Virulence and transmissibility of H1N2 influenza virus in ferrets imply the continuing threat of triple-reassortant swine viruses

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Efficient worldwide swine surveillance for influenza A viruses is urgently needed; the emergence of a novel reassortant pandemic H1N1 (pH1N1) virus in 2009 demonstrated that swine can be the direct source of pandemic influenza and that the pandemic potential of viruses prevalent in swine populations must be monitored. We used the ferret model to assess the pathogenicity and transmissibility of predominant Korean triple-reassortant swine (TRSw) H1N2 and H3N2 influenza viruses genetically related to North American strains. Although most of the TRSw viruses were moderately pathogenic, one [A/Swine/Korea/1204/2009; Sw/1204 (H1N2)] was virulent in ferrets, causing death within 10 d of inoculation, and was efficiently transmitted to naive contact ferrets via respiratory droplets. Although molecular analysis did not reveal known virulence markers, the Sw/1204 virus acquired mutations in hemagglutinin (HA) (Asp-225-Gly) and neuraminidase (NA) (Ser-315-Asn) proteins during the single ferret passage. The contact-Sw/1204 virus became more virulent in mice, replicated efficiently in vitro, extensively infected human lung tissues ex vivo, and maintained its ability to replicate and transmit in swine. Reverse-genetics studies further indicated that the HA_{225G} and NA_{315N} substitutions contributed substantially in altering virulence and transmissibility. These findings support the continuing threat of some field TRSw viruses to human and animal health, reviving concerns on the capacity of pigs to create future pandemic viruses. Apart from warranting continued and enhanced global surveillance, this study also provides evidence on the emerging roles of HA_{225G} and NA_{315N} as potential virulence markers in mammals.

severe disease | interspecies transmission | evolution | viral segments

Swine influenza A viruses of the H1N1, H1N2, and H3N2 subtypes remain endemic in major swine populations globally. Pigs play a pivotal role in the ecology of influenza A viruses by allowing productive replication of viruses from both the avian and mammalian lineages. Cellular receptors containing α2,3- and α2,6-linked sialic acids in the pig trachea are thought to be responsible for this dual susceptibility, which can drive interspecies transmission by the accumulation of mutations (antigenic drift) that permit replication in a new host, and through reassortment of the segmented viral genomes of different viruses within coinfected swine (1, 2). As such, pigs have been described as "genetic mixing vessels," or opportune intermediate hosts for the generation of novel and potentially dangerous viruses.

Despite the apparent importance of pigs in the genetic evolution of influenza A viruses, surveillance in domestic swine has been abysmal. The unanticipated emergence of the novel swine-origin pandemic H1N1 (pH1N1) influenza virus in 2009 illustrates the need for effective, systematic global surveillance in swine populations. The pH1N1 virus uniquely comprises segments from North American triple-reassortant swine (TRSw) H1 viruses (six genes) and Eurasian avian-like swine viruses [neuraminidase (NA) and matrix genes] (3, 4). Although most pH1N1 infections were mild, the virus was highly transmissible and could replicate deep in the lungs (5). Introduction of known virulence factors into the pH1N1 virus did not significantly alter its disease phenotype (6, 7), suggesting the presence of unknown molecular determinants of pathogenicity and transmissibility.

The nearest TRSw virus precursor of the pH1N1 virus has predominated in North America since 1998 (8). Its continued circulation in swine herds has generated a range of further reassortants that have caused sporadic human infections (9, 10) but have not apparently sustained transmission among humans. To date, the global spread of pH1N1 is the most extensive known human dissemination of a swine-origin influenza virus. North American lineage TRSw viruses have been also detected in Asian swine populations (11), and H1N2 and H3N2 viruses of this lineage have recently predominated in South Korea (12). The occurrence of the 2009 pandemic also implies that influenza viruses prevalent in swine are plausible pandemic candidates and in the absence of adequate surveillance data, it remains unknown whether other swine influenza subtypes are potentially transmissible and virulent in mammalian hosts. Therefore, we examined the zoonotic potential of representative Korean TRSw H1N2 and H3N2 influenza viruses genetically related to North American strains; we found that some of these circulating TRSw viruses are pathogenic and readily transmissible in ferrets, a recognized model of human influenza infection (13, 14). Through plasmid-based reverse genetics, we also showed that the NA_{315N} substitution in combination with the HA_{225G} substitution plays an important role in the pathogenicity and transmissibility of the TRSw H1N2 A/Swine/Korea/1204/2009 (Sw/1204) virus, indicating that these mutations are potential virulence markers in mammals.

Results

Pathogenicity of TRSw H1N2 and H3N2 Influenza Viruses in Ferrets and Mice. North American-like TRSw H1N1, H1N2, and H3N2 viruses have been found exclusively in Korean pig herds for at least a decade (reviewed in ref. 11). More recently though, we have reported that H1N2 viruses (with classical swine-like HA) of the same overall genotype and two distinct H3N2 (with antigenically divergent HA) viruses were more commonly isolated after a national swine vaccination program late in 2005 (*SI Appendix*, Fig. S1) (12). To examine the pathogenic potential of contemporary swine viruses in South Korea before the 2009

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pandemic, we studied TRSw H1N2 [A/Sw/Korea/1130/2009 (Sw/1130) and Sw/1204] and H3N2 [A/Sw/Korea/CY05/2007 (Sw/CY05) and A/Sw/Korea/CY07/2007 (Sw/CY07)] viruses isolated from pig abattoirs. All field viruses were recovered from swine lung homogenates inoculated in Madin-Darby canine kidney (MDCK) cells. Stock viruses were prepared and propagated one more time in MDCK cells and then titered before the conduct of the study.

Ferrets (n = 3 per group) were inoculated intranasally and intratracheally with $10^{5.5}$ 50% tissue culture infectious doses ($TCID_{50}$)/mL of the respective viruses and observed for 14 d. By virus titration of nasal washes in MDCK cells, most of the TRSw strains demonstrated generally comparable growth kinetics and viral titers in inoculated ferrets. The Sw/1130 (H1N2), Sw/CY05 (H3N2), and Sw/CY07 (H3N2) viruses reached moderate peak titers in nasal washes (6.3×10^5 to 1.6×10^6 $TCID_{50}$/mL); these upper respiratory titers were undetectable by 7 d postinoculation (dpi) (Table 1), and no remarkable clinical disease signs were noted. In contrast, the Sw/1204 (H1N2) virus caused severe disease in all inoculated ferrets (weight loss ≥18%, sneezing, labored breathing, rhinorrhea, lethargy, and high fever), starting at 3 dpi (*SI Appendix*, Table S1). The Sw/1204 virus replicated efficiently in the upper respiratory tract, producing high nasal-wash titers for up to 7 dpi with a mean peak titer of 1.6×10^7 $TCID_{50}$/mL on day 5 postinfection (Table 1). All inoculated ferrets had seroconverted at 18 dpi. In mice, all of these TRSw viruses caused moderate morbidity (measured by weight loss) after intranasal inoculation with 10^5 $TCID_{50}$/30 μL and grew without prior adaptation (*SI Appendix*, Table S2), but Sw/1204 persisted the longest in mouse lungs and induced substantially greater weight loss.

Two of the ferrets inoculated with Sw/1204 became hypothermic (≤36 °C) at 7 dpi, where one eventually died and the other was euthanized prior to death; the remaining ferret was humanely euthanized at 10 dpi because of nonrecovery from severe clinical disease, with ∼25% weight loss. One inoculated ferret from each of the other virus groups was also humanely euthanized at 7 dpi, and lungs, brains, spleens, kidneys, and colons from all killed ferrets were harvested for virologic examination. Notably, only Sw/1204 was detected in lung tissue homogenates (6.3×10^4/g mean titer) (Table 1).

In ferrets inoculated with the Sw/1204 virus, gross pathology showed a "liver-like" lung appearance with prominent edema accompanied by mucopurulent exudates (Fig. 1 *A* and *B*). Histopathologic findings confirmed severe inflammation with peribronchial and interstitial immune cell infiltrates, consistent with the abundant detection of viral antigen in alveolar cells by immunohistochemistry (Fig. 1*C*). Because natural bacterial flora in the respiratory tract could potentiate increased damage during virus infection, the possible contribution of bacterial coinfection to the observed severe pulmonary lesion in Sw/1204-infected ferrets cannot be ruled out. Regardless, Sw/1130, Sw/CY05, and Sw/CY07 induced moderate lung histopathology relative to Sw/1204 (*SI Appendix*, Fig. S2).

Respiratory Droplet Virus Transmission. At 1 dpi, each of the three inoculated ferrets were individually placed in an isolator adjacent to a naive ferret. Each of the respiratory droplet (RD)-contact animals was separated from the donor animal by two stainless steel grids 5 cm apart, preventing direct-contact but permitting airborne transmission. Only the Sw/1204 virus was transmitted via RD to all three naive contact ferrets (Table 1). One of these RD contacts succumbed to infection at 12 d postexposure (dpe) and another was subsequently humanely euthanized because of signs of severe illness and weight loss. One Sw/CY07 RD-contact ferret demonstrated seroconversion at 17 dpe without detectable virus shedding, indicating inefficient RD transmission.

Identification of Molecular Markers Associated with Pathogenicity and Transmissibility. Despite differences in subtypes of both surface glycoproteins, all viral segments of the isolates in this study are genetically similar, clustering together with the North American TRSw lineage (ref. 12 and present study). The predicted amino acid sequence identities between the triple-reassortant internal gene (TRIG) segments of these viruses were >91% (*SI Appendix*, Table S3) and none had undergone genetic reassortment with other viruses of different lineage. A full-genome sequence analysis of all TRSw viruses was performed to identify molecular correlates of virulence and transmissibility in mammalian hosts. All four field TRSw isolates contained the mammalian host-adaptive 591-Arg residue in PB2 (15); PB1-F2 proteins also appear to be present but only Sw/CY07 had the characteristic 66-Ser residue (16). Although few amino acid differences were noted between corresponding viral segments of each virus, none of these appear to be associated to previously described virulence markers or determinants of pathogenicity (*SI Appendix*, Table S4) (16). The deduced amino acid sequence of the parental WT-Sw/1204 HA1 molecule indicated the presence of two glycosylation sites (residues 90 and 122, H3 numbering) at the globular domain. Surprisingly, virus recovered from each of the three Sw/1204 RD-contacts, designated CT-Sw/1204, consistently revealed two simultaneous substitutions compared with the parental WT-Sw/1204: aspartic acid (D)-to-glycine (G) at position 225 [H3 numbering in HA and serine (S)-to-asparagine (N) at residue 315 in NA (N2 numbering)]. HA_{225} resides within the 220-loop of the HA receptor-binding pocket, indicating that modification on this position may affect receptor-binding specificity (*SI Appendix*, Fig. S3*A*). NA_{315} appears to be near the vicinity of the active site of NA but this position is not directly associated with previously identified sites responsible for sialidase activity (*SI Appendix*, Fig. S3*B*) (17). Viral RNA samples directly prepared from the daily nasal washes collected from all inoculated (donor) and RD-infected ferrets were additionally analyzed. The HA_{225G} substitution was found in all of the sampled nasal washes and sequencing indicated that it was gradually acquired during the course of infection in the donor ferrets (*SI Appendix*, Fig. S4*A*). In contrast, the NA_{315N} substitution appeared to initially evolve genetically in two of the donor ferrets; it eventually arose simultaneously in all naive contacts after RD transmission (*SI Appendix*, Fig. S4*B*).

Table 1. Nasal shedding and transmission of TRSw influenza viruses in ferrets

Virus	Subtype	Abbreviation	Mean nasal wash titer* (SD)				Lung titer*	RD Contact	
			1 dpi	3 dpi	5 dpi	7 dpi		Virus detected	Sero con.
A/Sw/Korea/1130/2009	H1N2	Sw/1130	6.3×10^5 (0.5)	6.3×10^4 (0.3)	1.6×10^5 (0)	—	—	0/3	0/3
A/Sw/Korea/1204/2009	H1N2	Sw/1204	7.9×10^5 (0.3)	2.5×10^6 (0.1)	1.6×10^7 (0.1)	2.5×10^5	6.3×10^4 (0.3)	3/3	1/1[†]
A/Sw/Korea/CY05/2007	H3N2	Sw/CY05	1.6×10^5 (0)	5.0×10^5 (0.5)	3.2×10^4 (0.1)	—	—	0/3	0/3
A/Sw/Korea/CY07/2007	H3N2	Sw/CY07	1.0×10^6 (0.7)	1.6×10^6 (0.3)	6.3×10^4 (0.5)	—	—	0/3	1/3

Three ferrets were inoculated with $10^{5.5}$ $TCID_{50}$/mL of each virus. Three naive ferrets were placed in RD contact. Nasal washes were obtained on alternating days from inoculated ferrets and daily from contact ferrets. Tissues were collected at 7 dpi and seroconversion (Sero con) was determined in all inoculated and contact ferrets at 18 dpi (17 dpe).

*Viruses were titrated in MDCK cells; dashes (—) indicate titers < 0.7 $\log_{10}$ $TCID_{50}$ per milliliter or per gram. Tissues were collected at 7 dpi.

[†]The only surviving RD contact of Sw/1204-infected ferrets.

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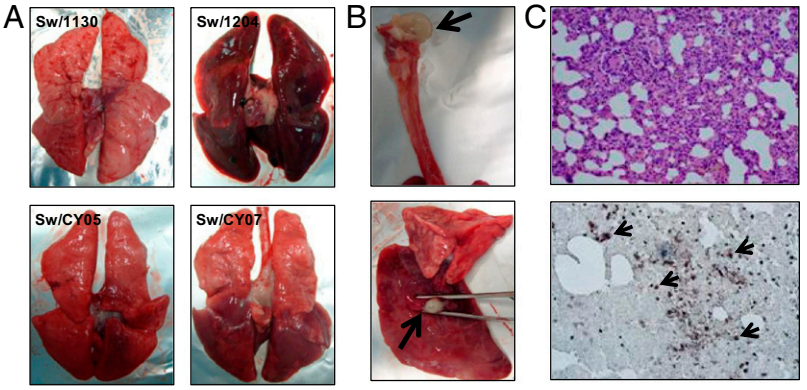

Fig. 1. Lung pathology caused by TRSw viruses in inoculated ferrets. Lung of ferrets inoculated with $10^{5.5}$ $\log_{10}$ $TCID_{50}$/mL of either TRSw H1N2 (Sw/1130 and Sw/1204) or H3N2 (Sw/CY05 and Sw/CY07) viruses were harvested at 7 dpi. (*A*) Gross morphologic features. (*B*) Mucopurulent exudates (arrows) were noted in the lungs of Sw/1204-inoculated ferrets. (*C*) Histopathology in the lungs of ferrets inoculated with SW/1204 was examined by H&E staining (*Left*, magnification 200×) and by immunostaining with polyclonal NP antibody (*Right*, magnification 400×). Arrows indicate positive reaction.

These two mutations (HA_{225G} and NA_{315N}) were not found in viruses isolated from mouse lung tissue homogenates, suggesting that they were positively selected in the ferret model.

To directly compare the transmission kinetics between the parental WT-Sw/1204 and a representative CT-Sw/1204 virus (all contact viruses had consensus simultaneous mutations in HA and NA as stated earlier), we inoculated additional groups of three ferrets in a similar regimen as above. Although the two viruses appeared to induce comparable virulence, mortality in CT- Sw/1204–inoculated ferrets was noted a day earlier relative to the WT-Sw/1204–inoculated group (Fig. 2). Furthermore, CT-Sw/1204 was more readily transmitted (as early as 1 dpe) among naive contacts compared with WT-Sw/1204, which required ∼4–5 dpe for RD transmission. Thus, CT-Sw/1204 maintained pathogenicity and notably enhanced ferret-to-ferret RD transmission.

Replication of WT- and CT-Sw/1204 in Vitro and ex Vivo. The growth properties of the WT-Sw/1204 and CT-Sw/1204 viruses were examined in MDCK, differentiated human bronchio-epithelial (HBE) and lung epithelial (A549) cells to compare any variation in their replication kinetics in vitro (Fig. 3*A*). For comparison, the other TRSw viruses were also tested in this particular experiment (*SI Appendix*, Fig. S5). Between the WT-Sw/1204 and CT-Sw/1204, no substantial differences in viral titers were observed in MDCK cells at 33 °C and 37 °C, the typical temperatures of the upper nasal and lower lung regions, respectively. Although both viruses demonstrated substantial growth in representative cells of the lower respiratory tract, CT-Sw/1204 appeared to have a growth advantage over WT-Sw/1204 in HBE and A549 cells at both temperatures, particularly in HBE cells at 33 °C (titers differed from those of WT-Sw/1204 by a factor of 10^2 to 10^3 in these cells and by a factor of $\sim 10^{1.2}$ in A549 cells) (Fig. 3*A*, *Left*). Although the other three field TRSw viruses [Sw/1130 (H1N2), Sw/CY05 (H3N2), and Sw/CY07 (H3N2)] demonstrated efficient replication in MDCK cells, variable growth patterns were observed in HBE and A549 and none could reach peak titers higher than the field WT-Sw/1204 virus ($\leq$ 2.7 $\log_{10}$ $TCID_{50}$/mL) (*SI Appendix*, Fig. S5). Therefore, these results suggest that CT-Sw/1204 could grow more efficiently than the TRSw field isolates, including the WT-Sw/1204 virus, in human respiratory epithelial cells and under thermal conditions representative of the upper and lower airways.

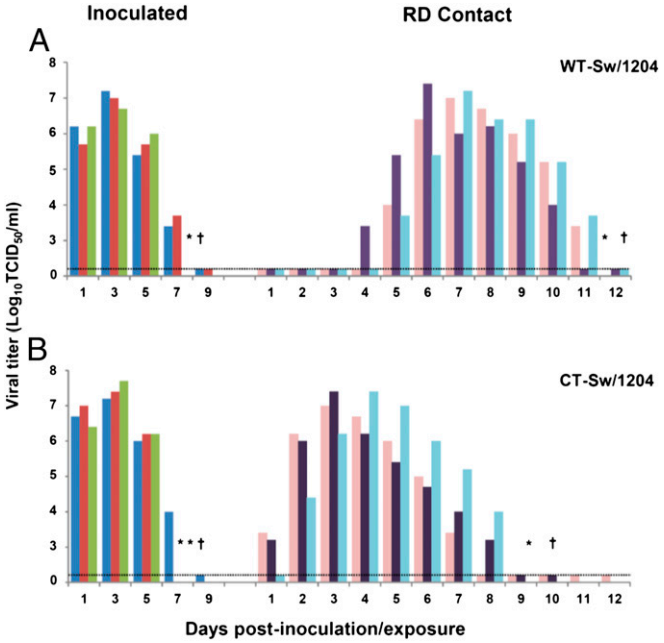

Fig. 2. Pathogenicity and transmissibility of CT-Sw/1204 virus. Groups of three ferrets were inoculated with $10^{5.5}$ $TCID_{50}$/mL of WT-Sw/1204 (*A*) or CT-Sw/1204 (*B*) and RD contacts were placed individually adjacent to the inoculated ferrets. Nasal-wash titers ($\log_{10}$ $TCID_{50}$/mL) are shown for individual ferrets. The limit of virus detection was 0.7 $\log_{10}$ $TCID_{50}$/mL as represented by the dotted lines (*succumbed to death; †killed).

We then compared the viral replication and infectivity of WT-Sw/1204 and CT-Sw/1204 ex vivo in freshly biopsied human lung tissue samples. The prototype pH1N1 virus A/California/4/2009 (CA/04, H1N1), which is known to grow efficiently in human lungs (5), was included in this analysis. After incubation with supplemented RPMI-1640 (negative control), no virus was detected by immunostaining with an anti-nucleoprotein (NP) antibody, indicating that the tested tissues had not been exposed to influenza viruses. The CA/04 (H1N1) virus demonstrated NP-positive reaction in alveolar cells (Fig. 3*B*) and efficient replication (Fig. 3*C*), as previously reported (18). The WT-Sw/1204 virus showed minimal infectivity and yielded the lowest titers of the three viruses tested. In contrast, CT-Sw/1204 was extensively detected in alveolar cells and grew to high titers (comparable to those of CA/04) at 36 hpi (Fig. 3*C*). These results are consistent with the severe lung pathology and high viral titers observed in ferrets.

HA_{225G} and NA_{315N} Contribute to Enhanced Transmission and Virulence. To assess the role of the two substitutions on viral pathogenicity and transmissibility, we generated mutant viruses in which the HA_{225G} or NA_{315N} substitution was introduced into Sw/1204 through a plasmid-based reverse-genetics (Rg) system (19). Rg WT-Sw/1204 and Rg CT-Sw/1204 were additionally generated. Growth properties of the recombinant viruses in vitro indicated that Rg WT-Sw/1204 and Rg CT-Sw/1204 displayed replication patterns comparable to their parental virus counterparts (Fig. 3*A* and *SI Appendix*, Fig. S6). For the single mutant viruses, Rg Sw/1204 HA_{225G} demonstrated slightly higher viral titers, whereas those of Rg Sw/1204 NA_{315N} were comparable relative to Rg WT-Sw/1204 in HBE and A549 cells (*SI Appendix*, Fig. S6).

The replication efficiency and pathogenicity of the Rg Sw/1204 HA_{225G} and Rg Sw/1204 NA_{315N} viruses did not differ substantially from those of the parental WT-Sw/1204 and CT-Sw/

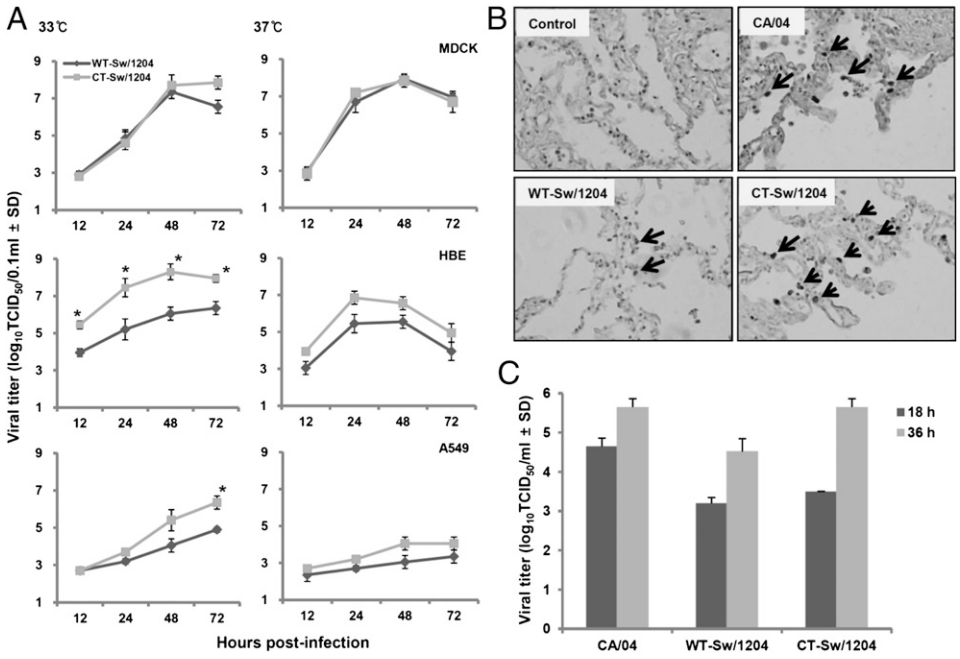

Fig. 3. In vitro replication and ex vivo infectivity of WT-Sw/1204 and CT-Sw/1204 viruses. (*A*) Comparative growth curves in MDCK, HBE135-E6E7, and A549 cells at 33 °C (*Left*) or 37 °C (*Right*) after inoculation in triplicate at an multiplicity of infection of 0.001 or 0.1 $TCID_{50}$/cell, respectively (*$P <$ 0.05). (*B*) Immunohistochemical detection of viral NP antigen at 36 hpi in freshly obtained biopsy specimens of human lung tissue (magnification, 400×). Tissue sections incubated in supplemented RPMI-1640 only were used as negative control. (*C*) Virus titers at 18 and 36 hpi in human lung tissues inoculated ex vivo (in triplicate). Titers are the mean and SD.

1204 (Fig. 2); two of three inoculated ferrets died or were humanely killed within 10 d (Fig. 4*A*). However, the Rg Sw/1204 HA_{225G} virus was detected in the nasal washes of RD contact ferrets at 1–2 dpe and reached peak titers earlier than the Rg Sw/1204 NA_{315N} virus in contact ferrets (2–4 dpe). Moreover, the HA_{225G} mutant induced apparently more severe gross lung lesions than the NA_{315N} mutant (Fig. 4*A*, *Right*).

To compare disease phenotypes in the mouse and ferret models, mice were inoculated intranasally with 10^5 $TCID_{50}$ of Rg WT-Sw/1204, single-mutant (Rg Sw/1204 HA_{225G} and Rg Sw/1204 NA_{315N}) and double-mutant (Rg CT-Sw/1204) viruses. The Rg CT-Sw/1204, Rg Sw/1204 $HA_{225G,}$ and Rg Sw/1204 NA_{315N} viruses induced signs of severe infection (ruffled hair, labored breathing) and caused death as early as 4 dpi (Fig. 4*B*) but Rg CT-Sw/1204 was most lethal, killing 8 of the 10 mice during the 14-d observation period [50% mouse lethal dose (MLD_{50}) = 4.6]. The Rg Sw/1204 HA_{225G} and Rg Sw/1204 NA_{315N} mutants were slightly less virulent (MLD_{50} = 4.7 and 5.0, respectively), causing ∼60–70% mortality. In contrast, 9 of 10 mice survived inoculation with the Rg WT-Sw/1204 virus (MLD_{50} = 5.9) (Fig. 4*B*). Therefore, the CT- and single-mutant Sw/1204 viruses were more pathogenic in mice than Rg WT-Sw/1204, correlating with their replication efficiency in ferrets. Collectively, these observations indicated that the HA_{225G} and NA_{315N} modifications may each have contributed to virulence and transmission. In contrast to ferrets, mice infection did not promote the HA or NA mutation in either mutant virus inoculums. Regardless, no revertant viruses or any other mutation was observed in both species.

We also sought to determine the impact of the identified mutations on viral replication and transmission in swine.Intranasal inoculation of the Rg (WT , single-mutant, and CT-Sw/1204) viruses (at titers 10^6 $TCID_{50}$/mL) caused mild signs of morbidity with no remarkable differences among viruses. Although there were some variations in peak titers and length of shedding in different animals, all Rg viruses tested replicated well in the inoculated animals and were transmitted to direct-contact animals with similar kinetics (*SI Appendix*, Table S5, and Fig. S7). Viruses recovered from inoculated and transmitted pigs did not contain additional amino acid modifications. Although thickening of alveolar septa was noted in some regions of Rg CT-Sw/1204-infected lung, examination of lungs harvested at 5 dpi from each group revealed viral NP detection and generally moderate pathological lesions that were indistinguishable between viruses (*SI Appendix*, Fig. S8).

The role of the NA_{315N} on enzyme kinetics and inhibitor susceptibility was additionally investigated. Consistently, possession of both the NA_{315N} and HA_{225G} substitutions (as seen in Rg CT-Sw/1204) induced substantially improved catalytic and thermostable NA activity relative to Rg WT-Sw/1204, indicating that the NA_{315N} is important, but not sufficient, to maintain enzyme kinetics (*SI Appendix*, Text S2, Fig. S9 and Table S6). Regardless, all Rg Sw/1204 viruses tested remained susceptible to NA inhibitors available for antiviral treatment of influenza infection (*SI Appendix*, Table S6).

Discussion

The failure of influenza experts to anticipate the emergence of the swine-origin pH1N1 2009 virus illustrates both the confounding unpredictability of influenza A viruses and the role that swine can play as putative "genetic mixing vessels." In this study, the ferret model was used to assess the pathogenicity and transmissibility (i.e., the putative zoonotic potential) of four representative contemporary TRSw influenza viruses. Most of these viruses caused mild disease and were inefficiently transmitted through the air, consistent with previous reports in ferrets (20, 21) and restricted epidemiology of TRSw influenza viruses in humans (10). However, the Sw/1204 H1N2 virus replicated well in both the ferret upper and lower respiratory tracts, induced severe viral pneumonia, was highly lethal, and was efficiently transmitted via respiratory droplets. It is noteworthy that ferrets inoculated with the reconstituted 1918 pandemic virus succumbed within 11 d of infection (22), but they did not succumb in this manner to the pH1N1 2009 virus (23) or other genetically related TRSw H1 influenza viruses (21, 22). Therefore, our findings suggest that some field isolates from swine may also show zoonotic potential in humans.

Sw/1204 is closely related phylogenetically to TRSw viruses commonly circulating in North America. The HA of Sw/1204 belongs to the genetically drifted γ-cluster of classical H1 swine-like viruses (*SI Appendix*, Fig. S1), which include the pH1N1 2009 virus. This cluster of TRSw H1 viruses appears to have crossed the animal–human interface sporadically since 2005 in the United States (9, 10), becoming successfully established in human populations only in 2009 and, therefore, remains a threat to public health. Because the Sw/1204 virus was obtained from pig abattoir, its pathogenesis in swine hosts was relatively unknown. Our data indicated that Sw/1204 is well-adapted in pigs with no remarkable clinical signs of disease. Acquisition of the HA_{225G} and NA_{315} mutations upon ferret replication did little to alter disease

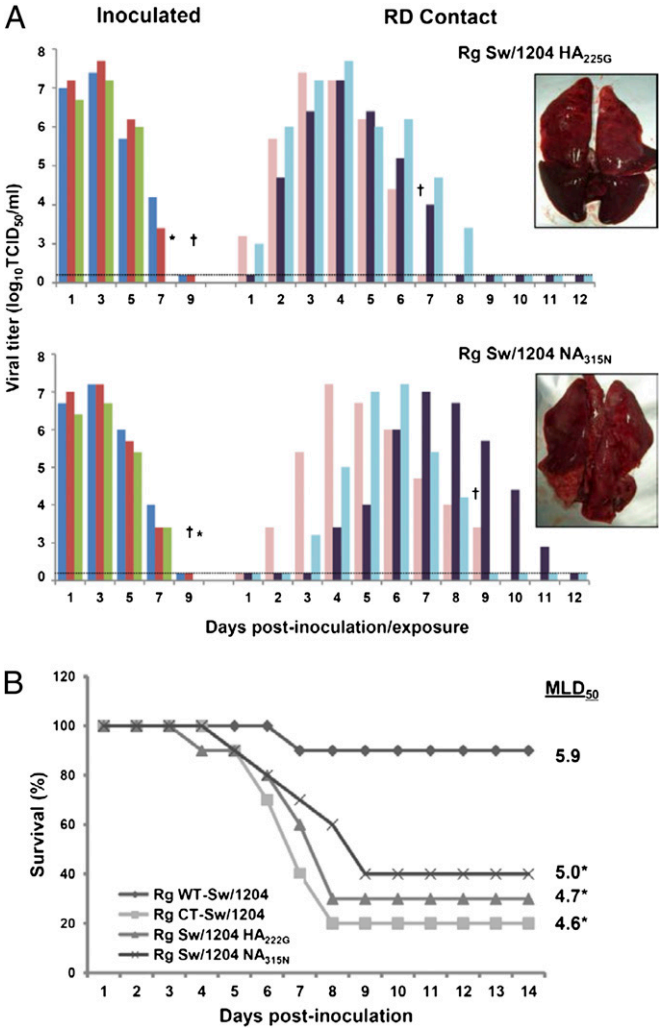

Fig. 4. Effect of the HA_{225G} and NA_{315N} mutations on transmissibility in ferrets and virulence in mice. (*A*) Three ferrets were inoculated with Rg-Sw/1204 HA_{225G} (*Upper*) or Rg-Sw/1204 NA_{315N} (*Lower*) and naive ferrets were individually placed in RD contact with the respective inoculated ferrets. Virus titers are expressed as $\log_{10}$ $TCID_{50}$/mL. The limit of virus detection was 0.7 $\log_{10}$ $TCID_{50}$/mL, as indicated by the dotted lines (*succumbed to death; †euthanized). *Right* panels show representative gross lung pathology caused by the mutant viruses. (*B*) Virulence of Rg viruses in mice as shown by survival rates and MLD_{50} after inoculation with 10^5 $TCID_{50}$/mL of virus in 30 μL. Mice were monitored daily for 14 dpi (*$P < 0.05$).

phenotype, replication, and transmission in swine. Such asymptomatic infection might therefore allow further genetic evolution of Sw/1204-like viruses or facilitate the inconspicuous spread of another potentially swine-origin virus with plausible clinical implications among humans. By hemagglutination inhibition (HI) assays, serologic evaluation of sera raised against pH1N1 2009 viruses demonstrated notable cross-reactivities (~160 HI titers) toward the Sw/1204 virus (*SI Appendix*, Table S7), indicating that postexposure to or immunization with pH1N1-like vaccines is likely beneficial. However, it still remains to be confirmed whether such cross-reactivities would essentially provide protection and prevent zoonotic transmission of Sw/1204-like viruses.

We observed that Sw/1204 viruses acquired the simultaneous HA_{225G} and NA_{315N} mutations in ferrets infected by aerosol contact and that these mutants showed enhanced transmissibility with sustained virulence. Because these residues were not observed in the parental isolates or in virus retrieved from the lungs of infected mice, these de novo viral mutations appear to have been positively selected only in ferrets. In addition, single-mutant Rg viruses also indicated their mutability, particularly HA_{225G}, in the context of the Sw/1204 in these models. We further noted that the HA_{225G} residue enhanced virus growth in ferret lungs, consequently inducing marked lung pathology. The CT-Sw/1204 virus also became virulent in BALB/c mice known to express predominantly α2,3-sialic acid in their lungs (24), and demonstrated enhanced infectivity and growth in human lung tissues, indicating potentially productive pulmonary replication. Notably, severe viral pneumonia attributed to efficient virus attachment to α2,3-sialic acid glycoconjugates deep in the lungs have been observed in H5N1 (25) and HA_{225G} variants of the pH1N1 (5, 26–30) virus infections. Moreover, it has been demonstrated that avian-type receptor-binding ability because of HA_{225G} increased pathogenicity of the pH1N1 2009 virus in nonhuman primates (31).

In contrast to the 1918 pandemic virus (22), the HA_{225G} mutation in CT-Sw/1204 did not appear to reduce RD transmissibility; instead, we speculate that it may have promoted dual receptor-binding specificity, complementing results observed with its introduction to a prototype pH1N1 virus (32). Parental CT-Sw/1204 and Rg Sw/1204-HA_{225G} were readily disseminated into naive contact ferrets via RDs, despite evident replication in the lower respiratory tract. Examination of replication kinetics in relevant human epithelial cell models also revealed that CT-Sw/1204 replicated more competently than the WT-Sw/1204 counterpart under conditions simulating both regions of the human respiratory tract. Surprisingly, however, naturally occurring HA_{225G} variants of the pH1N1 2009 virus do not appear to be transmitted efficiently in the field (33). It may be possible that molecular differences in and around the H1 receptor-binding domain account to the disparate effects of this mutation in different virus background. Nonetheless, our observation of the crucial impact of HA_{225G} on adaptation, pathogenicity, and transmission strengthens its proposed role as a molecular determinant of these characteristics among H1 viruses.

Less is known about the impact of the NA_{315N} substitution. A viral genome sequence search in GenBank revealed that only three other N2 viruses (human H3N2: accession no. CY011434; and swine H1N2, H3N2 strains: accession nos. CY085424, GU135893) have been found to carry the NA_{315N} residue (not in combination with HA_{225G}), and its role on pathogenicity, virulence, or transmissibility is yet unknown. Although its contribution in those aspects remains to be clearly elucidated, we found that presence of the NA_{315N} mutation alone resulted to significantly reduced sialidase activities. However, its combination with the HA_{225G} substitution promoted stable enzymatic activity. Therefore, we speculate that selection of NA_{315N} in ferrets might have been a compensatory modification necessary for the HA_{225G} mutation to restore functional balance between the surface glycoproteins in the context of the CT-Sw/1204 H1N2 virus. Couceiro and Baum (34) have demonstrated complementary HA and NA activity among H1N1 and H3N2 influenza viruses. Given that the HA_{225G} augments α2,3-sialic acid receptor-binding specificity, it is reasonable that an amino acid change that similarly enhances NA activity would provide a selective advantage for the virus in a new host, facilitating efficient release of progeny viruses from infected cells that efficiently bind the HA. Overall, the optimal balance between the affinity of binding between the virus and the cell receptor and the virus-releasing activity of the NA appears critical to virus replication (35), as well as efficient RD transmission, as recently observed with the pH1N1 2009 virus (21).

All of the Korean TRSw viruses tested here contain a TRIG cascade, typical of their North American virus precursors (11, 12). The absence of previously identified molecular markers also suggests that Sw/1204 possesses other factors (e.g., previously uncharacterized genetic determinants, unique gene constellation, and so forth), probably acquired during continuous circulation in swine herds, that modified pathogenicity and transmissibility. Not only the surface genes but also each of the remaining viral segments had been implicated in these traits (16). Introduction of HA_{225G} and NA_{315N} in the background of the Sw/1130 significantly enhanced virulence in mice (increased from ≥ 6.0–4.9 MLD_{50} and up to 30% weight loss) (*SI Appendix*, Fig. S10 *A* and

B). Virus replication of Rg SW/1130 HA_{225G}-NA_{315N} in the respiratory tract of inoculated ferrets increased (~10 times), consequently extending virus shedding up to 7 dpi (*SI Appendix*, Fig. S10*C*). Additionally, two of three contacts seroconverted with no evident viral detection, indicating potential RD transmission, albeit not as efficiently as Sw/1204. However, lethality was not observed in the Sw/1130 background, indicating the genetic relevance of the internal gene constellation of Sw/1204. Future work will therefore seek to define the individual contributions of the internal viral gene segments. Whatever these might be, we demonstrate here that selection of the HA_{225G} and NA_{315N} mutations exacerbated disease severity and transmissibility in the context of the Sw/1204 internal gene constellation.

Overall, we have presented evidence proposing the role of HA_{225G} and NA_{315N} as potential virulence markers of TRSw influenza viruses in mammalian hosts. Our findings further support the continuing pandemic potential of TRSw influenza viruses circulating in pigs. We also underscore the possibility that influenza viruses in swine populations can become a threat to animal and human health, as they generate novel viruses, reviving concerns over the capacity of pigs to potentially create pH1N1 2009-like influenza viruses in the future. Hence, continuous and efficient surveillance remains vital and beneficial. Such surveillance, together with studies in animal model systems, can help identify novel viruses with zoonotic potential and that pose health risks.

Materials and Methods

Replication and Transmission Studies. A complete description of the experimental process done in ferrets and pigs is provided in *SI Appendix, SI Materials and Methods*. Briefly, groups of three ferrets were inoculated intranasally and intratracheally with $10^{5.5}$ $TCID_{50}$ of each field TRSw virus isolate. At 1 dpi, one contact ferret was added to the other half of a cage containing one of the inoculated ferrets and separated by two stainless steel grids, allowing RD transmission without direct contact. Each test group consisted of three infected animals and three contacts. Signs of morbidity (e.g., weight, temperature, sneezing) and mortality were monitored daily for 14 d. Viral growth at the upper respiratory tract was tested by collection of nasal washes at indicated days and titration in MDCK cells. One ferret from each inoculated group was killed at 7 dpi (corresponding to the death of Sw/1204-infected ferrets) for viral pathology and tissue localization.

Two pigs were inoculated intranasally with 10^6 $TCID_{50}$ of Rg-WT-Sw/1024, Rg-Sw/1204-HA_{225G}, Rg-Sw/1204-NA_{315N}, or Rg-CT-Sw/1204-HA_{225G}/NA_{315N} virus. At 1 dpi, three naive pigs were added in direct contact with the inoculated animals, whereas two separate uninfected pigs were maintained as control. Nasal swab specimens were collected from all pigs up to 10 d and any signs of morbidity were recorded daily. An additional pig was infected for each virus for immunohistopathology at 5 dpi. Seroconversion in ferrets and pigs were examined at 18 and 14 dpi, respectively. All ferrets and pigs used in our study have been tested negative for influenza virus infection in NP ELISA (AbD Serotec) and SIV H1N1/H3N2 Ab Test (IDEXX) assays.

Virus preparation, titration, inoculation, and serologic testing were performed in an enhanced biosafety level 3 (BSL-3+) containment facility approved by the relevant national authorities. The use of mice, swine, and ferrets in this study adhered strictly to the animal handling policies mandated under the corresponding institutional and national guidelines of Chungbuk National University and St. Jude Children's Research Hospital Institutional Animal Care and Use Committees.

Site-Directed Mutagenesis and Rescue of Rg Viruses. All eight gene segments of the Sw/1204 virus was amplified and cloned into the pHW2000 vector (19). The characteristic mutations (HA_{225G} and NA_{315N}) found in the RD contact virus CT-Sw/1204 were each introduced into the plasmid construct of WT-Sw/1204 by site-directed mutagenesis (GeneTailor Site-directed Mutagenesis System; Invitrogen). Rescued viruses were fully sequenced to ensure the absence of unwanted mutations.

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