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Optimized clade 2.3.2.1c H5N1 recombinant-vaccine strains against highly pathogenic avian influenza

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A/Puerto Rico/8/34 (PR8)-derived recombinant viruses have been used for seasonal flu vaccines; however, they are insufficient for vaccines against some human-fatal H5N1 highly pathogenic avian influenza (HPAI) viruses (HPAIV) due to low productivity. Additionally, the polymerase basic 2 (PB2) protein, an important mammalian-pathogenicity determinant, of PR8 possesses several mammalian-pathogenic mutations. We previously reported two avian PB2 genes (01310 and 0028) related to efficient replication in embryonated chicken eggs (ECEs) and nonpathogenicity in BALB/c mice. In this study, we generated PR8-derived H5N1 recombinant viruses harboring hemagglutinin (attenuated) and neuraminidase genes of a clade 2.3.2.1c H5N1 HPAIV (K10-483), as well as the 01310 or 0028 PB2 genes, and investigated their replication and immunogenicity. Compared with a control virus harboring six internal PR8 genes (rK10-483), the recombinant viruses possessing the 01310 and 0028 PB2 genes showed significantly higher replication efficiency in ECEs and higher antibody titers in chickens. In contrast to rK10-483, none of the viruses replicated in BALB/c mice, and all showed low titers in Madin-Darby canine kidney cells. Additionally, the recombinant viruses did not induce a neutralization antibody but elicited decreased protective immune responses against K10-483 in mice. Thus, the highly replicative and mammalian nonpathogenic recombinant H5N1 strains might be promising vaccine candidates against HPAI in poultry.

Keywords: influenza A virus H5N1 subtype, polymerase basic 2 gene, reverse genetics, vaccines, virulence

Introduction

Influenza viruses are enveloped, segmented, single-stranded, negative-sense RNA viruses of the family *Orthomyxoviridae* and are divided into types A, B, and C. Influenza A viruses are further divided into 18 hemagglutinin (HA) and 11 neuraminidase (NA) subtypes, with wild aquatic birds serving as a reservoir of most of the known subtypes [27,38,39].

A high-growth A/Puerto Rico/8/34 (PR8) virus-based reverse-genetics system has been used for the generation of vaccine strains used in the prevention of seasonal flu; however, vaccines against infections of some H5N1 highly pathogenic avian influenza (HPAI) and pandemic H1N1 viruses have shown only 30% to 40% yields of seasonal flu vaccines [26,34]. To resolve this problem, internal PR8 genes, specifically matrix and polymerase basic 1 (PB1) genes, were replaced with other genes [1], and recently, six internal genes from high-yield PR8 were established for recombinant-vaccine generation in cell culture [28]. A mutation (E627K) was identified in H5N1 HPAI viruses (HPAIV) from human-fatal cases and demonstrated to be the key mutation in avian influenza viruses (AIV) capable of acquiring mammalian pathogenicity [8]. To date, dozens of mammalian-pathogenic mutations have been reported, with PR8 PB2 possessing E627K as well as other mutations [2,10,18,32]. Recently, we reported highly productive and nonpathogenic PB2 and nonstructural protein genes from Korean H9N2 low-pathogenic (LP) AIVs (LPAIVs) and generated an H5N1 LPAIV that was protective against lethal challenges of homologous and heterologous H5N1 viruses [13,16,17].

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To date, various clades of H5 HPAIV have been reported, with clade 2.3.2.1 still circulating in Asia [3,5,23,29]. Different clades of H5N1 vaccine strains have been developed, with > 113 billion doses used between 2002 and 2010 for H5N1 HPAI control [35,36]. The majority of inoculated vaccines were inactivated PR8-derived recombinant viruses grown in embryonated chicken eggs (ECEs) and emulsified in mineral oil adjuvant.

In this study, we generated PR8-derived H5N1 recombinant viruses harboring HA and NA genes of a clade 2.3.2.1c H5N1 HPAIV, A/mandarin duck/Korea/K10-483/2010 (K10-483), and combinations of A/chicken/Korea/KBNP-0028/2000 (0028) and A/chicken/Korea/01310/2001 (01310) PB2 genes and compared their replication efficiencies in ECEs and Madin-Darby canine kidney (MDCK) cells, as well as their pathogenicity in BALB/c mice. Further, we compared the immunogenicity and protective efficacy of the recombinant viruses in chickens and BALB/c mice.

Materials and Methods

Viruses, cells, eggs, and plasmids

The 01310 strain, which was passaged 20 times through specific pathogen-free (SPF) ECEs (VALO BioMedia, USA), was obtained from the Laboratory of Influenza Viruses at the Animal and Plant Quarantine Agency in Korea. The 01310 strain has been used as a vaccine strain to control H9N2 LPAIV outbreaks in Korea [6]. The 0028 strain is an attenuated and highly productive H9N2 LPAIV that does not induce embryonic death when incubated for 3 days in 10-day-old SPF ECEs [20]. The highly pathogenic K10-483 strain was isolated from a mandarin duck that migrated to Korea in 2010. This strain was classified into clade 2.3.2.1 together with the H5N1 HPAIV viruses that caused the fourth poultry outbreak in Korea from 2010 to 2011 [21]. The Hoffmann vector system was used to generate recombinant influenza viruses [11], which were passaged three times in 10-day-old SPF ECEs and then used for experiments. The 293T and MDCK cells were purchased from the American Type Culture Collection (USA) and maintained in Dulbecco's modified Eagle medium (Invitrogen, USA) supplemented with 5% fetal bovine serum (Invitrogen). The 293T cells were used to generate recombinant viruses through reverse genetics. All experiments with HPAIV were conducted in a biosafety level 3 facility at Konkuk University (Seoul, Korea) and approved by the Konkuk University Institute Biosafety Committee (KUIBC-2016-018).

HA gene mutagenesis

The high pathogenicity of HPAIV is determined by the proteolytic cleavage site of HA. We converted the multibasic amino acids of the cleavage site of K10-483 (RERRRKR) to monobasic amino acids of 0028 (ASGR) [30,31,33]. The ASGR fragment generated by using the ASGR primers listed in Table 1 was subsequently cloned into a T/A cloning vector (RBC Bioscience, Taiwan). The amplified fragment was sequenced by using polymerase chain reaction (PCR) primers on a 3730xl DNA analyzer (Cosmo Genetech, Korea). The sequences of the ASGR fragment were aligned and compared by using the BioEdit program (McMaster University, Canada). The HA1 and HA2 gene fragments of K10-483 without the cleavage site were amplified by using the primer sets HA-F/HA1-R and HA2-F/NS-R, respectively. The amplified HA1, ASGR, and HA2 fragments with overlapping ends were then used to generate the attenuated complete HA gene fragment [HA5(ASGR)], which was cloned into the pHW2000 vector as previous described [14]. The nucleotide sequence of

Table 1. Primers used in this study

Primer	Sequence (5′–3′)	Usage
HA-F	AGCAAAAGCAGGGG	HA1-fragment amplification/sequencing
HA1-R	GGGACATTCTCCGATAGTGA	
NS-R	AGTAGAAACAAGGGTGTT TT	HA2-fragment amplification/sequencing
HA2-F	GGACTGTTTGGAGCTATAGCA	
cmv-SF	TAAGCAGAGCTCTCTGGCTA	pHW2000-insert sequencing
bGH-SR	GGTGGCGTTTTTGGGGACA	
ASGR-F	TCCACAACATACACCCTCT	ASGR-fragment amplification/sequencing
ASGR-R	ATACCAACCATCTACCATTCCCT	
ASGR-P1	TCCACACACTACACCCTCTCACTATCGGAGAATGTCCCAAATATGTG	ASGR-fragment synthesis
ASGR-P2	TCGCAAGGACTAATTTGTTTGATTTCACATATTTGGGACATTCTCC	
ASGR-P3	AACAAATTAGTCCTTGCGACTGGGCTCAGAAATAGTCCTCAAGCAT	
ASGR-P4	ATAGCTCCAAACAGTCCCCTACCTGATGCTTGAGGACTATTTCTGA	
ASGR-P5	GGGGACTGTTTGGAGCTATAGCAGGTTTTATAGAGGGAGGATGGCA	
ASGR-P6	ATACCAACCATCTACCATTCCCTGCCATCCTCCCTCTATAAAA	

the attenuated HA gene was confirmed by sequencing with the primers cmv-SF and bGH-SR [14].

Recombinant-virus generation by reverse genetics

The pHW2000 bidirectional-transcription vector and eight plasmid vectors harboring eight PR8-genome segments were provided by St. Jude Hospital (USA), and the PR8-derived recombinant H5N1 viruses harboring HA5(ASGR) and NA of K10-483, but different PR8 PB2 genes (01310 and 0028), were generated by reverse genetics as previously described [11]. Briefly, 293T cells were cultured (1×10^6 cells/well in 6-well plates) and transfected with 300 ng of each plasmid by using Lipofectamine 2000 and Plus reagents (Invitrogen) in a final volume of 1 mL of Opti-MEM (Thermo Fisher Scientific, USA). After 3 h of incubation, 1 mL of fresh medium was added, and cells were incubated for 3 days, followed by the addition of 0.5 mg/mL trypsin (Thermo Fisher Scientific). After 12 h, the culture medium was harvested, and 200 μL of the medium were injected into 10-day-old SPF ECEs via the allantoic cavity. After incubation for 3 days, the allantoic fluid was harvested and tested via a hemagglutination assay using 1% (v/v) chicken red blood cells (RBCs) according to the World Health Organization (WHO) Manual on Animal Influenza Diagnosis and Surveillance. The genetic markers of each reassorted virus were confirmed by reverse transcription PCR and sequencing as previously described [12]. The recombinant viruses were referred to as rK10-483, rK10-483-PB2(01310), and rK10-483-PB2(0028).

Recombinant-virus titration

Each recombinant virus was serially diluted from 10^{-1} to 10^{-10} in 10-fold increments, and each dilution was inoculated into MDCK cells and five 10-day-old SPF ECEs to measure the titer of the recombinant viruses. The presence of AIVs in the allantoic fluid and MDCK culture medium was confirmed by performing the hemagglutination assay. The 50% egg infectious dose (EID_{50}/mL) and the 50% tissue-culture infectious dose ($TCID_{50}$/mL) in MDCK cells were calculated by using the Spearman-Karber method [9].

Pathogenicity and protective efficacy in BALB/c mice

Animal experiments were conducted in a biosafety level 3 facility at Konkuk University and approved by the Institutional Animal Care and Use Committee of Konkuk University (IACUC-KU16217). Pathogenicity, immunogenicity/antigenicity, and protective efficacy were assessed for the rK10-483, rK10-483-PB2(01310), and rK10-483-PB2(0028) viruses. Eleven female BALB/c mice (Orient Bio, Korea) were assigned to each experimental group. Mice were anesthetized with Zoletil (15 mg/kg; Virbac, France) and inoculated intranasally with 10^6 EID_{50}/50 μL to test for pathogenicity, immunogenicity, and protective efficacy. Negative-control (mock) mice were inoculated with the same volume of sterilized phosphate-buffered saline (PBS). Mortality and weight loss were observed every day for 14 days. For antigenicity and protection-efficacy testing, mice were challenged intranasally at 14 days post-inoculation (DPI) with K10-483, and body weight and mortality were monitored daily for 14 days post-challenge (DPC). When mouse body weight decreased by > 25% with severe clinical signs, the mouse was humanely killed by CO_2 asphyxiation. Blood was collected from the retro-orbital sinus at 0 DPI, 14 DPI, and 14 DPC.

Viral titration in the infected lungs of mice

Three mice from each group were killed at 3 and 6 DPI, and lung samples were collected. The lung samples were ground by using a Tissue Lyzer II (Qiagen, Germany) with 5 mm stainless steel beads, and 10% suspensions were prepared in PBS. After centrifugation at 2,000 × g for 10 min, the supernatants were stored at −70°C until use. The viral titers were measured as described above.

Virus neutralization tests

The virus neutralization (VN) test was performed as previously described [13,15], with slight modifications, to measure protective antibody in serum samples. Briefly, serum samples were serially diluted 2-fold in MDCK culture medium with 2 μg/mL tosyl phenylalanyl chloromethyl ketone-trypsin, and an equal volume of 200 $TCID_{50}$ of K10-483 was mixed into each diluted sample. After incubation for 1 h at 37°C, 100 μL of each mixture were inoculated onto an MDCK monolayer in a 96-well culture plate. After incubation for another hour at 37°C, the MDCK culture medium was removed and replaced with MDCK maintenance medium. After incubation in MDCK maintenance medium for 5 days, the highest dilution causing complete inhibition of cytopathic effects was recorded as the VN titer of each sample.

Immunogenicity of inactivated oil-emulsion vaccines in SPF chickens

Immunogenicity of inactivated oil-emulsion vaccines containing rK10-483, rK10-483-PB2(01310), or rK10-483-PB2(0028) viruses was tested by using SPF chickens. Harvested allantoic fluid of each virus was inactivated with binary ethylenimine, and the inactivated viruses were mixed with an oil adjuvant (ISA70) at a 3:7 (w/w) ratio and emulsified. Six SPF chickens (Namduck Sanitek, Korea) were assigned to each group, and the SPF chickens in each group were vaccinated via an intramuscular route with 1 mL of the appropriate oil-emulsion vaccine to evaluate immunogenicity and antigenicity. Negative-control (mock) chickens were inoculated with the same volume of sterilized PBS. Mortality was observed every day for 28 days, and blood was collected from the wing vein at 7 DPI, 14 DPI, 21 DPI, and 28 DPI. The hemagglutination

inhibition (HI) test was conducted according to the WHO Manual on Animal Influenza Diagnosis and Surveillance. Briefly, each collected serum sample was treated at 56°C for 30 min, followed by 2-fold sample dilution with PBS. A 25 μL aliquot of each diluted serum sample was then mixed with the same volume of 4 hemagglutinating unit rK10-483 virus and incubated at room temperature for 30 min, followed by addition of 25 μL of 1% (v/v) chicken RBCs and recording of the assay results after 30 min.

Statistical analyses

Body weight changes and viral titers were evaluated for statistical significance by using one-way analysis of variance and 95% confidence intervals statistical IBM SPSS (ver. 23.0; IBM, USA). A $p < 0.05$ was considered significant.

Results

Replication efficiency of recombinant viruses in ECEs and MDCK cells

The three reassorted H5N1 viruses, rK10-483, rK10-483-PB2(01310), and rK10-483-PB2(0028), were generated by reverse genetics, and their viral titers were measured in ECEs and MDCK cells (Table 2). The viral titers of rK10-483-PB2(01310) and rK10-483-PB2(0028) were significantly higher than that of rK10-483 in ECEs at 37°C and comparable to that of rPR8 ($p < 0.05$). Additionally, the viral titers of all H5N1 recombinant viruses exhibited steep decreases in MDCK cells; however, the EID_{50} to $TCID_{50}$ ratio for rK10-483 was less than those for rK10-483-PB2(01310) and rK10-483-PB2(0028).

Comparison of recombinant-virus pathogenicity in BALB/c mice

None of the mice inoculated with any of the H5N1 recombinant viruses exhibited mortality or loss of body weight (Fig. 1); however, mice inoculated with rPR8 showed body weight loss at 6 DPI and complete mortality at 8 DPI. The rK10-483 was re-isolated from the lungs of all inoculated mice, with viral titers of $10^{3.1}$ EID_{50}/0.1 mL and $10^{1.9}$ EID_{50}/0.1 mL at 3 DPI and 6 DPI, respectively. However, rK10-483-PB2(01310) and rK10-483-PB2(0028) were not isolated from the lungs of inoculated mice (Table 3).

Table 2. Viral titers of the PR8-derived H5N1 recombinant viruses

Virus	Viral titer	
	ECE (log_{10} EID_{50}/mL)*	MDCK (log_{10} $TCID_{50}$/mL)*
rPR8	8.95 ± 0.62	7.38 ± 0.85
rK10-483	7.70 ± 0.28†	5.25 ± 0.87†
rK10-483-PB2(01310)	9.15 ± 0.30	4.63 ± 0.63†
rK10-483-PB2(0028)	8.90 ± 0.40	5.38 ± 0.25†

ECE, embryonated chicken egg; MDCK, Madin-Darby canine kidney cell line; EID_{50}, 50% egg infectious dose; $TCID_{50}$, 50% tissue culture infectious dose. *The geometric mean of the log_{10} titer is shown with the SD. †Significant difference from the control (PR8) group value ($p < 0.05$).

Immunogenicity of recombinant viruses in SPF chickens

The HI-antibody titers of SPF chickens inoculated with rK10-483-PB2(01310) and rK10-483-PB2(0028) were higher than that of rK10-483-inoculated chickens during the observation period. Specifically, SPF chickens inoculated with rK10-483-PB2(01310) showed relatively high HI-antibody titers, significantly high at 7 DPI (3.4 ± 1.5), and reached the highest

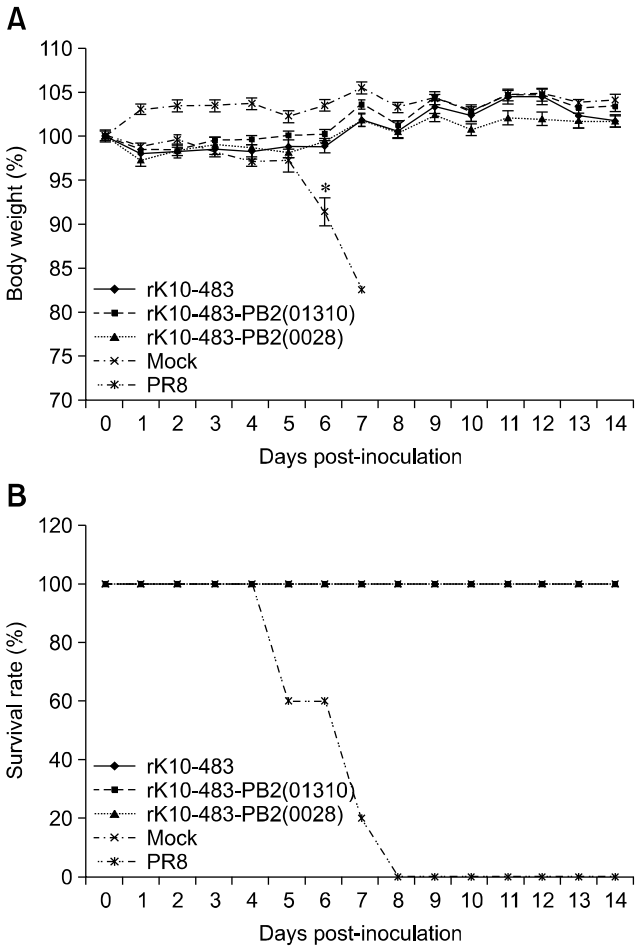

Fig. 1. Comparison of the virulence of H5N1 recombinant viruses in mice. Anesthetized BALB/c mice (5-week-old mice) were challenged with 10^6 EID_{50} of each virus (n = 5) or mock virus (n = 5; inoculated with the same volume of sterilized phosphate-buffered saline). Body weight (A) and mortality (B) were observed for 14 days. *Significant difference ($p < 0.05$) between PR8 and the other groups.

titer at 28 DPI (10.4 ± 1.5) (Table 4).

Protective efficacy of recombinant viruses against homologous H5N1 HPAIV

Serum samples of mice inoculated with rPR8, rK10-483, rK10-483-PB2(01310), rK10-483-PB2(0028), or mock (PBS) were collected at 14 DPI, and VN testing was performed. The results indicate that rPR8 and rK10-483 induced high VN-antibody titers of 64 and 160, respectively, in inoculated mice (Table 5). However, VN-antibody titers induced by rK10-483-PB2(01310), rK10-483-PB2(0028), and mock were not detected in mice. Additionally, the body weight of mice inoculated with rK10-483-PB2(01310) or rK10-483-PB2(0028) decreased significantly at 2–4 DPC and 2–7 DPC after lethal challenge with K10-483, but recovered after 4 DPC and 6 DPC, respectively (Fig. 2). By contrast, mice inoculated with rK10-483 showed no body weight loss during the observation period. Additionally, lethal challenge with K10-483 caused 100% mortality in the mock group, but 0% to 20% mortality in the rK10-483, rK10-483-PB2(01310), and rK10-483-PB2(0028) groups. Therefore, pre-inoculation with rK10-483, rK10-483-PB2(01310), or rK10-483-PB2(0028) was sufficient to protect mice from mortality following lethal challenge with rK10-483.

Discussion

Highly pathogenic H5 and H7 viruses contain multibasic amino acids at the proteolytic cleavage site of HA, with replacement of this cleavage site with monobasic amino acids resulting in decreased AIV pathogenicity [30,31,33]. The amino acids constellation at the cleavage site of 0028 (ASGR) is encoded by codons that do not harbor single point mutations resulting in translation to basic amino acids such as R or K. Therefore, we hypothesized that all recombinant H5 viruses possessing ASGR at the HA cleavage site in this study would exhibit an attenuated potential to attain increased pathogenicity and that the ASGR sequence would not affect viral replication in ECEs.

H9N2 LPAI viruses have become endemic [7,22,24,25], with the 01310 strain successfully used for inactivated-vaccine production in Korea [6]. Recent pairwise comparison with the internal genes of strains 01310 and 0028 revealed that recombinant PR8 viruses possessing the 01310 and 0028 PB2

Table 3. Replication of PR8-derived H5N1 recombinant influenza viruses in the lungs of BALB/c mice

Virus*	Virus re-isolation in mouse lung†			
	3 DPI		6 DPI	
rPR8	3/3‡	4.3§	3/3	4.1
rK10-483	3/3	3.1‖	0/3	1.9‖
rK10-483-PB2(01310)	0/3	< 0.5¶	0/3	< 0.5¶
rK10-483-PB2(0028)	0/3	< 0.5¶	0/3	< 0.5¶
Mock (PBS)	0/3	< 0.5¶	0/3	< 0.5¶

DPI, days post-inoculation; PBS, phosphate-buffered saline. *BALB/c mice (5-week-old mice) were infected via the intranasal route with $10^{6.0}$ EID_{50}/50 μL of recombinant virus. †Lung tissues were sampled at 3 DPI and 6 DPI. ‡Viruses isolated from infected mice. §Viral titers from pooled lung tissues (log_{10} EID_{50}/mL). The detection limit of the titer was < 0.5. ‖Significant difference from the control (PR8) group value ($p < 0.05$). ¶Significant difference from the control (PR8) group value ($p < 0.05$).

Table 5. Virus neutralization titers in mice infected with recombinant viruses

Virus*	Neutralization-antibody titer†
rPR8	640
rK10-483	160‡
rK10-483-PB2(01310)	< 10§
rK10-483-PB2(0028)	< 10§
Mock (PBS)	< 10§

PBS, phosphate-buffered saline. *BALB/c mice (5-week-old mice) were infected via an intranasal route with $10^{6.0}$ EID_{50}/50 μL of recombinant rK10-483 viruses, and blood samples were collected at 2 weeks post-inoculation. †The detection limit of the VN titer was < 10. ‡Significant difference from the control (PR8) group value ($p < 0.05$). §Significant difference from the control (PR8) and rK10-483 group ($p < 0.05$).

Table 4. Hemagglutination inhibition (HI) titers in chickens vaccinated with inactivated H5N1 viruses

Virus*	HI titer (log_2)†				
	0 DPI	7 DPI	14 DPI	21 DPI	28 DPI
rK10-483	0.0	0.4 ± 0.9	6.0 ± 1.7	9.0 ± 1.9	8.8 ± 1.5
rK10-483-PB2(01310)	0.0	3.4 ± 1.5‡	8.2 ± 1.3	10.2. ± 1.6	10.4 ± 1.5
rK10-483-PB2(0028)	0.0	1.6 ± 0.5	7.2 ± 1.3	10.4 ± 1.8	9.2 ± 1.7

HI, hemagglutination inhibition; DPI, days post-inoculation. *Specific pathogen-free chickens (3-week-old chickens) were vaccinated via an intramuscular route with 1 mL of inactivated H5N1 viruses. †The geometric mean of the log_2 titer is shown with the SD. ‡Significant difference from the other two groups ($p < 0.05$).

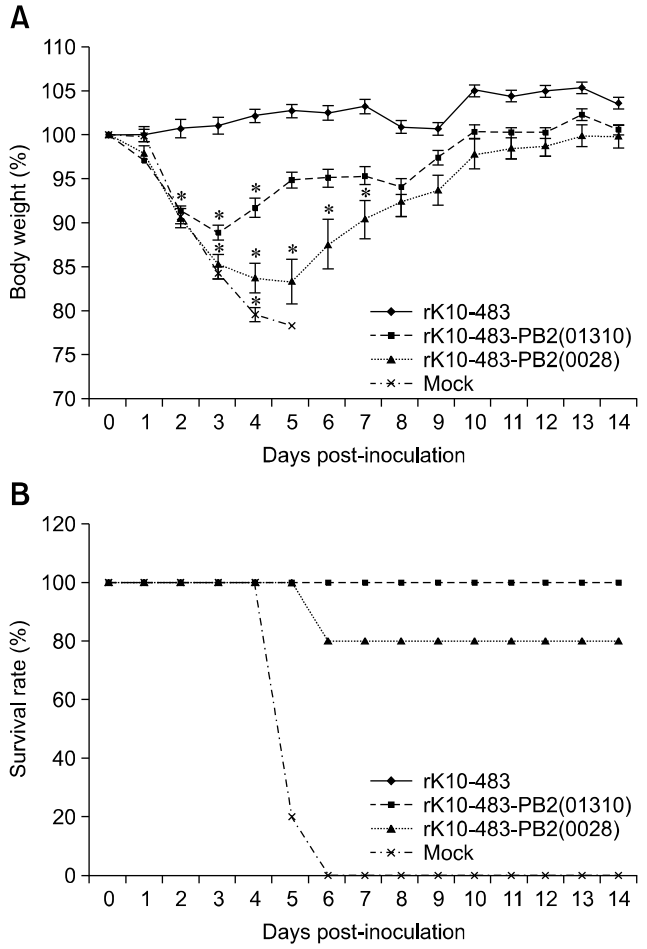

Fig. 2. Comparison of the protective efficacy of recombinant H5N1 viruses against homologous highly pathogenic avian influenza H5N1 viruses. BALB/c mice (5-week-old mice) were inoculated with each virus (n = 5) or the mock virus (n = 5). Anesthetized mice were then challenged with A/mandarin duck/Korea/K10-483/2010 (H5N1), whereas the negative-control (mock) mice were inoculated with the same volume of sterilized phosphate-buffered saline. Body weight (A) and mortality (B) were observed for 14 days. *Significant difference ($p < 0.05$) between the rK10-483 and other groups.

genes exhibited decreased viral pathogenicity in mice and increased replication efficiency in ECEs [13,17]. In the present study, inoculation with rK10-483-PB2(01310) and rK10-483-PB2(0028) resulted in significantly higher viral titers in ECEs compared with that resulting from rK10-483 inoculation. Moreover, rK10-483-PB2(01310) and rK10-483-PB2(0028) induced higher HI-antibody titers in vaccinated chickens relative to those measured following rK10-483 inoculation. The higher antibody titers induced by vaccines composed of rK10-483-PB2(01310) or rK10-483-PB2(0028) might be the result of higher antigen levels in the vaccines. Clade 2.3.2.1 viruses have evolved to several subclades, with rK10-483 classified into clade 2.3.2.1c according to the updated nomenclature for H5N1 HPAIV [40]. Most of the PR8-derived H5N1 recombinant viruses have been generated to grow to high titers; however, a clade 2.3.2.1a virus showed relatively low viral and HA titers [28]. The viral and HA titers of PR8-derived H5N1 viruses might depend upon HA clades, and low rK10-483 titers could be explained; however, different replication efficiencies might be related to HA function and/or HA and NA balance. Therefore, further study of the relationship between HA amino acid sequence and low viral titer will be valuable.

All of the H5N1 recombinant viruses used in this study exhibited lower viral titers in the mammalian cell line (MDCK) than the titers in ECEs. However, the replication efficiency associated with each recombinant virus differed in the animal experiments. Although all H5N1 recombinant viruses resulted in no mortality or body weight loss, but only rK10-483 was re-isolated from the lungs of inoculated mice at 3 DPI and 6 DPI. These results support those from the VN and homologous protection tests. Additionally, neutralization antibodies against K10-483 were only detected in mice inoculated with rK10-483, and only mice inoculated with rK10-483 were completely protected from K10-483-related body weight loss and mortality. However, the protection of mice inoculated with rK10-483-PB2(01310) and rK10-483-PB2(0028) in the absence of the VN antibody might be explained by the natural and cellular immunities induced by HA and other internal proteins, respectively [4,19,37]. Therefore, the replacement of PR8 PB2 with nonpathogenic 01310 or 0028 PB2 might be sufficient to completely attenuate the pathogenicity of reassorted recombinant viruses in mice, as has been previously reported [13]. Our results showing the ability of the 01310 and 0028 PB2 genes to generate highly productive and nonpathogenic vaccine strains constitute the successful reproduction of previous findings [13,14]

In summary, we generated PR8-derived H5N1 recombinant viruses harboring HA (attenuated) and NA genes of a clade 2.3.2.1c H5N1 HPAIV (K10-483), as well as 01310 or 0028 PB2 genes. Moreover, we investigated the replication and immunogenicity of these recombinant H5N1 viruses. Our results confirm that the 01310 and 0028 PB2 genes are capable of generating highly productive and nonpathogenic vaccine strains and that these highly replicative and nonpathogenic clade 2.3.2.1c H5N1 vaccine strains might be promising vaccine candidates against HPAI in poultry.

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Conflict of Interest

The authors declare no conflicts of interest.

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