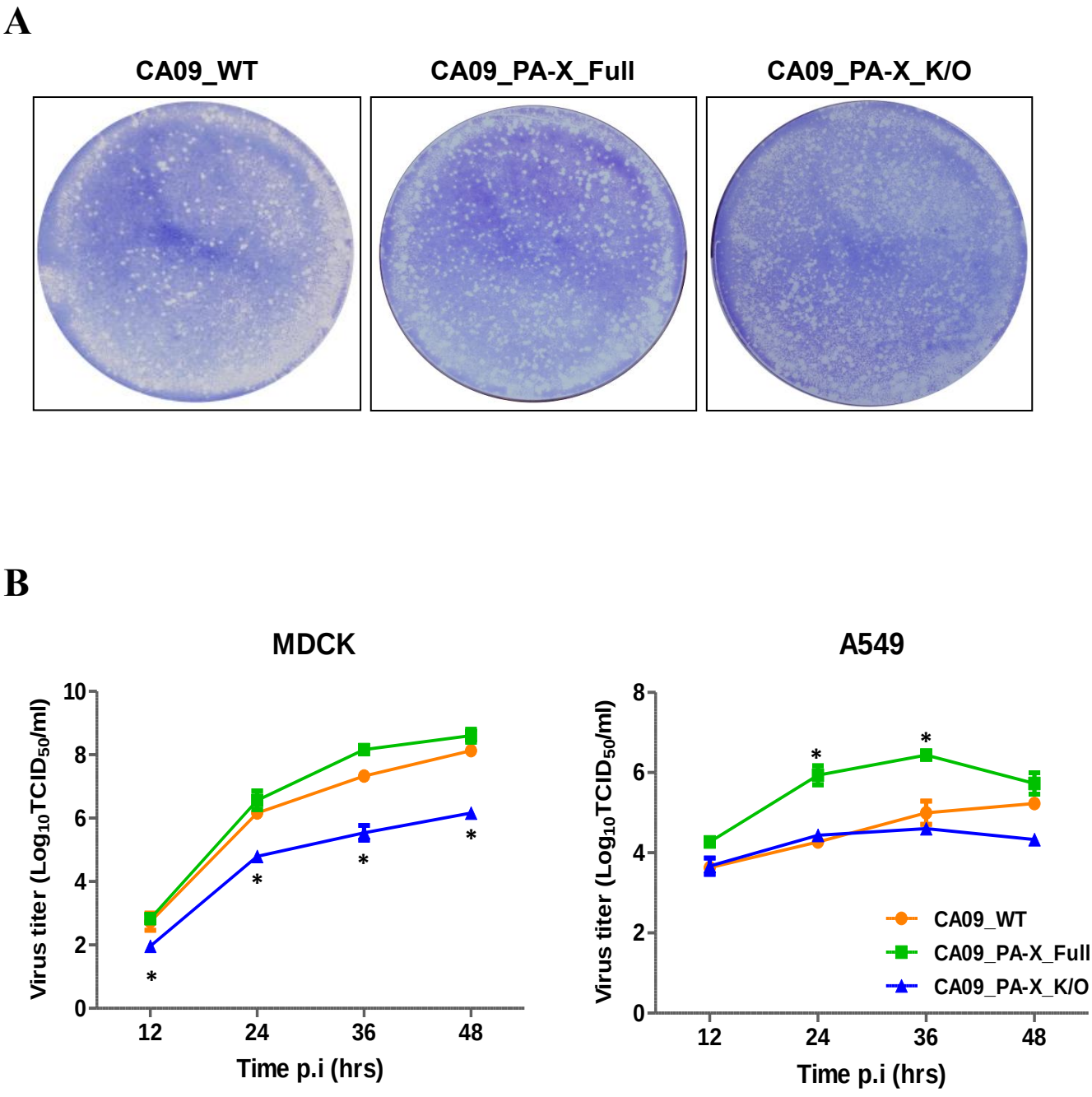


Figure 3-1 (A) Schematic structure diagram of PA and PA-X proteins. PA-X protein contains the N-terminal 191aa identical to PA protein and a PA-X unique C-terminal domain (grey color box) of 41 or 61aa produced by +1 ribosomal frameshifting. 2009 pH1N1 virus expresses truncated PA-X protein with 41aa in the C-terminal domain due to a stop codon (UAG) at position 42. To generate the CA09_PA-X_Full virus, a single amino acid substitution from UAG (stop codon) to UGG (W) at position 42 in the C-terminal domain of PA-X protein was introduced. Three nucleotide mutations in frameshifting motif were introduced from UCC UUU CGU to UCC UUC AGA to inhibit PA-X expression. The bold letters indicate the nucleotide substitutions. (B) The expression of indicated PA-X proteins was determined by western blotting analysis from transfected 293T cells with pCAGGS_PA-X_WT, pCAGGS_PA-X_Full or pCAGGS_PA-X_K/O plasmids. Mock control was transfected with the empty pCAGGS plasmid.

Figure 3-2 Impact of different expressions of PA-X protein on plaque morphology, growth kinetics and polymerase activity



C

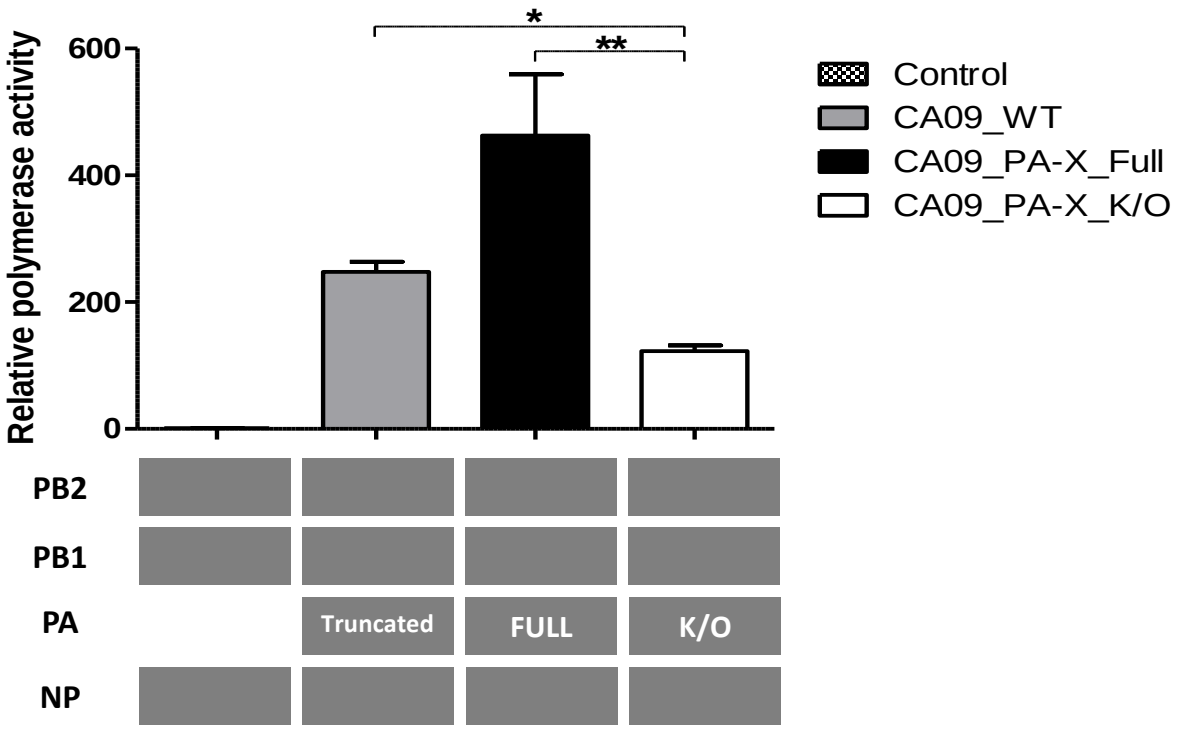
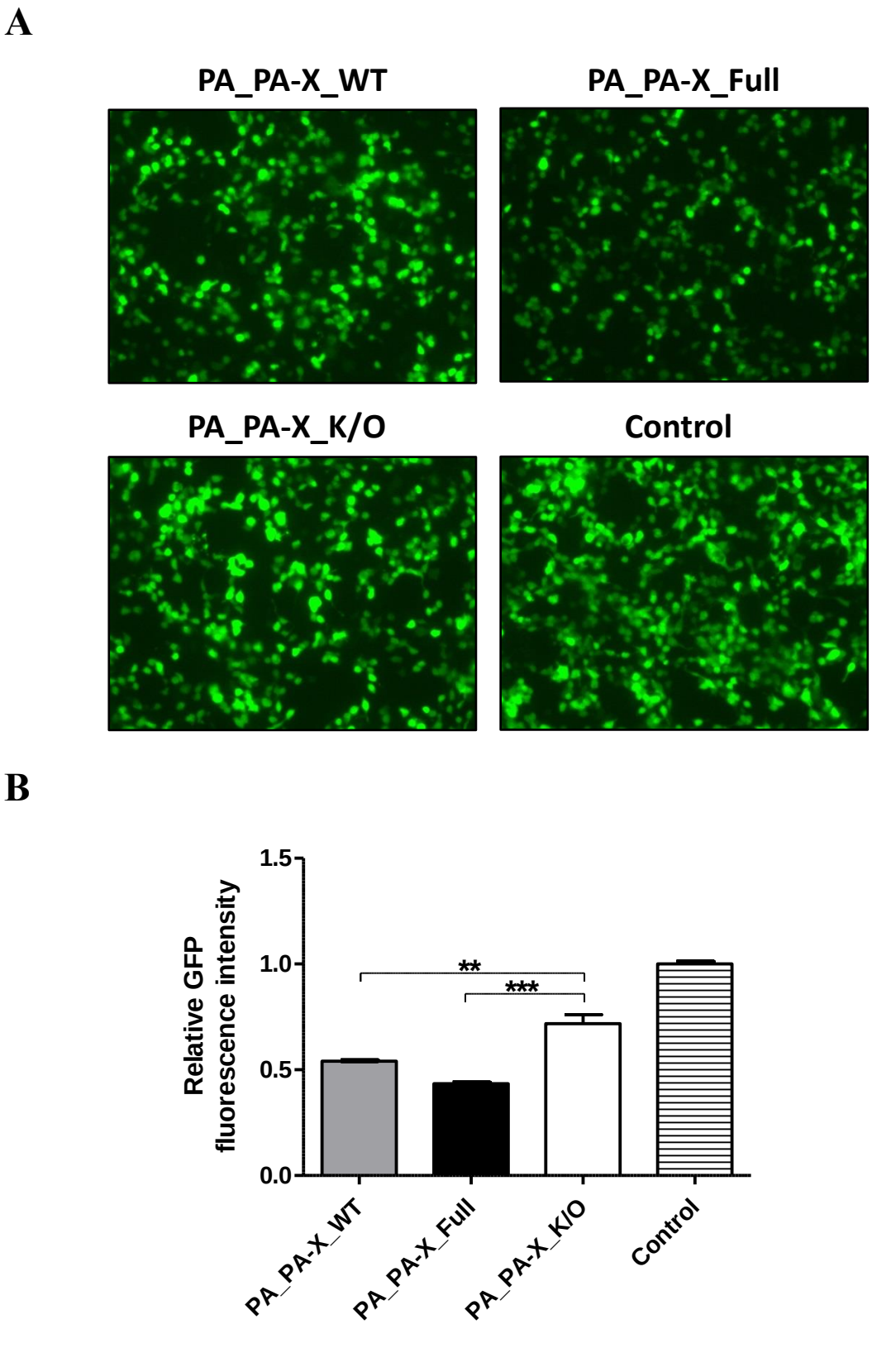


Figure 3-2 (A) Plaque sizes formed by recombinant viruses in MDCK cells at 3 days post-infection (dpi). (B) Growth kinetics of indicated recombinant viruses in MDCK or A549 cells infected at an MOI of 0.01. Each data point on the curve indicates the means of the results in triplicate, and the error bars indicate standard errors of the mean (SEM). The asterisks (*) indicate a statistically significant difference with the other groups. (C) Comparison of polymerase activities of vRNPs with indicated different expressions of PA-X protein in 293T cells. The grey color boxes indicated transfected genes. The asterisks (*) indicate a statistically significant difference between groups (*: $p<0.05$ and **: $p<0.01$).

Figure 3-3 Effects of different expressions of PA-X protein on co-transfected GFP expression



C

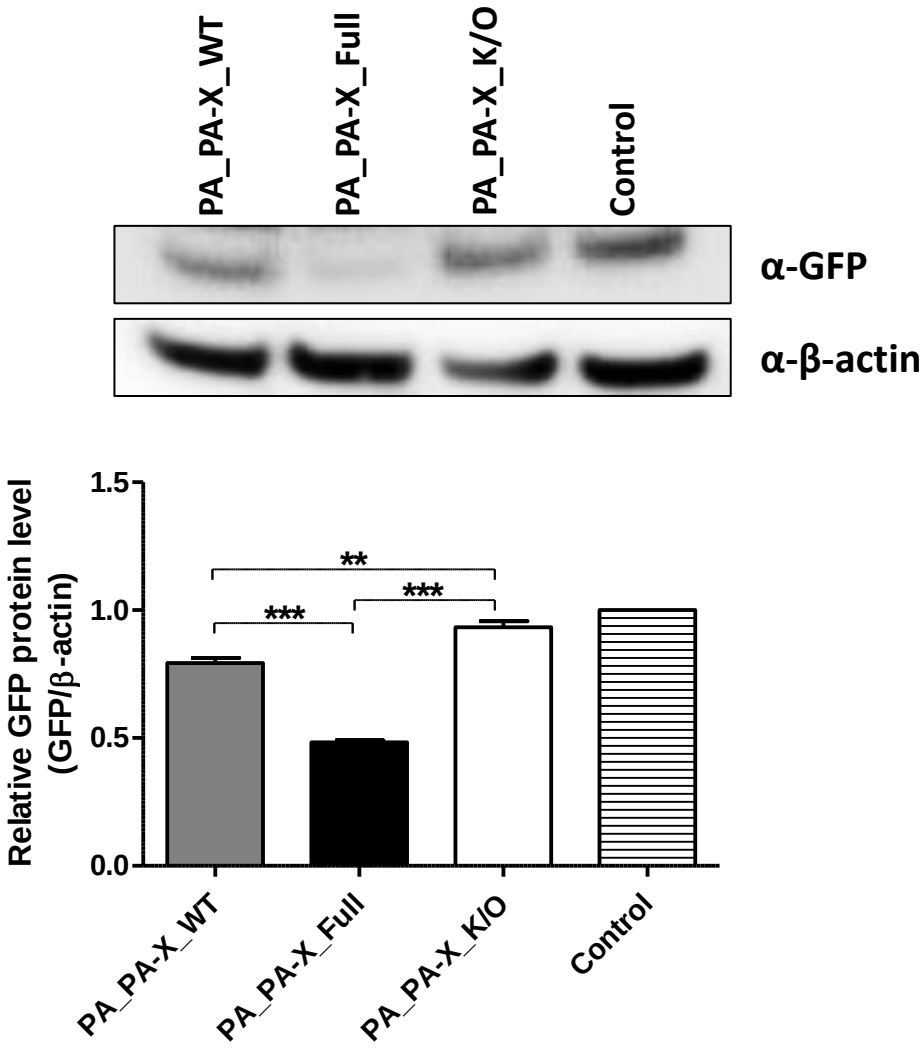


Figure 3-3 293T cells were co-transfected for 24h with GFP expression plasmid and indicated PA expression plasmids encoding different PA-X forms. (A) Fluorescence images of GFP expression at 24h post transfection with indicated PA expression plasmids. The control was co-tranfected with GFP expression vectors with empty pCAGGS plasmid. (B) Relative GFP fluorescence intensity of each group compared to the control cells. (C) Relative expression levels of GFP proteins compared to the control cells using western blotting and densitometry. The GFP protein expressions were normalized to β-actin expression. The asterisks (*) indicate a statistically significant difference between groups (*: $p<0.05$. **: $p<0.001$ and ***: $p<0.0001$).

Figure 3-4 Impact of different expressions of PA-X protein on pathogenicity of CA09 virus in BALB/c mice

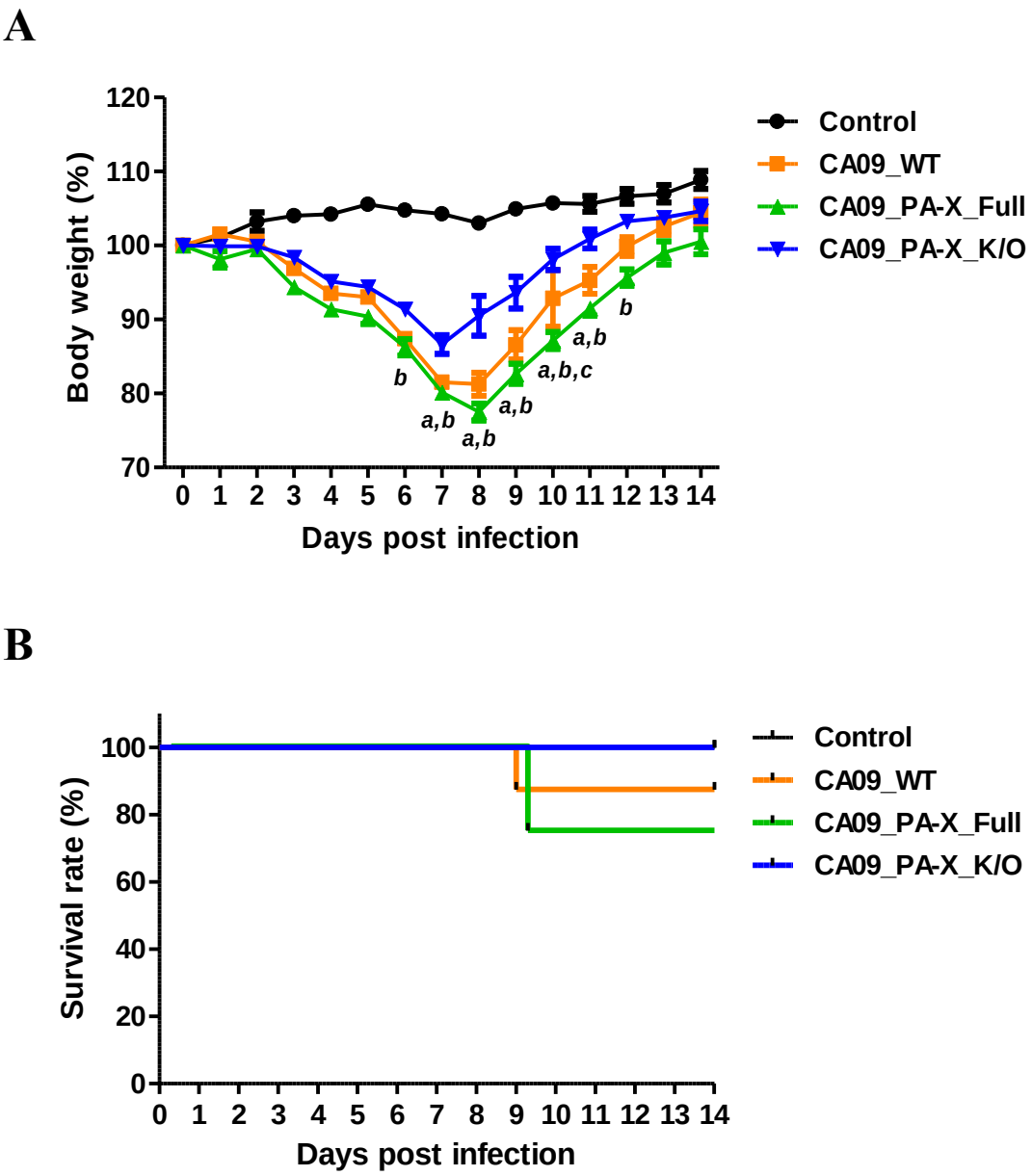


Figure 3-4 (A) Average body weights of surviving mice in each group up to 14 dpi are represented as percentages of the original weight on day 0. *a*,*b* and *c* on the value point of each day address significant differences ($p<0.05$) between infected groups (*a*: CA09_WT and CA09_PA-X_K/O, *b*: CA09_PA-X_K/O and CA09_PA-X_Full, *c*: CA09_WT and CA09_PA-X_Full). The error bars indicate standard errors of the mean (SEM). (B) Survival rate of mice infected with indicated viruses. No significant difference was observed for mortality rate between groups.

Figure 3-5 Virus replication and histopathological scores in lungs of infected mice with either wild type CA09 or its mutated viruses

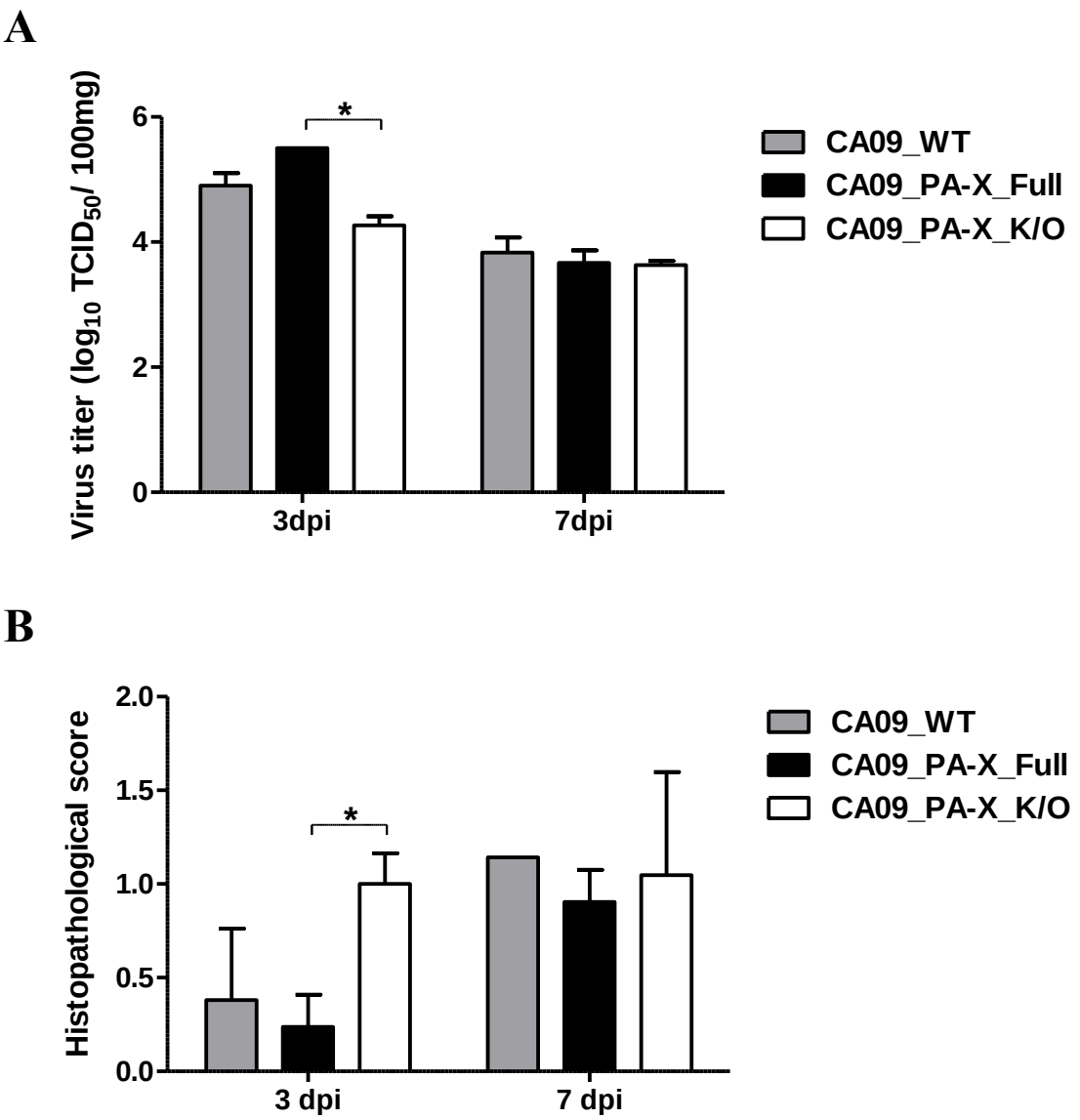


Figure 3-5 (A) Virus titers in lungs of infected mice were determined at 3 and 7 dpi by calculating the 50% tissue culture infective dose ($TCID_{50}$)/ml in MDCK cells. (B) Microscopic lung scores are presented as average scores ± SEM of three mice in each group at 3 and 7 dpi. Lungs were graded on the following seven criteria: Subjective percentage of lung involved in the histological section examined (4 point scale of 1-4); airway epithelial necrosis, neutrophilic airway inflammation, peribronchiolar lymphocyte cuffing, interstitial pneumonia, airway epithelial hyperplasia (all on a 3 point scale of 1-3); and lastly the presence and absence of hyaline membranes (2 point scale, 0=absent and 1=present). The asterisks (*) indicate a statistically significant difference between groups (*: $p<0.05$). The error bars represent standard errors of the mean (SEM).

Figure 3-6 H&E and IHC staining of mouse lung sections at 3 dpi

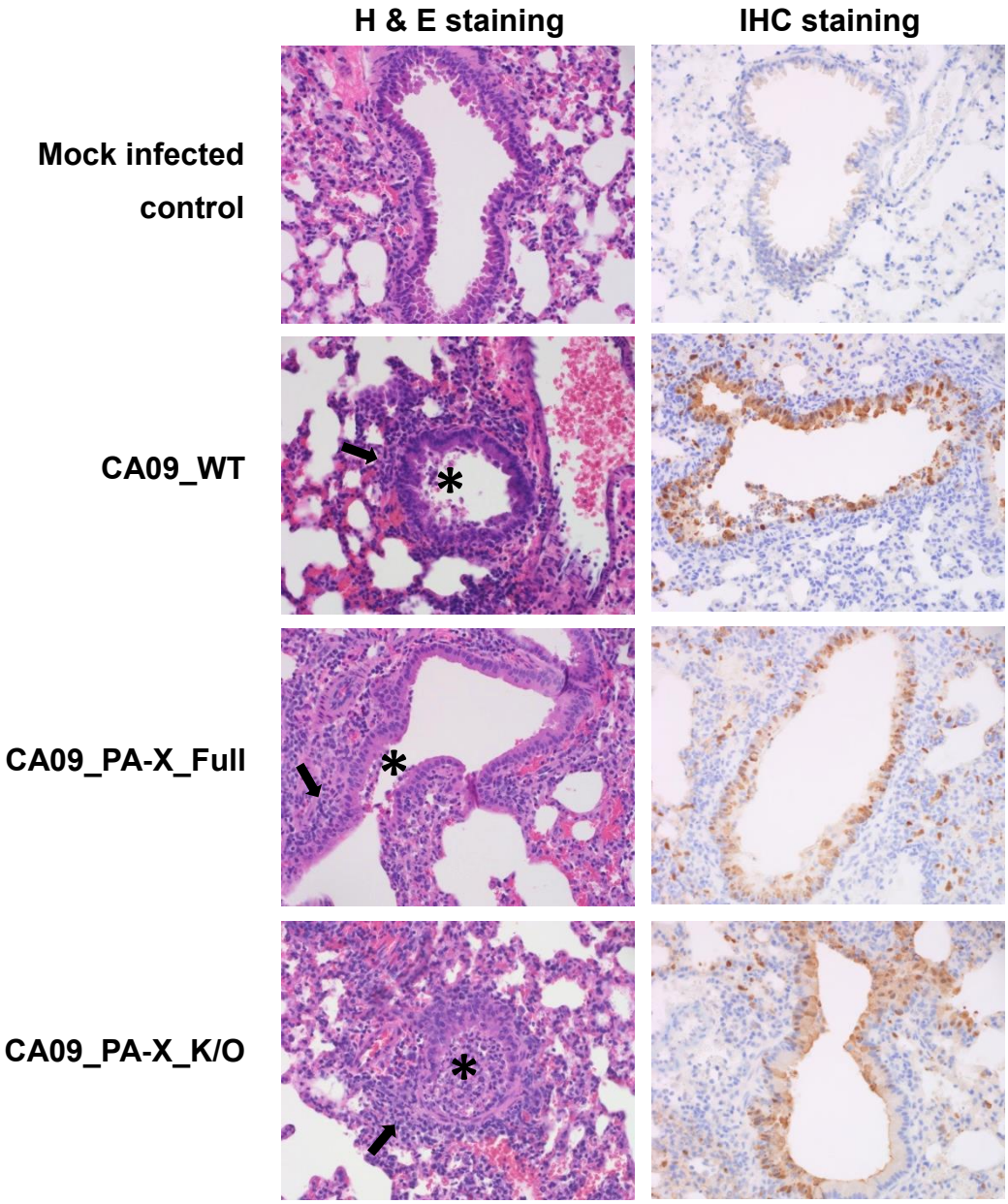


Figure 3-6 H&E stained sections of mouse lungs infected with indicated viruses showed typical influenza pneumonia. Mock infected control: There are no lesions in the bronchiole. There is no antigen deposition in the H1N1 immunohistochemistry. CA09_WT and CA09_PA-X_Full: A small number of neutrophils are present in the bronchiole lumen (asterisk) and there is mild cuffing of the bronchiole by lymphocytes (arrow). IHC cytoplasmic staining with H1N1 antibody is present in the cytoplasm of bronchioles and pneumocytes (brown staining). CA09_PA-X_K/O: The bronchiole is filled with neutrophils (asterisk) and there is segmental loss of bronchiole epithelium and moderate cuffing of the bronchiole by lymphocytes (arrow). Adjacent alveolar lumina are expanded by lymphocytes and histocytes. IHC cytoplasmic staining with H1N1 antibody is present in the cytoplasm of bronchioles and pneumocytes (brown staining).

Figure 3-7 The mRNA expression levels of pro-inflammatory cytokines and IFNs in lungs of mice infected with either wild type CA09 or its mutated viruses

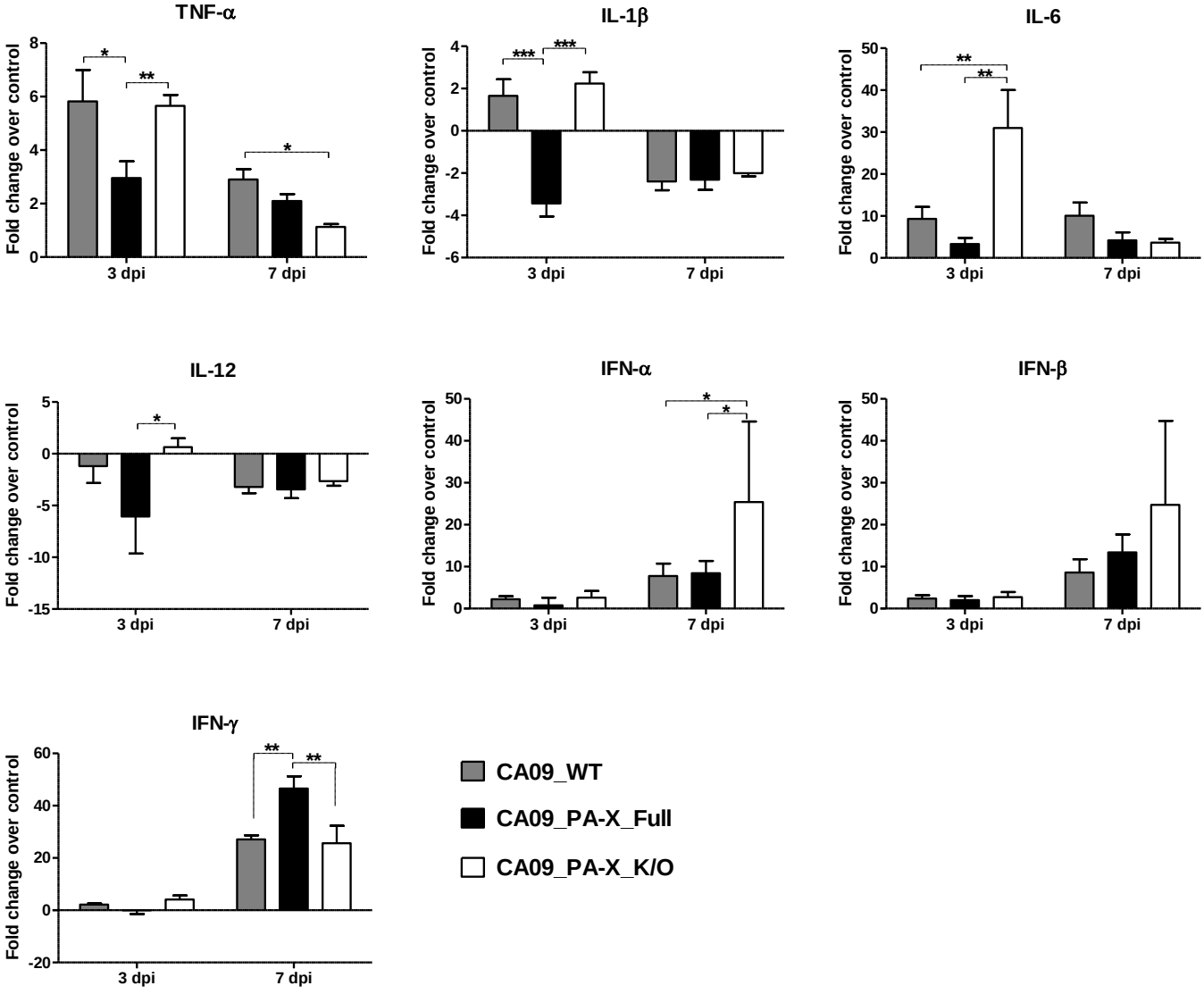


Figure 3-7 The mRNA expressions of the indicated mouse cytokines and IFNs were quantified from lungs of infected mice by using qRT-PCR analysis. The expression fold change of each group was calculated relative to the control group after normalization to 18S ribosomal RNA internal control using $2^{-\Delta\Delta Ct}$ method. Data represent the average values ± SEM of three mice in each group on the days indicated. The asterisks (*) indicate a statistically significant difference between groups (*: $p<0.05$. **: $p<0.01$ and ***: $p<0.001$).

Chapter 4 - General discussion and future prospects

IAV is a significant human and animal pathogen that constantly circulates worldwide and causes considerable public health and economic problems. Despite abundant efforts to control IAV infection, there is no absolutely effective anti-influenza therapeutics or vaccines to date. One of the main reasons for this difficulty is the continued genetic mutations and evolution of IAVs. To overcome the limited genomic information and enhance the protein coding capacity, IAVs have evolved molecular strategies including ribosomal scanning, alternative spicing and frame-shifting (Firth & Brierley, 2012; Vasin *et al.*, 2014; Yewdell & Ince, 2012) to translate multiple viral proteins from a single gene segment. This mechanism further complicates the pathogenesis of IAV. The eight segmented IAV genes were thought to encode 10 viral proteins, until a new IAV protein, PB1-F2, was identified in 2001 (Chen *et al.*, 2001). Since 2001, seven additional viral proteins, PB1-N40 (Wise *et al.*, 2009), PA-X (Jagger *et al.*, 2012), NS3 (Selman *et al.*, 2012), PA-N155, PA-N182 (Muramoto *et al.*, 2013), M42 (Wise *et al.*, 2012), and PB2-S1 (Yamayoshi *et al.*, 2016) have been discovered. However, the roles of newly discovered proteins and their contributions to IAV pathogenesis still remain to be elucidated. In this dissertation, we focused on studies to investigate the effects of two novel IAV proteins, PB1-F2 and PA-X, on viral pathogenicity *in vitro* and *in vivo*.

PB1-F2 protein is a small accessory protein of IAV with various functions. Although PB1-F2 protein is considered an important virulence marker of IAV, its effects on viral pathogenicity vary depending on viral strain and host species. In the mouse model, PB1-F2 protein enhanced the viral pathogenicity of mouse-adapted H1N1 viruses (A/Puerto Rico/08/1934 and A/WSN/1933) and 1918 pandemic H1N1 virus (McAuley *et al.*, 2007; Zamarin *et al.*, 2006). In the H5N1 HPAIVs, PB1-F2 protein increased viral virulence in ducks,

while negligible effects of PB1-F2 protein were observed on viral pathogenicity in mice and attenuated virulence was found in chickens with PB1-F2 protein in H5N1 HPAIVs (Leymarie *et al.*, 2014; Schmolke *et al.*, 2011). PB1-F2 protein expression in TR H3N2 SIV or 2009 pH1N1 displayed minimal impact on viral pathogenicity in swine models (Pena *et al.*, 2012a; Pena *et al.*, 2012b) while deletion of PB1-F2 in TR H3N2 SIV induced earlier clinical signs and effective viral transmission in turkeys (Deventhiran *et al.*, 2015). The N66S substitution in PB1-F2 was previously described to contribute to increased virulence in mice (Conenello *et al.*, 2007; Schmolke *et al.*, 2011). On the other hand, this substitution in 2009 pH1N1 virus had no significant impact on virulence in mice and 66S in PB1-F2 protein played only a minor role in viral virulence in swine and ducks (Deventhiran *et al.*, 2015; Pena *et al.*, 2012a; Schmolke *et al.*, 2011). In Chapter 2, we showed that PB1-F2 protein expression in virulent H1N1 TR SIV has moderate effects on viral pathogenicity in swine, while PB1-F2 expression does not impact the viral replication and virulence in mice when compared with the PB1-F2 deficient virus. Consistent with findings of previous studies, the substitution N66S in PB1-F2 protein of virulent H1N1 TR SIV plays a critical role in high lethality in mice, while there was no effect on pigs.

Another novel IAV accessory protein, PA-X, has shown a diverse range of functions with virus strain and host specificity. PA-X protein in 1918 H1N1and HPAIV H5N1 viruses reduced viral virulence in mice, while PA-X protein worked as a virulence factor in the H9N2 virus (Gao *et al.*, 2015b; Gao *et al.*, 2015c; Hu *et al.*, 2015; Jagger *et al.*, 2012). Interestingly, two previous studies showed opposite results of the impact of PA-X protein in 2009 pH1N1 virus in different mouse strains: Gao *et al.* found that loss of PA-X expression enhanced viral virulence in BALB/c mice, whereas Hayashi *et al.* reported that inhibition of PA-X expression attenuated viral growth in C57BL/6 mice in comparison with the wild type virus (Gao *et al.*, 2015b; Hayashi *et al.*,

2015). Other studies investigated the role of 20aa at the C-terminal end of PA-X protein in viral virulence. Gao *et al.* observed that full-length PA-X protein with additional 20 aa at the C-terminal end increased viral pathogenicity of pH1N1, HPAIV H5N1 and H9N2 viruses in mice when compared to truncated PA-X protein (Gao *et al.*, 2015a). On the other hand, Xu *et al.* demonstrated that truncated PA-X protein in TR H1N2 SIV improved viral replication and pathogenicity in swine compared to full-length PA-X protein (Xu *et al.*, 2016). Recently, several studies revealed that the first 15 aa in the PA-X C-terminal domain is important for PA-X shutoff activity and subcellular localization; in particular six basic aa in the PA-X C-terminal region play a critical role in PA-X shutoff activity (Hayashi *et al.*, 2016; Oishi *et al.*, 2015). In Chapter 3, we evaluated the contribution of different expressions of PA-X protein including full-length, truncated and PA-X deficient forms, on viral pathogenicity of 2009 pH1N1 virus *in vitro* and in mice. Consistent with a previous report (Hayashi *et al.*, 2015), our results indicate that either full-length or truncated PA-X expression increased viral replication and pathogenicity *in vitro* and in mice compared with the PA-X deficient virus. In addition, full-length PA-X with additional 20aa at the C-terminal end showed stronger effect on viral virulence in comparison with truncated PA-X form, which is consistent with the previous study (Gao *et al.*, 2015a). Although most studies observed the same PA-X host shutoff activity in common with our data, the contribution of PA-X to viral pathogenicity appears to have a diverse range of consequences.

While some studies described critical roles of PB1-F2 and PA-X as virulence factors of IAVs (Hayashi *et al.*, 2015; McAuley *et al.*, 2007; Zamarin *et al.*, 2006), others found opposite or no effect on viral virulence (Hu *et al.*, 2015; Jagger *et al.*, 2012; Pena *et al.*, 2012b; Schmolke *et al.*, 2011). Possibly, the discrepancies between these studies are attributed to the various factors such as virus strains used, different infection doses and genetic diversity together with

host factors including different host species, immune status and genetic background. Collectively, the outcome of IAV infections is diverse and unpredictable because the pathogenicity of IAV is dependent on various factors including virus and host factors. Therefore, a comprehensive understanding of the interactions between virus and host is required and more studies are needed to reveal the underlying mechanism of roles of PB1-F2 and PA-X in viral pathogenicity.

Genetic analysis has shown that most avian influenza viruses express the full-length PB1-F2 and PA-X proteins, whereas particular mammalian IAVs such as swine and human IAVs frequently contain the truncated forms of these proteins (Pasricha *et al.*, 2013; Shi *et al.*, 2012; Zell *et al.*, 2007). Notably, currently circulating 2009 pH1N1 virus expresses both truncated PB1-F2 and PA-X proteins. This fact implies that truncation of these proteins are associated with evolution and adaptation of IAVs in these mammalian hosts. Thus, future studies are required to monitor the variations in length and substitutions of functional genetic markers in these proteins to predict virulence change and the pandemic potential.

Many studies have described the multifunctional roles of PB1-F2 protein in IAV pathogenesis including pro-apoptotic function, cytokine modulation and inducing secondary bacterial infection (Chen *et al.*, 2001; Le Goffic *et al.*, 2010; Leymarie *et al.*, 2014; McAuley *et al.*, 2007; Zamarin *et al.*, 2005). PA-X protein has also been shown to have multiple functions such as anti- or pro-apoptotic functions, modulating inflammatory response and suppressing host protein synthesis (Gao *et al.*, 2015b; Gao *et al.*, 2015c; Hayashi *et al.*, 2015; Hu *et al.*, 2015; Jagger *et al.*, 2012). Since these two proteins behave in a similar way or opposite way during virus infection, they might create a synergistic effect or a mutually antagonistic effect on IAV pathogenesis. Several other IAV proteins have also been shown to have comparable or dissimilar

functions to PB1-F2 and PA-X functions. NA, NP, M1 and NS1 proteins are known to be related to influenza-induced host cell death (Halder *et al.*, 2011; Rossman & Lamb, 2009; Schultz-Cherry *et al.*, 2001; Tripathi *et al.*, 2013; Zhirnov *et al.*, 2002). NS1 protein acts as an IFN antagonist and modulates host immune response with several mechanisms (Hale *et al.*, 2008; Mibayashi *et al.*, 2007; Talon *et al.*, 2000; Wang *et al.*, 2000). In addition, NS1 and PA proteins have been described that they are related to host shutoff activity by interfering with cellular mRNA processing or degradation of the cellular RNA polymerase II (Fortes *et al.*, 1994; Nemeroff *et al.*, 1998; Qiu & Krug, 1994; Rodriguez *et al.*, 2007; Sanz-Ezquerro *et al.*, 1995; Vreede & Fodor, 2010). Therefore, more knowledge is required about how these proteins interact and work together to maintain the balance of IAV pathogenicity for the optimal viral replication.

IAVs undergo continuous genomic mutations which result in complexity and diversity of sequences. Moreover, the protein coding capacity of IAV genomes has not been completely determined. Therefore, it is possible that unknown novel proteins of IAV might constantly be identified and evolve to interact with other IAV proteins or host factors to influence viral replication. More studies are needed to identify and characterize these proteins.

Taken together, newly discovered IAV proteins, PB1-F2 in virulent TR H1N1 SIV and PA-X in 2009 pH1N1 virus, are involved in modulating host immune response and affecting viral replication and pathogenicity. It is necessary to monitor the prevalence and genetic alterations of these proteins in currently circulating IAVs and investigate new roles of these proteins as well as effects on the host for a better understanding of their contributions to influenza virus pathogenesis. It would be possible that uncovering the role of these newly

discovered IAV proteins will help to develop new strategies and potential targets for effective anti-influenza therapeutics.

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