Review article

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The significance of avian influenza virus mouse-adaptation and its application in characterizing the efficacy of new vaccines and therapeutic agents

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Due to the increased frequency of interspecies transmission of avian influenza viruses, studies designed to identify the molecular determinants that could lead to an expansion of the host range have been increased. A variety of mouse-based mammalian-adaptation studies of avian influenza viruses have provided insight into the genetic alterations of various avian influenza subtypes that may contribute to the generation of a pandemic virus. To date, the studies have focused on avian influenza subtypes H5, H6, H7, H9, and H10 which have recently caused human infection. Although mice cannot fully reflect the course of human infection with avian influenza, these mouse studies can be a useful method for investigating potential mammalian adaptive markers against newly emerging avian influenza viruses. In addition, due to the lack of appropriate vaccines against the diverse emerging influenza viruses, the generation of mouse-adapted lethal variants could contribute to the development of effective vaccines or therapeutic agents. Within this review, we will summarize studies that have demonstrated adaptations of avian influenza viruses that result in an altered pathogenicity in mice which may suggest the potential application of mouse-lethal strains in the development of influenza vaccines and/or therapeutics in preclinical studies.

Keywords: Influenza A virus, Mouse adaptation, Molecular determinant, Vaccination, Virulence, Serial passage

Introduction

Most influenza A viruses, including 16 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes, have been found in migratory waterfowl, thus, waterfowl is believed to be a natural reservoir [1,2]. The influenza virus exhibits a restricted host species barrier in the avian host [2] and in general, infection to other mammals including humans with avian influenza virus is limited. A few subtypes have successfully transcended and developed a capacity to establish themselves in mammalian hosts, primarily based on the genetic nature of the RNA genome (e.g., eight segments and error-prone) which leads to mutations conferring the ability to cross the host barriers. There have been three worldwide flu pandemics in the 20th century (the Spanish flu [H1N1] in 1918; the Asian flu [H2N2] in 1957; the Hong Kong flu [H3N2] in 1968). Most of the causatives were reported to be originated from avian species and have caused pandemics after reassortment between the new avian influenza virus and the human influenza

virus [3]. In June 2009, the World Health Organization (WHO) declared the novel influenza A (H1N1) virus as the first pandemic of the 21st century [4]. Molecular epidemiologic studies have shown that viruses prevalent in birds, pigs and humans have been reassorted in unknown events and then transmitted to humans [5]. Although the same subtype as the seasonal H1N1 virus was predominant at the time, most people under the age of 65 could be infected by the pandemic virus because they did not have immunity due to serologically differentiated immunogenicity [6]. The pandemics occurred when the novel subtypes of viruses or parts of genes (especially HA, NA) were introduced into humans through genetic reassortment. However, genetic reassortment, which is generally known to be the mechanism of past pandemics, is insufficient to account for the ability to overcome interspecies barriers. Before an influenza virus becomes established in new hosts, it must overcome host-specific factors, including the ability to adapt, survive, and thrive in another host; these adaptations are conferred as the result of genetic mutations and are thus also required for the virus to transcend the interspecies barriers. Although there is still little evidence of human-to-human transmission, a variety of avian influenza viruses (H5N1, H5N6, H6N1, H7N3, H7N7, H7N9, H9N2, and H10N8) have been infecting humans directly from avian species [7-12]. In particular, human infections caused by H5N1, H5N6, and H7N9 are rapidly increasing and mortality rates reach 30%–50% [13] indicating the urgent need for vaccines that effectively protect humans against these emerging viruses.

Recently, various animal models (e.g., mouse, ferret, and guinea pigs) have been established for studying influenza proliferation, pathogenesis, and transmissibility. Ferrets can experience influenza-like respiratory illnesses after infection similar to those observed in humans and can spread viruses through direct contact and aerosols. Therefore, the pathogenicity and transmissibility of influenza virus can be evaluated using the ferret model [14]. Unlike ferrets, guinea pigs do not cough or sneeze after influenza infection but have been successfully used to study viral transmission by direct contact and aerosol transmission models [15,16]. It was shown in the 1930s that swine and human influenza viruses can infect laboratory mice and have since been used extensively in influenza pathogenesis studies [17]. Although the mouse model may not fully mirror the clinical signs and behaviors of influenza infection in humans, there are numerous studies showing the suitability of mouse models (e.g., ability to study infections with various influenza viruses, low cost), and thus, it is still an important animal model in influenza research. Above all, laboratory mice play an important role in preclinical animal-model studies designed to evaluate the efficacy of newly developed vaccines and drugs, studies that are a priority given the growing incidence of human infections caused by novel avian influenza viruses and the urgent need to identify treatment and/or prevention options. However, the lack of appropriate mouse-lethal influenza viruses makes it difficult to assess the exact effects of vaccines or treatments, thus slowing development. Although some influenza viruses may exhibit high virulence without prior adaptation to the mouse [18], most viruses exhibit low susceptibility to mice since they are non-natural hosts of influenza. Therefore, an abundance of studies have been conducted to evaluate the efficacy of vaccines or inhibitors by first generating more virulent strains of low pathogenicity viruses by continuous passage in mouse lungs [19-22]. In addition, mouse-adaptation studies of a variety of avian influenza viruses have been used to discover the molecular determinants of influenza viruses, which are important for adapting to new hosts [17]. Key mutations, such as E627K in the PB2 gene which contributes to the ability of avian influenza viruses to: (1) infect to mammalian hosts (including humans) [8,23-25] and (2) exhibit high replicative properties, were identified via the mouse-adaptation approach [26-28]. Therefore, in this review, we will focus on avian influenza viruses isolated from birds or other mammalian hosts to determine what molecular determinants are identified through mouse-adaptation studies. Furthermore, we will summarize and discuss the significant use of laboratory mice for generating mouse-lethal viruses that may contribute to the development of vaccines or therapeutic agents.

Adaptation Using Avian Influenza Viruses in Mice

Since the first isolation of avian influenza virus in chickens (A/Brescia/1902 [H7N7]) in the beginning of 20th century, a variety of influenza ecology studies have shown that each type of influenza A viruses can be isolated from aquatic birds [2]. Some of the avian viruses caused previous pandemics and established in human. Furthermore, in 1997, the highly pathogenic avian influenza (HPAI) H5N1 virus infected humans in Hong Kong and caused severe diseases with high mortality [29]. Since the beginning of the 21st century, the number of molecular studies designed to identify changes capable of facilitating the interspecies transmission of avian

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influenza into human has dramatically increased. This increase is largely associated with studies aiming to adapt the virus in mammalian hosts, particularly mice. Recently, many avian influenza subtypes (e.g., H5, H6, H7, H9, and H10) have affected humans. Therefore, these subtypes of avian influenza viruses have been most commonly used to study their adaptation in mice and to determine what changes facilitate their ability to become highly virulent in the mammalian host.

Mouse adaptation of avian H5 subtypes and the molecular determinants mediating virulence

From the time of the first human infection by the HPAI H5N1 virus (1997), until March 2017, there are 856 laboratory-reported cases of human infection and a 53% mortality rate [13]. Despite no evidence of sustained human-to-human transmission, the high rate of H5N1 interspecies transmission and pathogenicity has encouraged investigation into its potential to cause a pandemic. Even though not all HPAI H5N1 viruses show high virulence in mammalian hosts, generally, the H5N1 has able to replicate at high titer and been found to be lethal to mice prior to adaption [18,30]. The pathogenic characteristics of these HPAI H5N1 viruses did not require the generation of mouse-adapted virulent variants to preclinically evaluate the efficacy of new vaccines or therapeutic agents. Thus, most mouse-adaptation studies have been conducted to investigate the potential molecular determinants facilitating direct human infection by avian influenza viruses. Some HPAI H5N1 viruses which were avirulent in mice become highly virulent as early as in a single replication in mice when they acquire the E627K substitution within the PB2 gene [27,31,32]. The PB2-E627K mutation appeared as early as 4 days after inoculation and replaced E to K completely by day 6 after virus infection [32]. This mutation has been proposed to: (1) be associated with the high virulence of H5N1 virus in mice and (2) an enhancement in the viral replication efficiency in the mouse upper respiratory tract [24,33]. Further, the mutation was found more in H5N1 human isolates than avian [34,35] suggesting it is a mammalian-adaptive mutation. On the other hand, mouse-adaptation studies with HPAI H5N1 variant do not always lead to the acquisition of a virulence phenotype in the host. HPAI H5N1 human isolate (A/Vietnam/1194/04) with attenuated virulence in mice after passages in mice lung involved an E190G substitution within the HA gene resulting in an altered binding specificity [36].

Due to the high virulence of HPAI H5N1 virus in mice, it is unnecessary to generate mouse-adapted variants to evaluate new vaccines or therapeutic agents. However, the use of HPAI viruses should be conducted in an appropriate facility (i.e., higher than Biosafety Level 3 laboratory handling) by highly qualified individuals. This requirement limits the accessibility for researchers who are developing new prophylactic and therapeutic measures. Therefore, the generation of low pathogenic avian influenza (LPAI) H5 virus strains virulent in mice may confer a benefit through mitigation of some of these infection risks. Thus, studies have been conducted with LPAI H5N2 viruses adapted into mice by lung-to-lung sequential passages [28,37]. Unlike HPAI H5N1 viruses that are already lethal prior to adaptation and elevate the virulence in mice within a single passage [27,31,32], LPAI H5N2 viruses need multiple passages, (up to 23 serial lung-to-lung passages) to obtain highly virulent phenotypes in mice, suggesting that HPAI H5N1 has more intrinsic properties resulting in virulence in the new host. Several amino acid changes were observed in mouse-adapted H5N2 variants including PB2-E627K and PA-T97I that played a critical role in enhanced replication and virulence in mice [28]. Since the initial identification of PA-T97I mutation in association with enhanced polymerase activity, viral replication in vitro and/or virulence in mice, further studies have been conducted to observe other substitutions that relates with mammalian adaptation [26,35,38,39].

Despite the highly stable HA and NA combination (H5N1) HPAI virus which has been maintained for more than a decade [40], novel combinations of HPAI H5Nx viruses generated by natural reassortment have been widely circulating recently [40-42] since the first outbreak of HPAI H5N5 variant in 2008 [43]. In recent years, the HPAI H5N8 virus has spread worldwide and crossed from Eurasia to North America for the first time. This HPAI H5 virus had led to various H5Nx variants with genes native to North America, posing a great threat to humans. Although the H5Nx variants showed high pathogenicity to chickens [44], most viruses isolated from avian species have maintained low pathogenicity in mammalian hosts [45,46]. However, the emergence of novel H5 variants retaining characteristics of the HPAI form and spreading rapidly with massive genetic evolution increases the need for pandemic preparedness. In addition, studies reported that H5Nx variants rapidly acquire virulence phenotype after passages in mouse model [35,47-49]. Mouse-adaptation of HPAI H5N2 [47,49], H5N6 [48], and H5N8 [35] viruses have been investigated and found to involve a variety of substitutions, some of which are previously reported and some of which are newly identified via these studies. These substitutions may

potentially be associated with the increased virulence in mammalian hosts. Of note, previously reported mutations (i.e., PB2-E627K, -Q591K, -D701N, and PA-T97I) also occurred in HPAI H5Nx viruses as observed in other viral strains after mouse adaptation [17]; a novel PB1-P708S mutation was also found in two H5N8 strains and shown to elicit increased viral replication and virulence in mice alone or synergistically with other virulence markers (PB2-E627K or -D701N) [35]. Interestingly, although most H5Nx viruses adapted in mice are clustered in clade 2.3.4.4 and/or shared more than 99% nucleotide sequence homology, they acquired the polymorphic and distinctive genetic markers (either PB2-E627K or PB2-Q591K/D701N) [35,48,49]. A single mutation such as PB2-E627K can confer a dramatic enhancement of virulence of HPAI H5 viruses in mice [27,31] but also the combination of other multiple mutations can also lead to an increase in virulence [27,35].

Mouse-adaptation of avian H7 subtypes and the molecular determinants mediating virulence

Although all of H7Nx subtypes have been isolated from wild birds, H7N1 [50], H7N3 [51], and H7N7 [52] subtypes have shown high pathogenicity to poultries. Recent studies demonstrated that many LPAI H7 viruses isolated from avian species in Eurasia and North America caused mortality in mice prior to adaptation [53-55], suggesting a potential public health risk. Furthermore, various avian H7 subtypes in combination with N2, N3, N7, and N9 have been introduced into humans through exposure to domestic poultry [7]. Particularly, there was a massive increase in human infections with LPAI H7N9 virus in China (more than 1,300 cases reported as of March 2017) with 30%-40% mortality. And most recently, the HPAI form of the H7N9 virus were found and has also infected humans [56]. The H7 avian subtype, together with H5, actively affects humans and possesses high potential to cause a pandemic.

Like HPAI H5N1, many H7 viruses, including LPAIs, are lethal to mice prior to adaptation [53]; many studies have demonstrated the adaptation of both HPAI [57,58] and LPAI [59,60] H7 viruses in mice to identify genetic determinants of virulence in a mammalian host. Both HPAI and LPAI H7 viruses acquired high virulence in three to seven passages in mice lungs [57,59,60]; this is slower than HPAI H5N1, but faster than LPAI H5N2 viruses. Moreover, all viruses classified as LPAI (including the H7N9 subtype) expanded their tissue tropism beyond the target organ (i.e., lungs) to brain, liver, spleen, kidney, and intestine after mouse adaptation [59-61]. Most studies of avian H7 mouse adaptation observed conserved PB2-E627K substitution [57,59,60]; this single change was sufficient to enhance the virulence of mouse-adapted H7 viruses. In addition, the PB2-E627K substitution was also observed in an equine H7N7 virus after mouse adaption and it is associated with an appreciable increase of virulence in mice [62]. A lethal mouse-adapted descendant SC35M originated from HPAI H7N7 (SC35) obtained several mutations (i.e., PB2-D701N, S714R, and NP-N319K) after mouse adaptation and combinations of these mutations synergistically increases viral polymerase activity, growth *in vitro* and virulence in mice [58]. Interestingly, lethal mouse-adapted H7N9 variants acquired none of the well-defined adaptive markers (e.g., PB2-E627K or -D701N), but instead obtained other 14 mutations including T97I and Q556R in PA gene [61] which was previously reported in other subtypes [28,63]. However, precise function of the mutations occurring in H7N9 needs to be verified in further studies.

Mouse adaptation of avian H9 subtypes and the molecular determinants mediating viral virulence

Since the first isolation of the avian influenza H9N2 subtype from turkeys in the Northern part of United States in 1966 [64], the subtype virus has been circulating among wild birds and domestic poultry in several Asian countries [10,65]. The influenza H9N2 subtype has also been identified in domestic mammals and humans [65-67] and has posed two major concerns: (1) the virus occasionally infects humans (although it is usually not fatal) and (2) the virus has donated its genetic material into the other avian influenza viruses such as HPAI H5N1 [68], H5Nx [69], H7N9 [70], and H10N8 [71], all of which cause major human infection and are suggested as candidates for the next pandemic.

Of all H9Nx subtypes, H9N2 has primarily been used to understand the mechanism of interspecies transmission and increased virulence in mammalian hosts (e.g., mice and ferrets) due to the prevalence of this subtype and its repeated introduction into humans [67]. In general, H9N2 influenza viruses isolated from avian hosts demonstrate no disease or mild disease activity in mice, although they have been shown to replicate in mouse lungs [72-74]. Therefore, the generation of lethal mouse-adapted variants may further contribute to the field's ability to evaluate the efficacy of the new vaccines and therapeutic agents against H9N2 influenza virus. Abundant mouse-adaptation studies have demonstrated the ability to identify strains with increased virulence in mice [75-81].

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Table 1. List of avian influenza viruses serially passaged in mice lung and their altered genetic and pathogenic properties after adaptation

Subtype	Parental virus	Pathogenicity index[a]	Mouse strain	No. of passages (detectable virulence[b])	MLD_{50} of MA-variant	No. of AA mutations (considerable mutations[c])	Tissue distribution	Reference
H5N1	Ck/Yamaguchi/7/04	HPAI	BALB/c	1 (1)	0.9 $log_{10}EID_{50}$	2 (PB2-E627K; HA-M531I)	Lu, Br, Sp, Li, Ki	[31]
	Wdk/Hunan/021/05	HPAI	BALB/c	15 (1)	0.5-2.2 $log_{10}EID_{50}$	15 (PB2-E627K, -L89V, -G309D, -T339K, -R477G, -I495V, -A676T)	Lu, Br, Li, Sp, Ki	[27]
	Vietnam/1203/04	HPAI	BALB/c	1 (1)	ND	1 (PB2-E627K)	Respiratory organ	[32]
H5N2	Ck/Hebei/1102/10	HPAI	BALB/c	15 (5)	2.5 $log_{10}EID_{50}$	5 (PB2-Q591K, D701N)	Lu, Hr, Li, Sp, Ki, Br	[47,82]
	Dk/Zhejiang/6DK19/13	HPAI	BALB/c	10 (ND)	ND	4 (PB2-E627K; PB1-I181T; HA-A150S; NS2-E69G)	Lu, Sp, Ki, Li, Br, Hr	[49]
	Mdk/Pennsylvania/10218/84	LPAI	ND	23 (23)	1.75 $log_{10}EID_{50}$	3 (HA-L320P, -S203F, -E273G)	Lu	[37]
	AB/Korea/W81/05	LPAI	BALB/c	17 (10)	2.7 $log_{10}TCID_{50}$	8 (PB2-E627K; PA-T97I)	Lu	[28]
H5N6	Dk/Zhejiang/6D2/13	HPAI	BALB/c	10 (ND)	2.68 $log_{10}EID_{50}$	5 (PB2-E627K; PA-A343T; HA-A150V; NA-R143K, -G147E)	Br, Sp, Li, Ki, Hr, Lu	[48]
H5N8	Mdk/Korea/W452/14 Em/Korea/W468/15	HPAI	BALB/c	5 (2)	0.5-4.8 $log_{10}PFU$	14 (PB2-E627K, -D701N, -Q591K; PB1-P708S; PA-T97I)	Lu, Br, Hr, Li, Sp, Int	[35]
H7N1	BP/HuNan/414/10	LPAI	BALB/c	5 (ND)	1.75-2.25 $log_{10}EID_{50}$	6 (PB2-E627K; HA-E114K, -G205E, -G218E; NA-S350N)	Lu, Br, Li, Sp, Ki, Int	[59]
H7N7	Ck-egg adapted Seal/Massachussetts/1/80	HPAI	BALB/c	11 (ND)	2.8 $log_{10}PFU$	9 (PB2-D701N, S714R; NP-N319K)	Lu	[58]
	Ck/Netherlands/621557/03	HPAI	BALB/c	3 (3)	2.7 $log_{10}TCID_{50}$	1 (PB2-E627K)	NT, Tr, Lu, Ileum, Jejun, Ki, Li, Sp, Hr, Br	[57]
	LWFG/HuNan/412/10	LPAI	BALB/c	7 (ND)	2.75-3.5 $log_{10}EID_{50}$	7 (PB2-E627K; PB1-R118I; PA-L550M; HA-G214R; NA-S372N)	Lu, Br, Li, Sp, Ki, Int	[60]
H7N9	Shanghai/2/2013	LPAI	BALB/c	10 (ND)	1.5-3.5 $log_{10}EID_{50}$	14 (PA-T97I, K328R; HA-A107T, -L226Q, -R354K; NP-A284T, -M352I; NA-M26I, -G389D)	Lu, Br, Li, Ki, Sp, Int	[61]
H9N2	Ck/Jiangsu/7/02	LPAI	BALB/c	ND (ND)	ND	25 (PB2-E627K)	Hr, Ki, Lu	[75]
	Ck/Hubei/01/99	LPAI	BALB/c	8 (5)	ND	9 (PB2-E627K)	Li, Lu, Sp, Ki, Hr	[76]
	Ck/Shandong/16/05	LPAI	BALB/c	8 (8)	2.8 $log_{10}PFU$	5 (PB2-M147L, -E627K)	Lu	[77]
	Dk/Jiangsu/1/08	LPAI	BALB/c	18 (5)	2.5 $log_{10}EID_{50}$	8 (PB2-F404L)	Lu	[78,79]
	Ck/Shandong/Li-2/10	LPAI	BALB/c	5 (4)	3.5 $log_{10}EID_{50}$	3 (PB2-E627K; HA-N313D, -N496S)	Lu	[80]
	Ck/Korea/163/04	LPAI	BALB/c	98 (ND)	ND	23 (PB2-E627K; HA-N158S)	Lu	[81]
H6N1	Mdk/SanJiang/275/07	LPAI	BALB/c	8 (ND)	2.75 $log_{10}EID_{50}$	3 (PB2-E627K; PA-T97I)	Lu, Br	[83]
H6N6	Sw/Guangdong/K6/10	LPAI	BALB/c	12 (8)	3.89 $log_{10}PFU$	5 (HA-H156N, -S263R; PA-I38M; PB2-E627K)	Lu	[84]
	Dk/Hubei/5/10	LPAI	BALB/c	8 (4)	3.4 $log_{10}EID_{50}$	17 (PB2-E627K)	Lu, Sp, Ki	[85]
H10N7	Ck/Zhejiang/2CP8/14	LPAI	BALB/c	10 (9)	ND	3 (PB2-E627K; PA-T97I)	Lu, Br, Li, Ki, Sp, Hr	[38]
H10N8	Em/Hunan/3-9/07	LPAI	BALB/c	7 (3)	ND	7 (PB2-E627K; PA-F277S, -C278Q)	multiple organs	[86]

[a]Defined by intravenous pathogenicity index criteria.
[b]The number of lung passages showing detectable virulence in mice.
[c]Mutations that the authors mentioned or proved important.
MLD, mouse lethal dose; MA, mouse-adapted; Ck, chicken; HPAI, highly pathogenic avian influenza; EID, egg infectious dose; Lu, lung; Br, brain; Sp, spleen; Li, liver; Ki, kidney; ND, not determined/defined; NS, not specified; Hr, heart; LPAI, low pathogenic avian influenza; TCID, tissue cultured infectious dose; Dk, duck; Mdk, mallard duck; PFU, plaque forming unit; Em, environment; Int, intestine; BP, Baer's Pochard; NT, nasal turbinate; Tr, trachea; LWFG, Lesser White-fronted Goose; Sw, swine.

Like the HPAI H5 and H7 viruses that rapidly adapted to mice and exhibit high virulence (Table 1), most avian H9N2 subtypes rapidly acquired high virulence within 8 serial passages in mice lungs (Table 1). It should be noted, however, that there has been no evidence reported suggesting that the avian H9N2 virus has evolved into a highly pathogenic form. Moreover, some mouse-adapted H9N2 variants were able to replicate in peripheral organs other than respiratory tissues, an attribute typically observed in HPAI viruses in mice [75,76]. This suggests the presence of intrinsic genetic properties that facilitate the ability of the virus to rapidly adapt and become highly virulent in mammalian hosts like the HPAIs. During mouse adaptation, most H9N2 viruses rapidly obtain the mammalian-adaptive mutation PB2-E627K which has been also found in pandemic viruses and human isolates, and this mutation has been shown to play a critical role in enhancing virulence in mice [75-77,80,81]. It was previously demonstrated that, in H9N2 viruses, the artificially conferred PB2-E627K mutation dramatically increased virulence without prior adaptation in mammalian hosts [80,87]. In addition, other substitutions (i.e., PB2-F404L, -M147L, HA-N313D, and -N496S) contributed to enhanced virulence in mice and were associated with increased activity of viral polymerase, replication, or enhanced receptor binding [77,78,80].

Mouse adaptation of other avian subtypes and the molecular determinants in viral virulence

A variety of avian influenza strains are widely circulating in China and some occasionally infects humans [88]. Novel LPAI strains (H6N1 and H10N8) have recently emerged in humans and H10N8 has been reported to have infect three humans and caused two deaths [89,90]. Similar to the H7N9 virus, H10N8 was also reassorted from the H10 and N8 genes that originated from wild birds with the internal gene backbone of H9N2 AIVs [71]. Furthermore, because the H10N8 virus, like the H7N9 virus, causes an asymptomatic infection in poultry, it is difficult for people to recognize the virus even if the chicken is infected. As a result, people have more chance to become infected with the virus that exists in the poultries. Interestingly, H6 virus isolated from humans possesses a unique combination of four key residues in the receptor binding site that have not been previously observed in human or avian influenza viruses [91] and some avian H6 strains were transmittable between guinea pigs by direct contact [92]. Therefore, the mechanism of avian-to-human interspecies transmission of the viruses needs to be addressed and new vaccines may need to be developed as a means of preparing for a potential pandemic.

Although some H6 subtype strains showed high virulence in mice [93,94], avian-isolated H6 viruses are generally associated with mild-disease signs in mice [85,92,94,95]. Avian H10 subtype influenza viruses also led to only mild-to-moderate disease signs in mice [96-98]; one of the H10N8 human isolates, however, was highly virulent in mice [99]. Thus, the generation of lethal mouse-adapted H6 or H10 variants will be beneficial to evaluate the efficacy of the new vaccines and therapeutic agents. Several studies on the adaptation of avian influenza H6 and H10 viruses in mice have been reported [38,83-86]. All of the LPAI viruses adapted to mice acquired high virulence in mice (Table 1). Some mouse-adapted H6 variants, from wildtype strains with limited replication capacity in the lungs of infected mice, did display expanded tissue tropism to brain, spleen, or kidney following adaptation [83,85]. The significant enhancement of virulence of all H6 and H10 viruses were observed within eight serial lung-to-lung passages and associated with the conserved PB2-E627K mutation that is commonly observed in the other subtypes of avian influenza viruses after mouse adaptation (Table 1). One of the H10N8 human isolates which resulted in death possessed the PB2-E627K mutation and displayed high virulence in mice [99]. Apart from the PB2-E627K substitution, PA-T97I was observed in one H6 and one H10 strain [38,83].

Evaluation of Protective Efficacy of Vaccines and Therapeutics by Challenging Mouse-Adapted Avian Influenza Viruses

Mouse-adaptation studies using human-originating strains of the influenza virus have been performed for more than 80 years [17]. Some mouse-adapted strains including A/Puerto Rico/8/1934(H1N1) (PR8) [100] have been widely used in various studies designed to (1) evaluate the efficacy of vaccines and therapeutics, (2) investigate host-virus interactions, and (3) identify immune-mediated mechanisms directed against influenza virus infection. Despite an established understanding of the mouse-specific pathogenesis and immunology of previously identified mouse-adapted strains, there remains a lack of knowledge of the immunogenicity and pathogenicity of recently emerging viruses in human (e.g., H5, H7, and H9). Therefore, the development and characterization of lethal mouse-adapted viruses that may adequately reflect the immunogenic and pathologic properties of these emerging

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viruses would help fill a substantial knowledge gap. In response to this need, there have been an abundance of studies designed to evaluate the effect of new vaccines and therapeutics and to investigate immunologic functions by using mouse-adapted avian influenza viruses.

Vaccination is one of the most effective and cost-effective interventions to prevent mortality and reduce morbidity associated with the influenza virus. However, the conventional inactivated/live-attenuated vaccines generate immunity only to specific strains and thus, need to be updated annually. Moreover, a recent increase in human infections with novel emerging viruses including avian influenza cannot be protected by conventional vaccines. Thus, universal vaccines designed to be more broadly reactive to various influenza strains and vaccines immunogenic to the specific emerging viruses are being developed [101]. Although the major purpose of mouse-adaptation studies with avian influenza were to investigate the molecular determinants altering virulence of avian virus in mammalian hosts, abundant studies have demonstrated the protective efficacy of various intervention approaches using the lethal mouse-adapted avian influenza viruses in mouse model [102-107]. Since a mouse-adapted H5N2 virus was originated from LPAI H5N2 [28] and exhibited high lethality in mice, it can therefore be used in BL-2 facility which makes it accessible and usable by more researchers interested in evaluating the efficacy of newly developing vaccines. There have been studies suggesting that a number of M2e-based vaccine candidates produce neutralizing antibodies that more broadly protect immunized mice from lethal mouse-adapted H5N2 [28] and H9N2 [81] challenges [106-109]. In addition, the mouse-adapted H5N2 virus also exhibited varying levels of lethality when mice were immunized with various vaccine candidates, suggesting an ability to identify effective candidates in the cold-adapted and NS1-truncated live-attenuated vaccine studies [102,104]. A DNA-vaccine in which the HA and NA genes obtained from avian H9N2 virus was used to immunize mice using electroporation was evaluated for its ability to convey protection by challenging lethal H9N2 virus generated by mouse adaptation [20].

The protective efficacy of therapeutic agents inhibiting influenza growth has been abundantly evaluated in mice by challenging with lethal mouse-adapted human influenza viruses [21,110-112]. Due to the high lethality of HPAI H5N1 virus in mice, the wild type virus has been used to evaluate the protective efficacy of therapeutic agents against this virus [21, 113]. However, for strains of avian influenza virus with low levels of virulence in mice (e.g., H9N2—which is also suggested as a pandemic potential), it would be beneficial to develop a virulent phenotype in mice in order to evaluate the efficacy of drug candidates against this subtype [21,113].

Conclusion

There are limitations to using adapted variants since, after multiple passages in mice; they may occasionally acquire an antigenicity that is distinct from wild type virus [37,114,115] meaning that they may represent an inappropriate lethal challenge model. For this reason, the antigenicity of mouse-adapted variant (compared to wild type) should be verified before the challenge experiment. The adaptation of avian influenza potentially generates variants with a virulent phenotype in humans, although mostly are attenuated. The aim of mouse-adaptation studies of most avian influenza is to better characterize the mechanism of genetic evolution in the mammalian host, which may potentially cause the next pandemic. However, genetic mutations identified from mouse-adaptation studies may be independent of their enhanced virulence in mice, since some mutations may occur during proliferation in laboratory cells or by other causes. However, based on data obtained from a vast amount of studies, we highlight that a number of mutations that occur commonly in mice have been identified through adaptation in mice, and some of these mutations also occur when avian influenza viruses infect humans. In addition, it was predicted that the number of passages needed to cause pathogenicity in mice is a subtype-dependent or dependent on the characteristics of HP/LPAI. For instance, some LPAI viruses can induce extra-pulmonary infection in mice after mouse adaptation. Therefore, it is considered that the avian virus may have low pathogenic potential in chickens, but cause high pathogenicity in mammalian host following adaptation.

Although cases of human infection caused by HPAI H5N1 decreased by 92% in 2016 [116] compared to 2015 (10 vs. 136 cases, respectively), human infection with novel HPAI H5N6 variants has been increasing in China since 2013 and 16 cases (including six deaths) have been recently reported as of April 2017 [13]. Due to the elevated pandemic potential of the H5Nx variants, the recently generated mouse-adapted HPAI H5Nx variants of which wild type viruses exhibit mild-to-moderate virulence in mice [35,47-49] will facilitate the evaluation of the efficacy of vaccines directed against H5Nx variants. Taken together, mouse-adaptation studies not only provide a com-

prehensive understanding of the evolution of avian influenza virus in mammalian hosts, but also can be used as an excellent tool to assess the efficacy of newly developing vaccines when new emerging viruses are introduced and thus, will play an important role in influenza research.

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