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Characterization of a Gallid herpesvirus 2 strain with novel reticuloendotheliosis virus long terminal repeat inserts

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Abstract A bacterial artificial chromosome clone, designated LCY, was constructed from a Gallid herpesvirus 2 (GaHV-2) isolate from a GaHV-2 and reticuloendotheliosis virus co-infected clinical sample. The LCY GaHV-2 insert was sequenced and found to consist of 175,319 nucleotides. LCY GaHV-2 open reading frames (ORFs) had a high sequence identity to those of reference strains. The major difference was that two REV long terminal repeats (LTRs), in the same direction, were inserted at the internal repeat short (IRs)/unique short (Us) and Us/terminal repeat short (TRs) junctions. In addition, the *a*-like sequence and UL36 were different from other strains. Phylogenetic analysis revealed that LCY was closely related to pandemic strains in China. A pathogenicity study and a vaccination-challenge test were performed on LCY and the reference strain, GA. The results showed that LCY induced gross Marek's disease (MD) lesions and mortality in 71.4 and 7.1% of chickens, respectively, which are lower rates than those observed for the reference strain GA (85.7 and 35.7%). The commercially available CVI988 vaccine provided complete protection against LCY and GA (100%). These results showed that the isolate exhibited lower pathogenicity in SPF chickens. This study revealed that a novel pattern of LTR inserts was found in the strain LCY and that the strain was of low virulence. The present work expands the available genetic information for GaHV-2 and will be useful for the control of MD in China.

Keywords Gallid herpesvirus 2 · Reticuloendotheliosis virus · Pathogenicity · Genomic sequence · Molecular characterization

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Introduction

Marek's disease (MD) is an avian disease that is prevalent worldwide and is caused by the Gallid herpesvirus 2 (GaHV-2), a member of the *Mardivirus genus* and the *Herpesviridae* family. In previous decades, MD brought great losses to the poultry industry due to the increasing virulence of GaHV-2 [1]. This increase in virulence may be the result of greater selective pressure applied by widespread and intensive vaccination. However, in the twentieth century, the study of GaHV-2 has been hindered by a lack of genetic information. Since the first determination of the GaHV-2 genome [2], more isolates have been sequenced, adding to the available genetic information and allowing researchers to study the function of GaHV-2 genes in detail.

In recent years, an increase in GaHV-2 prevalence has been reported in China despite widespread use of vaccination. Previous reports have indicated that the GaHV-2 isolates in China might be members of a separate clade, as

suggested by the sequence of the *meq* gene [3, 4], and demonstrated at the genome level that GaHV-2 has diverged over the last two decades in China and around the world [5]. However, genetic information on GaHV-2 isolates is limited. In addition, mixed infection of GaHV-2 and reticuloendotheliosis virus (REV) is common in China [6]. Previous reports have shown that mixed infection with REV may decrease the effectiveness of MD vaccines [7]. In addition, REV long terminal repeats (LTRs) can insert into the GaHV-2 genome at different sites and result in changes to the GaHV-2 phenotype [8, 9]. The GaHV-2 strain GX0101 contains a REV LTR insert. The rate of horizontal transmission of GX0101 appears to be significantly higher than that of GX0101△LTR, indicating the potential harm of REV LTR inserts to the poultry industry [10, 11]. In this study, a GaHV-2 strain was isolated from a GaHV-2 and REV co-infected clinical sample and was cloned into a bacterial artificial chromosome (BAC). The BAC clone containing the isolated GaHV-2 genome was named LCY. Using hot spot-combined PCR (HS-cPCR) [12], we detected REV LTRs inserted into the GaHV-2 genome in a novel pattern. In addition, to better understand the biological characteristics of LCY, the complete sequence and pathogenicity of LCY were determined and can be used for comparison with other reference strains.

Materials and method

Construction of the BAC clone and reconstruction of BAC-derived GaHV-2

An isolate was obtained from a GaHV-2 and REV co-infected flock from Shandong Province in November 2011. The isolate was passaged seven times and propagated for construction of the GaHV-2 BAC clone. Total DNA was extracted using sodium dodecyl sulphate-proteinase K extraction as previously described [13]. The BAC transfer vector (pUAB-gpt) used was previously constructed [14]. The construction and identification of the BAC clone containing the GaHV-2 genome was carried out as previously reported [14]. The BAC clones (designated LCY) extracted from *Escherichia coli* were purified using the QIAGEN Large-Construct Kit (Qiagen, Hilden, Germany) and were transfected into chicken embryonic fibroblasts (CEFs) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

DNA sequencing and determination of *a*-like sequence and the LTR inserts

DNA of *E. coli.* DH10B harbouring LCY was isolated as previously reported [13]. Sequence reads and quality scores were obtained using the Illumina HiSeq 2000 platform (San Diego, CA, USA) by Beijing Genomics Institute in the following manner. Briefly, a random 500 bp library of LCY DNA was constructed. After sequencing, data filtration, and analysis, the sequences were assembled to obtain the complete sequence.

Amplification of the *a*-like sequence was performed as described by Spatz and Silva [15, 16]. The LTR inserts were examined using HS-cPCR with one of four GaHV-2 primers and one of four REV primers [12, 17]. The sequences and sites of primers are shown in the Supplementary Material 1. The direction and position of the LTR inserts can be determined through PCR using four REV primers as well as GaHV-2 primers, which are located in the unique short (Us) region of the GaHV-2 genome near the internal repeat short (IRs)/Us junction or the Us/terminal repeat short (TRs) junction, respectively (Fig. 1). The PCR products were excised from a 1.0% agarose gel, purified using a gel extraction kit (OMEGA, Guangzhou, China), and cloned into the vector pMD18-T (TaKaRa, Dalian, China). The positive clones were sequenced by the Beijing Genomics Institute (Beijing, China).

DNA sequence analysis

The complete sequence of LCY was assembled using the SeqMan program (version 7.0; DNAStar, Madison, WI, USA) and manually edited. DNA sequences were analysed using DNAStar and web-based tools and software from NCBI. Using the default settings, homology searches were conducted using the NCBI programs blastP and PsiBlast. A neighbour-joining tree was drawn using the MEGA program, version 5.1 with 1000 bootstrap replicates [18].

Experimental infections

A total of 75 1 day-old SPF White Leghorn chicks, were purchased from the Experimental Animal Center of HVRI, CAAS, were divided into five groups, each containing 15 birds. The birds in the different groups were maintained in separate isolators with negative pressure, and food and water provided ad libitum. The chicks in groups CVI988-LCY and CVI988-GA were subcutaneously vaccinated on day 1 with CVI988/Rispens at a dose of 2000 PFU in 200 µl of diluent. Seven days post-vaccination (dpv), the chicks in groups LCY, CVI988-LCY, GA, and CVI988-GA were subcutaneously inoculated intra-abdominally with a LCY- or GA-containing dose of GaHV-2 (1000 PFU) in 200 µl of diluent. Sham-challenged chicks receiving the same volume of diluent by the same route served as the control. The birds were examined daily for clinical signs of infection. Post-mortem examination was performed on all birds that died or were killed humanely at the end of the experiment, 60 days post-challenge (dpc).

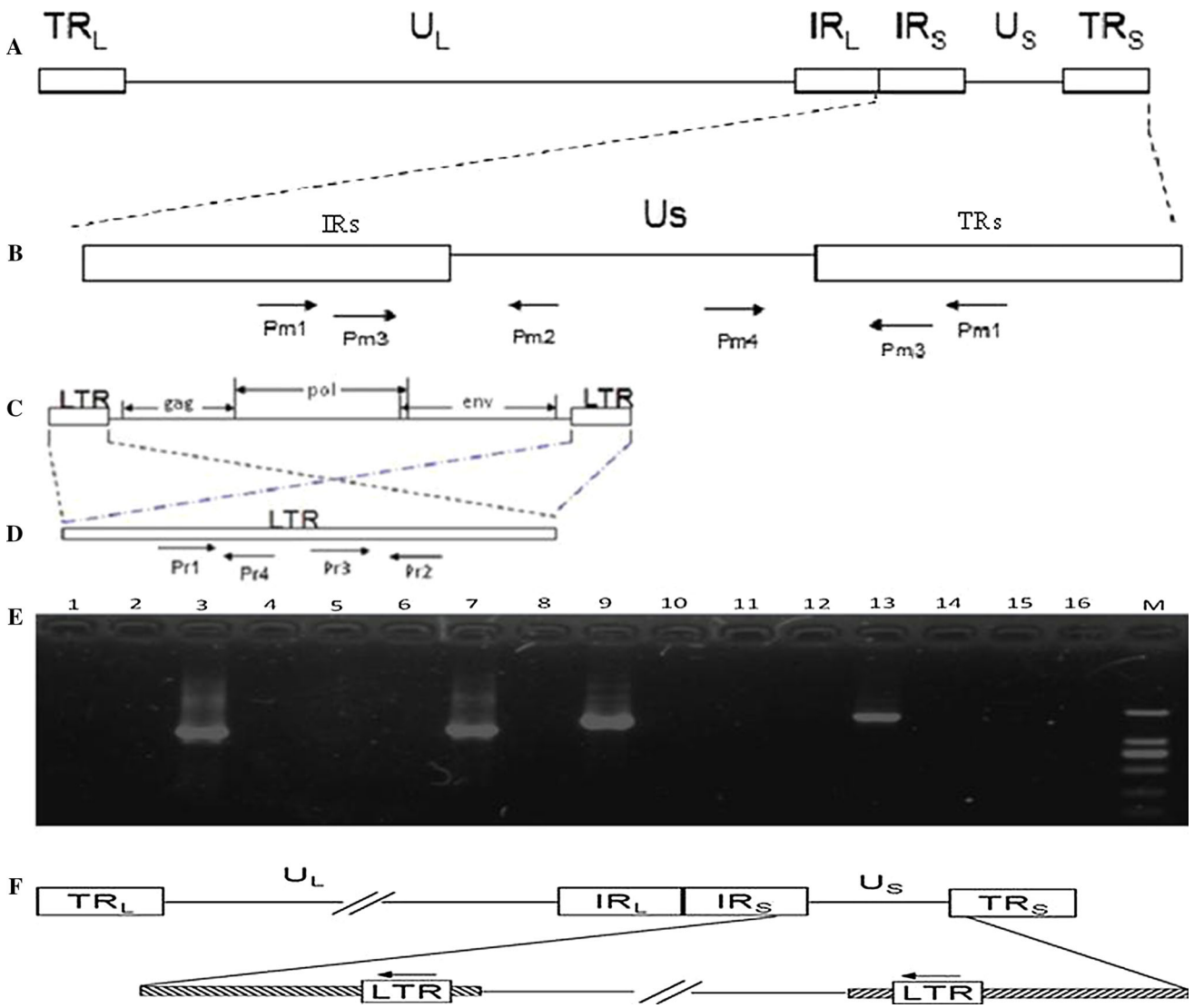

Fig. 1 REV insert position, number, and direction in the genome of the GaHV-2 strain LCY. **a** Structure of the GaHV-2 genome. **b** The sites of primers of the IRS/US and TRS/US regions of the GaHV-2 genome. **c** Structure of the REV genome. **d** The primer annealing sites in the LTR region of the REV genome. **e** Detection of REV LTR insert direction and number by PCR from uninfected CEF and LCY-infected CEF with different primers. *Lines 1* and *2* with primers M4 and R4; *Lines 3* and *4* with primers M4 and R3; *Lines 5 and 6* with primers M4 and R2; *Lines 7* and *8* with primers M4 and R1; *Lines 9* and *10* with primers M2 and R4; *Lines 11* and *12* with primers M2 and R3; *Lines 13* and *14* with primers M2 and R2; *Lines 15* and *16* with primers M2 and R1; and M DL 2000 DNA Marker. **f** Diagram of REV LTR insert position and direction in the IRs/Us and TRs/Us regions of the GaHV-2 genome

Results

Construction of the BAC clone and reconstruction of BAC-derived GaHV-2

Plaques were generated by co-transfection of chicken embryo fibroblasts (CEFs) with pUAB-gpt vector DNA and total DNA from the GaHV-2 isolate. Cells from these plaques were passaged seven times in the presence of selective medium and then the total DNA was extracted and electroporated into DH10B cells. This yielded five single chloramphenicol-resistant clones. One clone, LCY, was identified as harbouring the complete GaHV-2 genome. DNA from LCY was extracted and transfected into CEFs. Four days post-transfection, GaHV-2 specific plaques were produced, which were similar to those formed by the wild-type virus, proving the infectious ability of the LCY.

Genome organization and sequence characterization

The sequence of LCY was submitted to GenBank (KX290013) and was 182,684 bp long, including the BAC vector backbone. The inserted GaHV-2 genome was

175,319 bp. The unique long (U_L) region was 113,542 bp in length and the U_S region was found to be 11,161 bp in length. Both terminal repeat long (TR_L) and internal repeat long (IR_L) were 12,727 bp in length. However, the IRs and TRs differed in length, at 12,324 and 12,349 bp, respectively. The *a*-like sequence was 489 bp in length.

ORFs and single-nucleotide polymorphisms (SNPs) of the LCY genome were analysed and compared with those of GaHV-2 reference strains CVI988, Md5, RB-1B, LMS, and GX0101 [3, 19–21]. The Chinese strains, LCY, LMS, and GX0101 differed from Md5, RB-1B, and CVI988 by several non-synonymous mutations (Supplementary Material 2). The majority of LCY ORFs are most closely related to GX0101, which is a recombinant *GaHV-2* field strain with one REV LTR insert (Supplementary Material 3).

The LTR inserts in LCY in DH10B cells were verified by HS-cPCR (Fig. 1). Interestingly, two REV LTR inserts were found to be located at the IRs/Us junction and Us/TRs junctions of the GaHV-2 genome of LCY, which is consistent with recombination hot spots that introduce LTRs into the GaHV-2 genome [22]. Specifically, in LCY, one insert of 533 bp was found at the IRs/Us junction and was 2116 nt upstream of ICP4. Another insert of 533 bp was located 763 nt downstream of the Us/TRs boundary. Both of these LTRs were inserted in the same direction, which is a novel pattern (Fig. 1).

The *a*-like sequence, which contributes to the cleavage and packaging of viral DNA concatemers, varies among GaHV-2 strains [23]. The *a*-like sequence of LCY is 489 bp in length and is bracketed by DR1 sequences (GGCCGCGAGAGG). It contains a pac-1 site (GGGGGGGGGTGAAAATTTGGGGGG) and a pac-2 site (TTTTTTTTT ATACAGTGTGT). Short telomeric repeats (sTMR) with six repeats $(GGGTTA)_6$ are located between the DR1 and the pac-1 sites. Between the pac-1 and pac-2 sites, multiple telomeric repeats (mTMR) with nine repeats $(GGGTTA)_9$ are interspersed between islands of 13 bp repeats $(GGGTTCAGGCCTA)_4$.

The carboxyl terminus of UL36 is a variable region. Amino acid sequence alignment showed that the carboxyl terminus of LCY harbours four continuous repeat motifs (PTPAPK). In other strains, there are two or three repeats, but Md5 contains seven repeats.

Genomic phylogenetic relationship

A phylogenetic tree was constructed to show the relationship between LCY and the 20 GaHV-2 reference strains contained in GenBank database (Fig. 2). The results showed that LCY was most similar to GX0101, which was isolated in China. The pandemic strains isolated in China belong to one small clade, indicating that these strains may have evolved independently in China. In addition, consistent with a previous report [24], LCY and other new Chinese isolates show divergence from the previously isolated strains J-1 and 814, indicating recent evolution of GaHV-2.

Virulence studies

To test the pathogenicity of LCY, a pathogenicity study and a vaccination-challenge test were performed on LCY and a GaHV-2 reference strain, GA. The mortality and morbidity analysis included 73 chickens. Four weeks post-challenge, some diseased chickens in the challenged groups began to show clinical signs of disease, such as small size, depression, and drooping wings. Some of the remaining birds have developed MD (tumours predominantly in the liver) during the observation period. The overall mortality and morbidity rates are summarized in Table 1. The results show that the LCY strain induced gross MD lesions and mortality in 71.4 and 7.1% of SPF chickens, respectively, which are lower rates than those obtained for the GA strain (85.7 and 35.7%, respectively). None of the vaccinated chickens in the challenge or control group died or developed MD.

These results indicate that the virulence of LCY is lower than that of the reference strain. The CVI988 vaccine provides complete protection against it.

Discussion

Over the last several decades, GaHV-2 strains worldwide have diverged at the genomic level, and strains isolated in China after 2000 were closer related than previously isolated strains at both the gene and genome levels [5]. LCY and other strains that were isolated after 2000 form a distinct clade, and all of the new Chinese isolates are closely related. LCY has mutations at putatively functional loci that are characteristic of Chinese isolates, indicating that LCY is representative of Chinese isolates at the genomic level.

GaHV-2 and REV are two of the most widespread and persistent viral infections in chickens, and both induce T cell lymphomas. Many cases of co-infections with GaHV-2 and REV have been reported. It has been documented that the LTR of REV can integrate into the GaHV-2 genome after in vitro co-culture or in vivo co-infection [9, 17]. In this study, after four passages of the isolate, the REV LTR was shown to be inserted into the GaHV-2 genome using HS-cPCR. The pattern of insertion reminded us of RM1 in that during passaging in vitro, the LTR underwent duplication at an identical site in the TRs, presumably as a result of recombination between the IRs and TRs [25]. However, in contrast to RM1, both of the LTRs in LCY were in the same direction and were at a distance of 153 bp from the

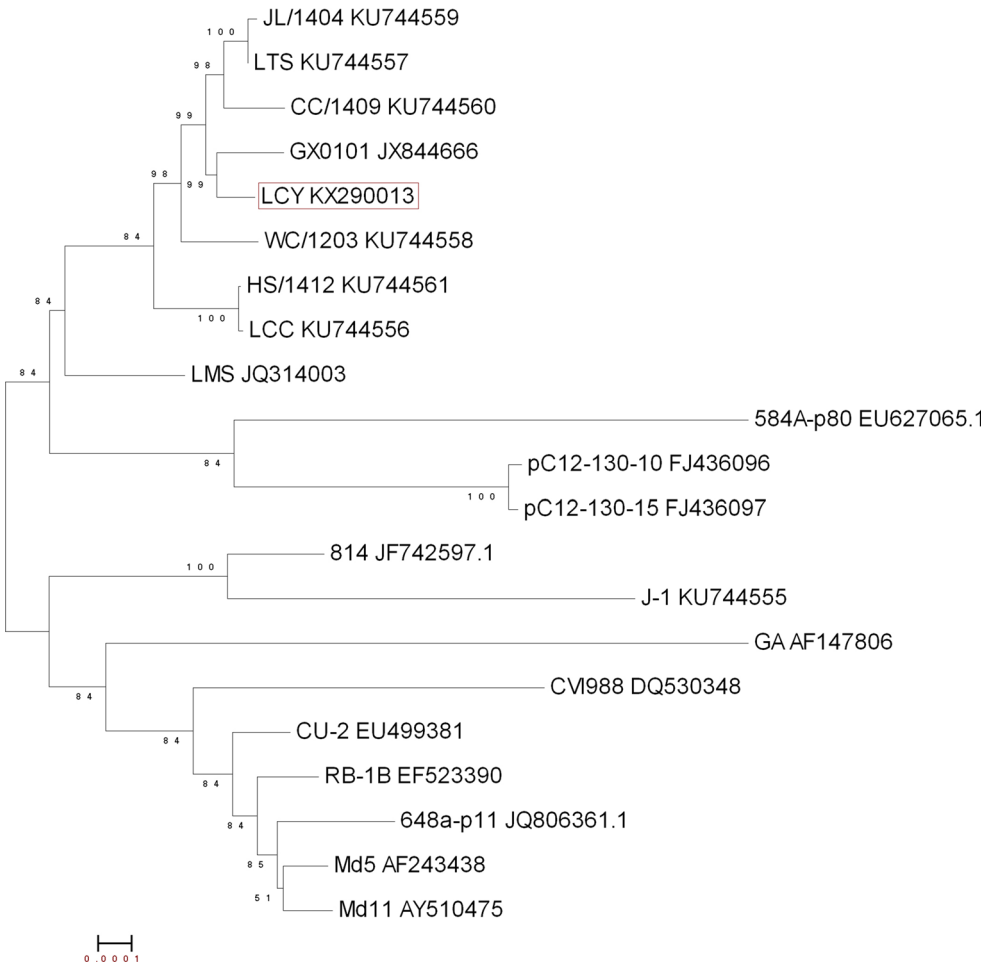

Fig. 2 Phylogenetic tree of the *meq* nucleotide sequences of GaHV-2 isolates and GaHV-2 reference strains

Table 1 Morbidity and Mortality at 60 days post-challenge in the various treatment groups

Groups	*N*	Morbility and mortality						PI
		Diseased cases (*n*)	Morbility (%)	Tumour cases (*n*)	Tumour incidence (%)	Dead cases (*n*)	Mortality (%)	
LCY	14	10	71.4	4	28.6	1	7.1	
CVI988-LCY	15	0	0	0	0	0	0	100
GA	14	12	85.7	12	85.7	5	35.7	
CVI988-GA	15	0	0	0	0	0	0	100
Control	15	0	0	0	0	0	0	

N number of chicks, *PI* protective index

corresponding site, indicating that the two LTRs inserted into the IR_S/U_S and U_S/TR_S sites. It is unclear if the two inserts were the result of independent recombination events or if some other mechanism was at play. However, it is certain that the LTR inserts induced the recombinant virus at a rate higher than that observed in the parent virus and promoted survival after serial passaging. In addition, the promoter character and enhancer activity of an LTR insert might activate nearby genes, including ICP4, even at distance of approximately 2 kb. As an immediately early (IE) gene, ICP4 is a major transcriptional regulator and is also a structural component of the GaHV-2 virion. Therefore, it is possible that the promoter activity of the LTR on the ICP4 gene induced the recombinant virus to be more proliferative. In addition, antisense transcripts of ICP4 were detected in latently infected cells and tumour cells [26, 27]. Therefore, the promoter activity of LTRs might affect the balance between ICP4 sense and antisense transcripts.

In our previous studies, we obtained a REV isolate, CY1111, from a co-infection with GaHV-2 and REV and

demonstrated that phylogenetically, based on the complete genome sequences of REV strains, CY1111 was most closely related to REV strains that were isolated from a variety of hosts [28]. Little variation may have taken place in the evolutionary process. Many reports have described the existence of REVs. It has been reported that REV infection alone, thus far, has not caused significant losses to the poultry industry [28–30]. In this study, a GaHV-2 isolate, LCY, was obtained from a co-infection isolate, and its complete genomic sequence was determined. A phylogenetic tree of GaHV-2 strains showed that pandemic strains in China are in one clade and that 53 SNPs within 26 genes are unique to Chinese isolates and absent from non-Chinese isolates. This indicates the possibility that pandemic strains evolved independently in China.

An unrooted phylogenetic tree was generated by the distance-based, neighbour-joining method using MEGA 5.1 software. The reliability of the tree was assessed by bootstrap analysis with 1000 replications. New isolates are indicated by open triangles.

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Author contributions C.J.L. and X.M.W. conceived and designed the study. Y.P.Z. and K.Y.B. carried out the experiments and wrote the manuscript. G.R.S. and H.C.L. analysed the data. H.Y.C. and Y.L.G. carried out the virulence studies. All authors read, revised, and approved the final manuscript.

Compliance with ethical standards

Conflicts of interest The authors declare that they have no competing interests.

Ethical approval The animal experiments were approved by the Animal Ethics Committee of the Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Sciences (CAAS) and were performed in accordance with animal ethics guidelines and approved protocols. The Animal Ethics Committee approval number was SYXK (hei) 2011-022.

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