ORIGINAL ARTICLE

Development and comparison of two H5N8 influenza A vaccine candidate strains

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Abstract

Avian influenza viruses circulating in birds have caused outbreaks of infection in poultry and humans, thereby threatening public health. Recently, a highly pathogenic avian influenza (HPAI) virus (H5N8) of clade 2.3.4.4 emerged in Korea and other countries and caused multiple outbreaks in domestic and wild birds, with concerns for human infection. To combat HPAI viral infections, novel vaccines are likely to be the most effective approach. Therefore, in this study, we generated H5N8 vaccine candidate viruses based on a Korean isolate (A/broiler duck/Korea/Buan2/2014). The vaccine candidate viruses were 2:6 reassortants expressing the two surface glycoproteins of A/broiler duck/Korea/Buan2/2014 on an A/Puerto Rico/8/34 (PR8) backbone generated by using an eight-plasmid-based reverse genetics system with or without replacement of the multi-basic amino acid cleavage motif (MBCM, a crucial pathogenic factor in HPAI virus) with a bi-basic amino acid cleavage motif (BBCM) in their HA. An H5N8 vaccine candidate virus containing the BBCM showed attenuated pathogenesis in embryonated eggs and exhibited less virulence in the infected mice compared with the wild H5N8 virus containing an MBCM. Vaccination with an inactivated preparation of the vaccine candidate virus protected mice from lethal H5N8 viral challenge. This is the first report of the development and evaluation of H5N8 vaccine strains (with an MBCM or BBCM) of HA clade 2.3.4.4 as vaccine candidates. Our findings suggest that H5N8 strains with a BBCM instead of an MBCM might be considered for H5N8 vaccine seed virus development or as a reference vaccine against H5N8 viral strains.

Abbreviations

BBCM	Bi-basic amino acid cleavage motif
HA	Hemagglutinin
HAI	Hemagglutination inhibition
Hpi	Hours postinfection
MBCM	Multi-basic amino acid cleavage motif
MDCK	Madin-Darby canine kidney
MID50	50% murine infectious dose
mLD50	50% murine lethal dose
NA	Neuraminidase
PBS	Phosphate-buffered saline
PR8	A/Puerto Rico/8/34
RBC	Red blood cells
SPF	Specific-pathogen-free
TCID	Tissue culture infectious dose

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Introduction

Highly pathogenic avian influenza (HPAI) viruses of the H5N1 subtype with hemagglutinin (HA) of A/goose/Guang-dong/ 96-like (GD/96) have been continually observed in wild and domestic bird populations since their re-emergence in 2003 [1]. Outbreaks of these HPAI viruses have caused high mortality in domestic poultry, resulting in economic losses in the poultry industry and related industries [2–4]. In addition to birds and animals, these H5N1 viruses can also infect humans and have a high fatality rate of approximately 53% [5].

Since 2010, new viruses of the H5Nx subtype having neuraminidase (NA) subtypes other than N1, including

H5N2, H5N3, H5N6, and H5N8, have emerged in wild and domestic birds in China [1]. Notably, all of these H5Nx reassortants belong to the HA clade 2.3.4.4 [1]. In 2010, the reassortant HPAI H5N8 virus (A/duck/Jiangsu/k1203/2010) belonging to the HA clade 2.3.4.4 of the GD/96 lineage was isolated from swabs taken from mallard ducks in a bird market of eastern China [6]. In 2013, an H5N8 virus of the HA clade 2.3.4.4 was identified in domestic ducks through surveillance of live-poultry markets in eastern China [7], and it has spread to Europe, the western and central USA, and Canada, where it has been detected in wild and domestic birds [8, 9]. In early 2014, HPAI H5N8 viruses were detected and caused outbreaks in poultry and wild birds in South Korea [10]. Despite control measures on HPAI-H5N8-infected poultry farms with culling of several million fowl, South Korea experienced sporadic outbreaks of H5N8 in wild and domestic birds from 2014 to 2015 [11]. During 2016–2017, clade 2.3.4.4 HPAI H5N8 viruses re-emerged in wild birds and poultry in South Korea, affecting at least 76 poultry farms [12]. In January 2017, clade 2.3.4.4 HPAI H5N8 viruses were isolated from a grey heron, and phylogenetic analysis confirmed that it was a novel reassortant virus of clade 2.3.4.4 H5N8 introduced by migratory birds from Qinghai Lake and Western Siberia [13].

Wild birds (ducks, geese, and terrestrial avian species) are thought to a play crucial role as a source of influenza A viruses. Moreover, wild birds are the primary reservoir in which influenza A virus reassortment occurs and from which the viruses flow into domestic fowl [14, 15]. Eradication of these viral reservoirs in nature is impossible because many wild birds and other animals are asymptomatically infected [14]. Furthermore, reports of human infections with H5N1, H7N2, H7N3, H7N7, H9N2, and H10N7 avian influenza viruses have provided clear evidence that avian influenza A viruses can be directly transmitted to humans [16–20].

Although cases of human infection with H5N8 have not been reported, circulation of H5N8 viruses may re-occur in future winter seasons, with a potential for human or pandemic infection by adaptive mutations and/or reassortment events with human or other avian influenza virus strains [21]. Therefore, active surveillance and monitoring of HPAI H5Nx viruses, along with continuous research and development of HPAI H5Nx vaccines, are the best strategies for the prevention and control of HPAI infections and pandemics in humans.

To facilitate this approach, we generated H5N8 vaccine candidate strains based on reassortant viruses possessing the *HA* and *NA* gene segments from the H5N8 Korean isolate A/broiler duck/Korea/Buan2/2014 and a safe A/Puerto Rico/8/34 (PR8) backbone of internal gene segments [22–24]. The presence of a multi-basic amino acid cleavage motif (MBCM) in the connecting peptide between HA1 and HA2 of HA in HPAI H5 and H7 viruses is associated with embryo-lethality and high pathogenesis in animals because it increases the tissue infection spectrum of these viruses, resulting in viral replication in multiple organs and consequent systemic disease and fatality in animals [25]. This presence of an MBCM in the HAs of H5- and H7-subtype viruses distinguishes HPAI viruses from low-pathogenic avian influenza viruses [26].

In this study, we aimed to obtain safer and more suitable H5N8 vaccine candidate strains for production in embryonated eggs by replacing the MBCM of H5H8 HA with a bi-basic amino acid cleavage motif (BBCM) in two out of three H5N8 vaccine candidate strains, in addition to reassortment with PR8. Using a Korean H5N8 isolate, we assessed the growth properties of three H5N8 vaccine candidates in embryonated eggs and in Madin-Darby canine kidney (MDCK) cells. In addition, we evaluated the pathogenesis of the vaccine candidates and the Korean isolate in a mouse model. Finally, we tested vaccine efficacy using a vaccine candidate strain with a BBCM instead of an MBCM. Our results provide important insights into the development of a reference vaccine against H5N8 viral strains.

Materials and methods

Viruses and cells

H5N8 Korean isolates (A/broiler duck/Korea/Buan2/2014 and A/breeder duck/Korea/Gochang1/2014, designated as Buan-wild and Gochang-wild, respectively, in Table 1) and the PR8 and H5N8 reassortants (Table 1) were propagated in the allantoic cavities of 10- to 11-day-old embryonated chicken eggs at 37°C. At 24–48 hours postinfection (hpi), the allantoic fluid was harvested and stored at –80°C until use. A tissue culture infectious dose 50 ($TCID_{50}$) assay was performed using monolayers of MDCK cells cultured in MEM with 10% fetal bovine serum (FBS). 293T cells for transfection assays were cultured in DMEM with 10% FBS. All experiments using H5N8 viruses, including work with mice, were performed using biosafety level 3 containment procedures and facilities.

Table 1 H5N8 wild and reassortant viruses

Virus	$TCID_{50}$/mL
Buan-wild	2.63×10^7
Gochang-wild	2.45×10^6
Buan-Full	6.03×10^6
Buan-LRET	2.63×10^6
Buan-QRET	1.00×10^6

Plasmids for reverse genetics

H5N8 reassortant viruses were generated using the eight-plasmid-based reverse genetics system with pHW2000 [27, 28]. Using Buan-wild viral RNA, the *HA* and *NA* genes of H5N8 were amplified by reverse transcription (RT)-PCR and incorporated into the pHW2000 plasmid. In addition, six internal genes from the PR8 virus were amplified by RT-PCR and cloned into pHW2000. The modified *HA* genes for Buan-LRET and -QRET were amplified in two parts by RT-PCR from Buan-wild viral RNA, using primers designed to replace the MBCM with a BBCM, as indicated in Fig. 1 (sequences available upon request). The PCR products were cloned into pHW2000 using ligation reactions as described previously [28]. All cloned plasmids were sequenced to ensure the absence of unwanted mutations.

Generation of H5N8:PR8 (2:6) reassortant viruses

The H5N8 reassortant viruses were generated by transfection with plasmid DNAs as described previously [27–29].

The viruses that were generated were 6:2 reassortants consisting of six genes from PR8 in conjunction with *NA* from H5N8 Buan and either the Buan-wild HA (Buan-Full) or a modified HA (Buan-LRET and Buan-QRET). 293T cells were transfected with the eight-plasmid mixture using Lipofectamine 2000 (ThermoFisher Scientific, USA) according to the manufacturer's protocol. At 3–4 days post-transfection, culture supernatants and cell lysates were collected in the presence of 1 µg of L-1-tosylamido-2-phenylethyl chloromethyl ketone–treated trypsin (Thermo Fisher Scientific) per mL and used to inoculate specific-pathogen-free

Korea/Buan2/2014 wildtype HA (Buan-wild)
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CCT CTA AGA GAA AGA AGA AGA AAA AGA ↓ GGA CTA TTT
Pro Leu Arg Glu Arg Arg Arg Lys Arg    Gly Leu Phe
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Korea/Buan2/2014 wildtype HA (Buan-Full)
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CCT CTA AGA GAA AGA AGA AGA AAA AGA ↓ GGA CTA TTT
Pro Leu Arg Glu Arg Arg Arg Lys Arg    Gly Leu Phe
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Modified HA (Buan-LRET)
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CCT CTA CGA GAA ACG              CGA ↓ GGA CTA TTT
Pro Leu Arg Glu Thr              Arg    Gly Leu Phe
    L   R   E   T
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Modified HA (Buan-QRET)
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CCT CAA CGA GAA ACG              CGA ↓ GGA CTA TTT
Pro Gln Arg Glu Thr              Arg    Gly Leu Phe
    Q   R   E   T
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Fig. 1 Replacement of pathogenic MBCMs with nonpathogenic BBCMs in HAs of H5N8 by PCR-based mutagenesis. Residues that were altered are underlined. The arrows indicate the sites at which HA1 and HA2 are cleaved. HAs of Buan-LRET and –QRET were generated from HAs of Buan-wild by PCR mutagenesis. Buan-Full had HAs and NAs identical to those of Buan-wild and six internal proteins and genes from PR8 virus

(SPF) embryonated eggs. The harvested fluids from the first passage in eggs were injected into fresh eggs; the H5N8 reassortant viruses were identified in the allantoic fluids from the second egg passage by limiting dilution in eggs as reported previously [29].

Viral growth curve *in vitro* and viral titration

To generate viral replication curves in MDCK cells and embryonated eggs, monolayers of MDCK cells and SPF embryonated eggs were inoculated with the designated viruses at 1.45×10^{-2} TCID$_{50}$ per cell (equivalent to a multiplicity of infection [MOI] of 0.01) and 145 TCID$_{50}$ per egg (equivalent to 100 pfu per egg) at 37°C, respectively, as reported previously [30]. Supernatants were harvested at 6, 12, 18, 24, 48, and 72 hpi for viral titration in MDCK cells. Allantoic fluids were collected at 6, 12, 24, 48, and 72 hpi for viral titration in embryonated eggs. Amounts of virus in the supernatants and allantoic fluids were determined by HA assays with 0.5% turkey red blood cell suspensions (50 µL) and checked using the 50% tissue culture infectious dose (TCID$_{50}$/ml) in MDCK cells based on the method of Reed-Muench [31]. For eggs, lethality was also observed during the inoculation.

HA and HA inhibition (HAI) assays

Fifty microliters of 0.5% turkey RBC suspension in PBS was added to 50 µL of a twofold dilution of the indicated virus in PBS, and the mixture was incubated at room temperature for 30 min to 1 h. HA titers were calculated as the reciprocal value of the highest virus dilution that caused complete hemagglutination of RBCs. For HAI assays, sera from mice were collected at the indicated times after vaccination and stored at –80°C until use. HAI assays were carried out as described previously [32]. In brief, mouse sera were treated with receptor-destroying enzyme to inactivate nonspecific inhibitors, with a final serum dilution of 1:10. The enzyme-treated sera were serially diluted twofold, and an equal volume of virus (8 HA units/50 µL) was added to each well in the microplates. The microplates were incubated at room temperature for 30 min to 1 h, followed by the addition of 0.5% turkey RBCs. The mixtures in the plates were gently mixed and incubated at room temperature for 30 min. HAI titers were determined as the reciprocal of the last dilution that contained turkey RBCs with no agglutination.

Microneutralization assays

Sequential twofold dilutions of the indicated heat-inactivated mouse sera were tested in microneutralization assays to measure the highest dilutions (neutralizing titers) of the antibodies that were able to neutralize the infectivity of 100

$TCID_{50}$ of the indicated viral strains on MDCK cells in 96-well plates as described previously [33]. After 3 days of incubation at 35°C, the cells were observed for the presence of cytopathic effects, and neutralizing titers were defined as the highest dilution to retain a confluent cell monolayer. Neutralizing titers below the limit of detection (< 1:10) were assigned a value of 1:5 (Table 2).

Pathogenicity of H5N8 viruses in mice

Six- to eight-week-old female BALB/c mice (Orient Bio, South Korea) were infected intranasally with 50 µL of H5N8 wild and H5N8 reassortant viruses individually under anesthetization with Avertin. The 50% murine lethal dose (mLD_{50}) and 50% murine infectious dose (MID_{50}) were calculated by inoculating 10 mice per group with serial tenfold dilutions, from 5×10^{-1} to 5×10^4 $TCID_{50}$, of the viruses. Three days after virus inoculation, after euthanasia, the lungs of four mice from each group were collected, and viral titers were measured by $TCID_{50}$ assay to calculate the MID_{50} in MDCK cells. Body weight changes and mortality rates in the remaining six mice were monitored daily for 14 days to calculate the mLD_{50}. Mice that lost more than 20% of their initial body weight were scored as dead and were euthanized according to institutional guidelines. MID_{50} and mLD_{50} were calculated by the Reed–Muench method [31]. All animal experiments were reviewed and approved by the KCDC Institutional Animal Care and Use Committee.

Preparation of inactivated virus concentrates for vaccination

H5N8 reassortant and PR8 viruses amplified in embryonated chicken eggs were incubated at 4°C for 7 days after the addition of 4% (w/v) paraformaldehyde to each sample to obtain a final paraformaldehyde concentration of 0.025%. After 7 days, inactivated viruses were purified by ultracentrifugation at 140,000 × g in a swing rotor at 4°C for 120 min. The virus pellets were washed gently with PBS, reconcentrated by ultracentrifugation, and resuspended in an appropriate volume of PBS as reported previously, with minor modifications [29, 30]. The protein amounts in the virus concentrates were determined by the Bradford method. A complete loss of viral infectivity was confirmed by incubation of the virus concentrates in embryonated eggs.

Immunogenicity and protective effects of vaccination with inactivated H5N8 reassortant virus in mice

Six- to eight-week-old female BALB/c mice (n = 10) were vaccinated intramuscularly with 30 µg/dose (100 µL) of each vaccine (inactivated virus concentrate of Buan-LRET or PR8) with 50 µL of aluminum hydroxide adjuvant, or mock vaccinated (PBS plus aluminum hydroxide adjuvant, 100 µL per dose). Two weeks after the individual vaccinations, sera (n = 3, pooled from 10 mice per group) were collected from the mice to evaluate the immunogenicity of the Buan-LRET vaccine against H5N8 wild viruses by HAI and microneutralization assays. In addition, 21 days after two vaccinations at 2-week intervals, mice were challenged with H5N8 wild virus (100 mLD_{50} of Buan-wild or 50 mLD_{50} of Gochang-wild). Mice infected with H5N8 wild virus were monitored daily for morbidity, as assessed by measuring body weight loss and survival for up to 15 days postinfection (dpi). Mice that lost more than 20% of their initial body weight were euthanized. To determine lung viral titers in infected mice, lung tissues were collected at 3 dpi and homogenized in 1 mL of MEM containing 1% antibiotics (penicillin and streptomycin). The titers of the infectious virus in lung homogenates were estimated by $TCID_{50}$ assay.

Table 2 HAI and neutralizing (NT) titers of sera from mice immunized with the H5N8 vaccine candidate virus against H5N8 wild viruses

Immunogen	HAI antibody titers against						NT antibody titers against		
	Buan-wild[a]			Gochang-wild[b]			Buan-wild[c]		
	Pre	First	Second	Pre	First	Second	Pre	First	Second
Buan-LRET	5, 5, 5	20, 20, 20	640, 640, 640	5, 5, 5	40, 40, 40	320, 320, 320	5, 5, 5	40, 20, 40	320, 320, 640
PR8	5, 5, 5	5, 5, 5	5, 5, 5	5, 5, 5	5, 5, 5	5, 5, 5	5, 5, 5	5, 5, 5	5, 5, 5
PBS	5, 5, 5	5, 5, 5	5, 5, 5	5, 5, 5	5, 5, 5	5, 5, 5	5, 5, 5	5, 5, 5	5, 5, 5

[a]HAI antibody titers against Buan-wild in mouse serum

[b]HAI antibody titers against Gochang-wild in mouse serum

[c]Neutralizing antibody titers against Buan-wild in mouse serum

Results

Generation of H5N8 reassortant viruses

After the cloning of HAs and NAs of H5N8 wild virus and PR8 internal genes into pHW2000 plasmids, the H5N8:PR8 (2:6) reassortant viruses were successfully rescued by transfection with the plasmids, based on plasmid-based reverse genetics (Fig. 1 and Table 1), and reassortant virus clones were purified and propagated by limiting dilution in embryonated eggs, yielding virus stocks with titers of $1.00–6.03 \times 10^6$ TCID$_{50}$/mL (Table 1). Buan-wild and Buan-Full, which had MBCMs in their HAs, yielded higher TCID$_{50}$ titers in egg cultivation than did H5N8 reassortants (Buan-LRET and Buan–QRET) with BBCMs (Table 1). The identities of the reassortant viruses were confirmed by Sanger sequencing and HAI assays with ferret serum, which inhibits the hemagglutination of H5N8 wild virus and neutralizes H5N8 wild virus, as described

previously [34]. Briefly, the *HA* and *NA* sequences of the reassortant viruses were identical to those of the corresponding pHW2000 plasmids. The antigenicity of the H5N8 reassortant viruses was not significantly altered by modifications in the *HA* gene or reassortment when compared with that of the Buan-wild virus. Post-infection ferret antisera raised against the H5N8 wild viruses specifically showed HAI titers (mostly 1:40) against H5N8 wild and reassortant viruses, whereas antisera against H1N1, H5N1, and H7N9 did not elicit antigenicity against the H5N8 wild and reassortant viruses (data not shown).

In-vitro and *in-ovo* characterization of H5N8 reassortant viruses in comparison with Buan-wild virus

To characterize the growth properties of the viruses, their growth kinetics were evaluated in embryonated eggs and MDCK cells. In the eggs, four strains (PR8, Buan-wild, Buan-Full, and Buan-QRET) reached peak titers (of 2^{11},
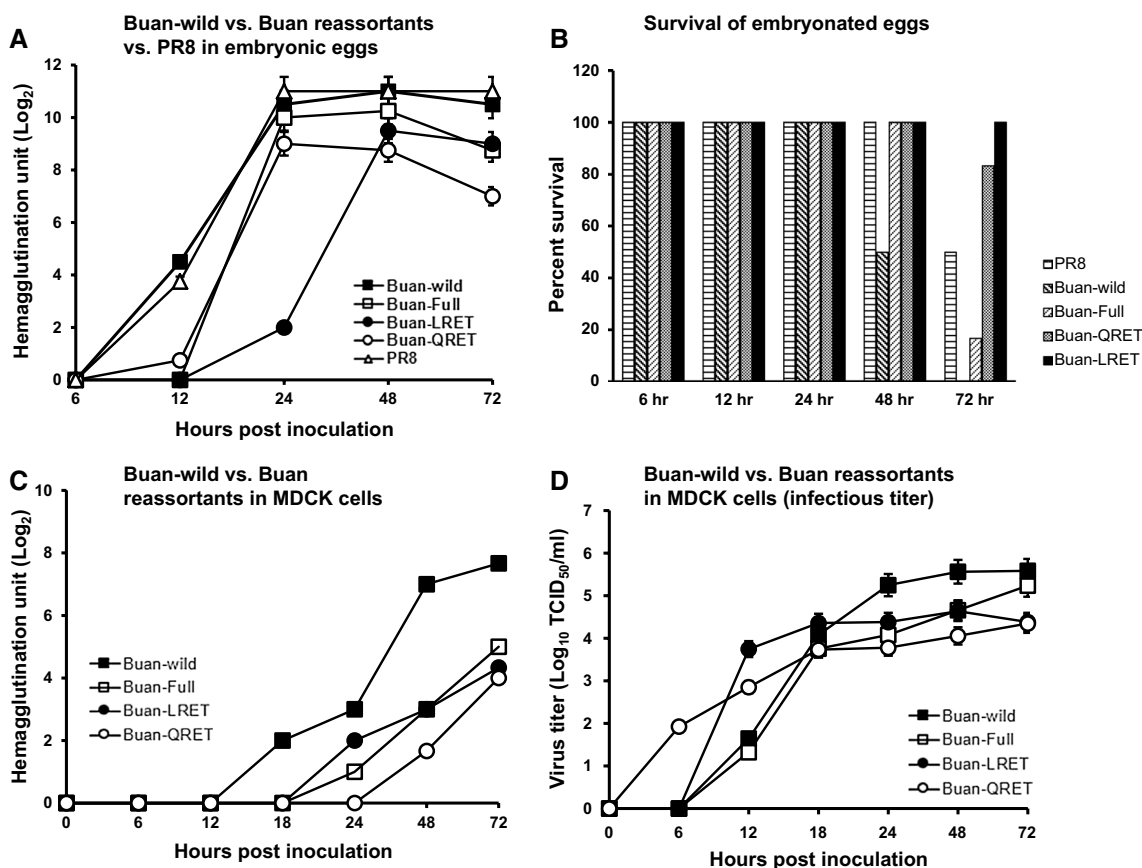

Fig. 2 Growth characteristics of Buan-wild and reassortant viruses in embryonated eggs (A) and MDCK cells (C and D). After the embryonated eggs were infected with the indicated viruses (B), the survival of the embryos was observed at 6, 12, 24, 48, and 72 hpi (n = 6 eggs per virus). HA activity in allantoic fluids of embryonated eggs or supernatants of MDCK cells was measured over time using turkey RBCs (A and C). (D) Infectious titers of the supernatants from MDCK cells were measured by TCID$_{50}$ assays. The data shown are averages, and error bars denote SEMs

$2^{10.5}$, 2^{10}, and 2^9, respectively) at 24 hpi, whereas Buan-LRET showed a peak titer (of $2^{9.5}$) at 48 hpi (Fig. 2A) instead of 24 hpi. Growth curves and HA units of Buan-wild virus were approximately equivalent to those of PR8 at all time points during egg cultivation. Buan-Full and Buan-QRET exhibited lower HA titers than Buan-wild and PR8 at 24 and 48 hpi.

During cultivation of the viruses in embryonated eggs, embryonic lethality was also observed to determine whether the removal or replacement of the MBCM affected embryo survival in embryonated eggs, which are required for the development of egg-based vaccines. At 48 hpi, Buan-wild, which has an MBCM, killed 50% of the embryos in the eggs, whereas the other viruses did not kill any embryos (Fig. 2B). At 72 hpi, Buan-wild and Buan-Full, which have MBCMs in their HAs, killed 100% and 83.3% of the egg embryos, respectively, whereas Buan-LRET and Buan-QRET, which have BBCMs in their HAs, killed 0% and 16.7% of the egg embryos, respectively (Fig. 2B). PR8 virus killed 50% of the embryos at 72 hpi (Fig. 2B). These results verified that removal of the MBCM decreases the embryonic lethality of influenza viruses. Furthermore, Buan-LRET is likely to be suitable as an H5N8 vaccine candidate strain because it did not kill egg embryos and grew well, with comparable HA titers ($2^{9.5}$) at 48 hpi in embryonated eggs.

In MDCK cells, H5N8 Buan-wild virus exhibited higher HA titers than reassortant viruses at 18–72 hpi, regardless of HA titers with turkey RBCs (Fig. 2C). In contrast to Buan-wild, Buan-LRET (BBCM) exhibited higher HA titers than Buan-Full (MBCM) and Buan-QRET (BBCM) at 24 hpi. At 72 hpi, the reassortant viruses replicated to HA titers of 2^4–2^5, approximately 3 log lower than that of Buan-wild (Fig. 2C).

Alternatively, cell-infectious titers were determined with MDCK virus cultures by $TCID_{50}$ assays. The patterns of the growth curves (Fig. 2D) on the basis of cell infectious titers were completely different from those generated on the basis of HA titers (Fig. 2C and D). At 12 hpi, Buan-LRET and Buan-QRET, which have BBCMs, exhibited the highest and second-highest titers ($10^{3.7}$ and $10^{2.8}$), respectively, when compared with the titers ($10^{1.6}$ and $10^{1.3}$) of other viruses with MBCMs in their HAs (Fig. 2D). However, viruses with MBCMs exhibited titers similar to those of viruses without MBCM at 18 hpi (Fig. 2D). Finally, Buan-wild exhibited the highest titers ($10^{5.2}$-$10^{5.6}$) at 24, 48, and 72 hpi when compared with other viruses ($10^{4.3}$-$10^{5.2}$) (Fig. 2D).

Pathogenicity of H5N8 reassortant viruses in mice

To investigate whether replacement of the MBCM with a BBCM in the HAs of H5N8 viruses reduces virus pathogenicity and replication in mice, as well as to select the least pathogenic H5N8 reassortant for production of a

safe vaccine against H5N8 viral infection, the mLD_{50} and MID_{50} of H5N8 wild and reassortant viruses were determined in a mouse model of influenza viral infection. To this end, BALB/c mice were infected intranasally with serially increasing doses of the viruses. Mice that received high doses (5×10^3 or 5×10^4 $TCID_{50}$) of Buan-wild and Buan-Full showed obvious body weight loss and high mortality (Fig. 3A, B, E, and F) accompanying clear clinical signs of influenza virus infection (ruffled fur, lethargy, and shivering). Therefore, as shown in Fig. 3A, B, E and F, H5N8 viruses (Buan-wild and Buan-Full) with MBCMs in their HAs were lethal in mice at doses of more than 5×10^3 or 5×10^4 $TCID_{50}$. Consequently, mLD_{50} values were 5×10^2 and 2.8×10^3 $TCID_{50}$, respectively. However, H5N8 reassortants (Buan-QRET and Buan-LRET) with BBCM exhibited no lethality in mice at any of the tested doses ($mLD_{50} > 5 \times 10^4$ $TCID_{50}$).

Similar to the mLD_{50} values in the infected mice, H5N8 reassortants (Buan-QRET and -LRET, having BBCMs), showed higher MID_{50} values (1.6×10^3 and 9.0×10^4 $TCID_{50}$, respectively) than H5N8 viruses (Buan-wild and -Full, having MBCMs, $MID_{50} = 1.5 \times 10^2$ and 8.5×10^2 $TCID_{50}$, respectively) (Fig. 3A–D). Collectively, these data indicated that the presence of the BBCM or removal of the MBCM in the H5N8 virus reduced viral pathogenesis and replication in mice (Fig. 3). Moreover, Buan-LRET exhibited the highest mLD_{50} and MID_{50} values in our study, implying that Buan-LRET showed the lowest pathogenicity of all of the tested H5N8 viruses (Fig. 2 and 3).

Antigenicity of and protection by vaccination with inactivated H5N8 reassortant virus as a vaccine in mice

To evaluate whether vaccination with the Buan-LRET virus elicited antibodies that could efficiently neutralize H5N8 wild viruses, mice were vaccinated with formalin-inactivated whole Buan-LRET, PR8 of H1N1 subtype, and PBS plus alum (as a negative control). Sera from each group were isolated at 2 weeks after the first and second vaccinations, and HAI and neutralizing titers of the sera were determined (Table 2). After the second vaccination with Buan-LRET, sera exhibited higher HAI and neutralizing titers (> 320 HAI and neutralizing titers) to H5N8 wild viruses than after the first vaccination, whereas sera collected from mice vaccinated with PR8 and PBS did not show any HAI or neutralizing activity toward H5N8 viruses (Table 2).

To investigate whether vaccination with the reassortant virus could protect mice from H5N8 viral challenge, mice were vaccinated with formalin-inactivated whole Buan-LRET, PR8, or PBS plus alum, challenged intranasally with 100 or 50 mLD_{50} of Buan- or Gochang-wild viruses, and were monitored for weight loss and survival for 15 days.
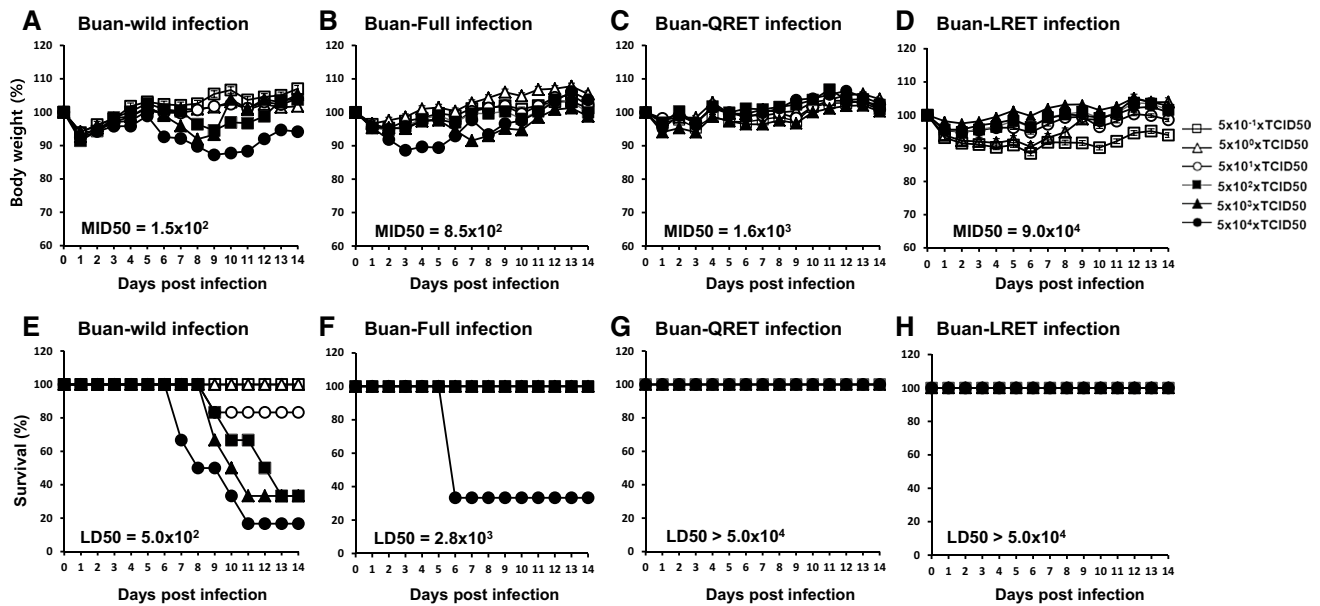
Fig. 3 Pathogenesis of Buan-wild and reassortant viruses in mice. Weight changes in mice after intranasal infection with the indicated viruses at doses ranging from 5×10^{-1} to 5×10^{4} $TCID_{50}$ per mouse (A–D). After viral infection, survival rates in mice were determined (E–H). The calculated values of mLD_{50} and MID_{50} of the viruses are indicated in the individual graphs. The data shown are averages, and error bars denote SEMs

Buan-LRET vaccination provided strong protection against lethal challenge with Buan- and Gochang-wild. In contrast, mice vaccinated with PBS plus alum succumbed to Buan and Gochang wild infections by day 7 and 5, respectively (Fig. 4B and E). Additionally, mice vaccinated with PR8 plus alum were killed at 7 and 8 dpi with Buan- and Gochang-wild viruses, respectively, although PR8 vaccination resulted in a slightly higher survival rate (1/6, 16%) following challenge with Buan-wild virus and delayed death following challenge with Gochang-wild virus, compared to mock vaccination (Fig. 4B and E). Buan-LRET-vaccinated mice showed an absence of virus in the lung at 3 dpi, whereas mice vaccinated with PBS and PR8 exhibited high lung viral titers at 3 dpi (Fig. 4C and F). These results correlated well with HAI and neutralizing titers against H5N8 viruses in vaccinated mice (Table 2).

Discussion

Since early 2014, several outbreaks caused by H5N8 viruses of novel reassortant HPAI (HA clade 2.3.4.4) have been reported in domestic and wild birds in South Korea, China, and Japan [1]. Furthermore, HPAI H5N8 viruses were detected in poultry holdings and various species of wild birds in numerous countries in Europe, North America, and Asia from 2014 to 2016 [9, 35]. Although no cases of human infection with H5N8 viruses have been reported worldwide [36] and H5N8 viruses are not transmitted efficiently in ferrets [37], the possibility of human infection with H5N8 viruses has not been excluded, and it should be noted that human infections with H5N6 viruses belonging to the HA 2.3.4.4 clade, such as H5N8, have been identified in China [38]. In addition, human population immunity against the recently detected H5Nx viruses of the HA clade 2.3.4.4 may be trivial or minimal. Thus, it is necessary to investigate whether vaccines may be effective countermeasures against viruses of this subtype or clade if they were to emerge as pandemic strains by obtaining further mutations or reassortments [21].

In this study, H5N8 vaccine candidate strains were generated by eight-plasmid-based reverse genetics [27, 39]. The H5N8 viruses used as vaccine candidates in this study expressed H5 HAs and N8 NAs of a Korean isolate of H5N8 (Buan-wild), and the remaining internal proteins and genes were derived from PR8. Viruses with the PR8 backbone are known to be generally safe for humans and birds and are not transmitted efficiently among mammals [22–24]. In some of the H5N8 viruses with the PR8 backbone in our study, the MBCM in their HA was replaced with a BBCM (Fig. 1) to yield a safer vaccine strain, because the MBCM in the HA of H5 subtype viruses, a crucial molecular marker of HPAI viruses, is an important determinant of pathogenicity in chickens and other animals [40]. Our results confirmed that the removal of the MBCM in HA of influenza H5 subtype viruses reduced viral pathogenesis in both mice and eggs. Alternatively, removal of MBCMs from HAs of vaccine strains increased
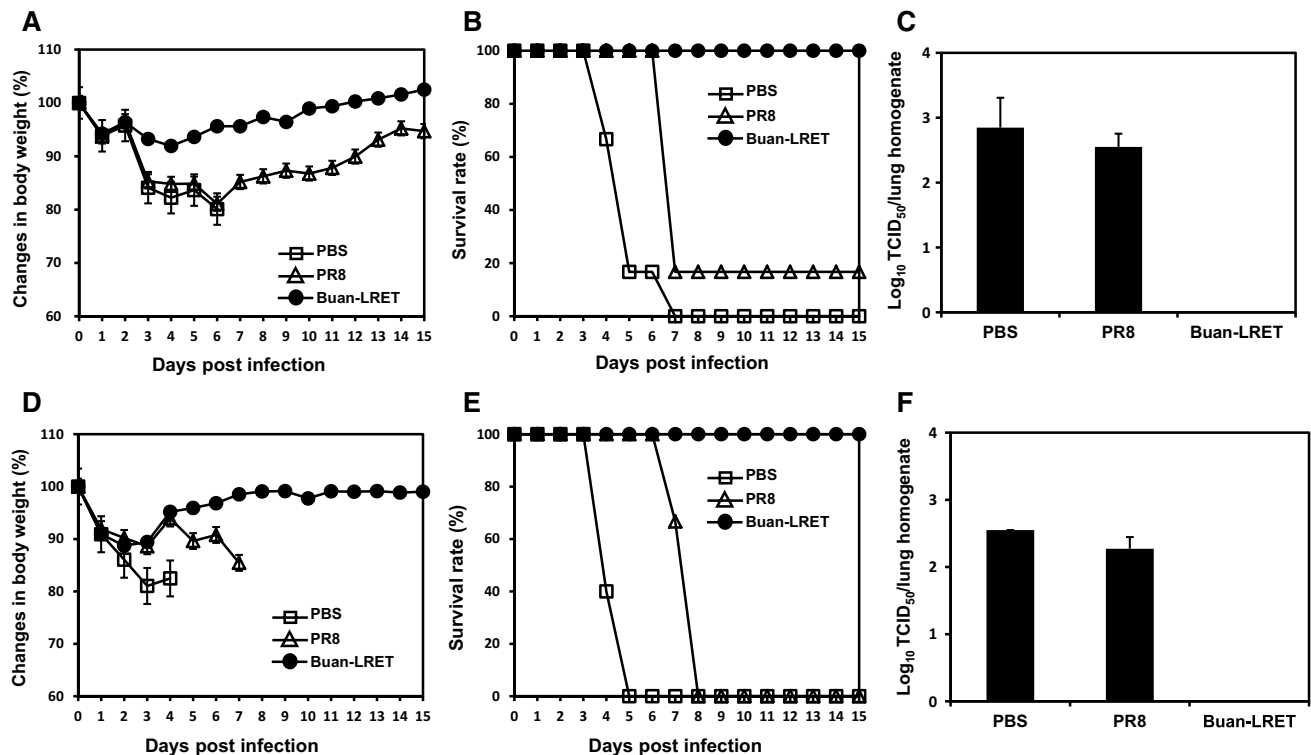
Fig. 4 Vaccination with an inactivated H5N8 virus (Buan-LRET) protects mice from lethal challenge with H5N8 wild viruses. Mice were challenged with lethal doses of live H5N8 Buan-wild (A–C) or H5N8 Gochang-wild viruses (D–F) 2 weeks after triple vaccination 2 weeks apart with inactivated Buan-LRET plus alum adjuvant. Mice were observed daily for 15 days, and body weight changes (A and D) and survival (B and E) were recorded. Lung viral titers of vaccinated animals at 3 dpi were evaluated by $TCID_{50}$ assay, and infectious titers of the animals were determined (C and F). The data shown are averages, and error bars denote SEMs

the safety of the influenza vaccine strains, consistent with previous studies [29, 41, 42].

Our study suggested that removal of the MBCM from H5 HA of clade 2.3.4.4 reduced the pathogenicity and replication efficiency of H5Nx viruses in embryonic eggs and mice, as has been shown for H5N1 viruses. Furthermore, H5N8 viruses (Buan-LRET and Buan-QRET) with BBCMs (replacing the MBCMs) exhibited different pathogenic, viral growth, and replication mechanisms in eggs and mice. This suggests that amino acid sequences in the connecting region (containing the cleavage site) between HA1 and HA2 may be important factors for determining the pathological and virological characteristics of H5Nx viruses. This also indicated that suitable and safe vaccine strains of the H5 subtype are likely to be generated by elaborate modifications of the connecting region by introducing alterations in the amino acid sequences in addition to replacement of a MBCM with the BBCM. Accordingly, the most suitable H5N8 virus, Buan-LRET, which has a BBCM and showed low pathogenicity and embryocidal activity in eggs, and low pathogenicity in mice, was selected as a vaccine candidate strain and subsequenctly used for successive vaccination of mice in our study (Fig. 4).

Following a single dose of vaccination with a formalin-inactivated concentrate of the selected vaccine candidate strain (Buan-LRET) and alum as an adjuvant, a few mice showed seroprotective HAI titers (1:40) against H5N8 wild viruses. Two-dose vaccination with the inactivated concentrates was required to elicit robust seroprotective HAI titers in mice. These results are consistent with previous reports on various H5 HA vaccines in which multiple doses and adjuvants were required to obtain seroprotective HAI titers and sufficient antibody responses against H5 HA subtype viruses in humans and animals [43–48] because H5 and H7 HA proteins intrinsically show low immunogenicity compared with H1 HA [49, 50]. Therefore, further studies are required to determine whether formalin-inactivated vaccines generated from H5N8 will be immunogenic in humans and can be used for industrial and commercial vaccine production. In addition, vaccine safety in ferrets and stability over multiple passages in embryonated eggs should be evaluated before commercialization of the H5N8 vaccine candidate strain is contemplated. Further, additional studies of the safety, immunogenicity, and dose-dependent responses of formalin-inactivated H5N8 vaccines in humans are needed for the preparation of vaccines to protect the human population

from pandemic influenza. In conclusion, the establishment of a reliable H5N8 vaccine candidate strain described in this study will be beneficial for future development of H5N8 vaccines.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval Animal experiments were authorized by the Institutional Animal Care and Use Committee of the Korea Centers for Disease Prevention and Control (approved numbers: KCDC-168-14-2A) and all experiments were performed according to the guidelines of this committee.

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