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# Attenuation of Marek's disease virus by codon pair deoptimization of a core gene

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ABSTRACT

Marek's disease virus (MDV) is an oncogenic alphaherpesvirus of *Gallus gallus*, the domesticated chicken. Control strategies rely upon vaccination with live attenuated viruses of antigenically similar avian herpesviruses or attenuated strains of MDV. Recent studies in other viruses have shown that recoding certain viral genes to employ synonymous but rarely-used codon pairs resulted in viral attenuation. We deoptimized two MDV proteins, UL54/ICP27 and UL49/VP22, and demonstrate that the more severely deoptimized variant of UL54 accumulates significantly less gene product *in vitro*. Using these UL54 deoptimized mutants, we further demonstrate that animals infected with the UL54-recoded recombinant virus exhibited decreased viral genome copy number in lymphocytes, reduced lymphoid atrophy and reduced tumor incidence. This study demonstrates that codon pair deoptimization of a single viral gene can produce attenuated strains of MDV. This approach may be useful as a rational way of making novel live attenuated virus vaccines for MDV.

## 1. Introduction

Marek's disease virus is an alphaherpesvirus and the type species of the genus *Mardivirus* (Marek's disease-like viruses). Historically the *Mardiviruses* were divided into serotypes 1–3, of which only serotype 1 is pathogenic in the domesticated chicken. It has been designated Gallid alphaherpesvirus 2 (GaHV2) in the 2016 release of Virus Taxonomy provided by the International Committee on Taxonomy of Viruses. In *Gallus gallus*, pathogenic strains of MDV cause a disease characterized by peripheral nerve enlargement due to leukocyte infiltration with concomitant paralysis, atrophy of the thymus and bursa with concomitant immunosuppression, and visceral tumor formation with a high mortality rate in susceptible birds (Biggs, 1975). Initial exposure to MDV probably occurs through inhalation of shed desquamated infected cells of the feather follicle epithelium, which are then engulfed by alveolar macrophages and/or dendritic cells of the new host (Addinger and Calnek, 1973). Thereafter MDV targets B cells and ultimately CD4+ ($CD3^+/CD4^+/CD8^-$ single-positive) T cells which are transformed and produce visceral tumors, although other T cell subsets may also be involved (Okada et al., 1997; Schat et al., 1991).

Marek's disease is ubiquitous in commercial flocks globally, necessitating a strategy of comprehensive vaccination *in ovo*. Because MDV is highly cell-associated (Biggs et al., 1968), vaccine strategies which rely upon eliciting antibodies against viral envelope glycoproteins are ineffective and live attenuated virus delivered in cryopreserved infected cells are the best available vaccination vector. Currently the most protective vaccine regimen consists of bi- or tri-valent preparations of the GaHV2 strain CVI988 ("Rispens") combined with herpesvirus of turkeys (HVT), with or without the SB-1 strain of GaHV3 (Gimeno, 2008). The CVI988 strain was created by serial passage of a low-virulence strain of MDV *in vitro* (Rispens et al., 1972), a non-rational method of attenuating live viruses for vaccine use which is still in use today (Lee et al., 2012). Because of the emergence of new and more pathogenic strains of MDV (Witter, 1997) and the likely future emergence of even more virulent strains of (Schat et al., 1982; Nair, 2005; Woźniakowski and Samorek-Salamonowicz, 2014), new vaccines are needed to ensure the continued protection of commercial flocks.

To date all organisms investigated from bacteria to vertebrate animals display a codon usage bias in their protein-encoding genes, in that certain codons are used more frequently than other synonymous codons (Sharp et al., 1988; Bennetzen and Hall, 1982; Ikemura, 1985). The same is true for codon pair usage bias (Moura et al., 2011; Chevance et al., 2014). The codon pair bias of a given gene can be altered to "fine tune" the rate of protein expression from that gene (Baker et al., 2015; Chevance et al., 2014; Quax et al., 2015) by optimization or deoptimization of its codon pair context. When applied to viral genes for the

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purpose of viral attenuation this practice is known as SAVE (synthetic attenuated virus engineering) (Ni et al., 2014). SAVE is a relatively new approach to the attenuation of wild type viruses for the creation of live attenuated vaccinal strains. The SAVE approach has been used successfully to attenuate a variety of viruses, including poliovirus (Coleman et al., 2008), respiratory syncytial virus (Nouën et al., 2014; Meng et al., 2014), influenza A virus (Nogales et al., 2014; Mueller et al., 2006), Chikungunya virus (Nougairede et al., 2013), papillomavirus (Cladel et al., 2013), adenovirus (Villanueva et al., 2016) and others (reviewed Mueller et al., 2010; Martínez et al., 2016). Although most of these viruses are RNA viruses, the papillomaviruses and the adenoviruses are small dsDNA viruses.

The mechanism(s) by which the SAVE approach ultimately results in altered gene expression of a particular viral gene and ultimately attenuation of viral replication is poorly understood and is still a matter for debate (Quax et al., 2015; Futcher et al., 2015; Plotkin and Kudla, 2010). Alteration of a gene's codon pair bias of necessity results in alteration of other related properties of the gene (*e.g.*, CpG incidence, see Tulloch et al., 2014), but how the effect is produced is currently unclear. Codon usage changes probably impact gene expression during both transcription and translation, at multiple levels and through multiple mechanisms (reviewed in Quax et al., 2015). When a wild type viral gene is replaced with a recoded gene (optimized or deoptimized) the effect upon viral replication in the resulting mutant virus is difficult to predict, and may be counter intuitive (*e.g.*, see Villanueva et al., 2016). In the absence of an accepted theoretical framework to explain how codon (or codon pair) recoding exerts its effect(s), the ultimate effect upon viral replication of a single recoded gene must be determined empirically.

We hypothesized that codon pair deoptimization of a single MDV gene might be sufficient to produce an attenuated virus. We used the GaHV2 isolate Md5 (Witter et al., 1980) as our base virus and targeted two genes for codon pair deoptimization: UL54/MDV068, which encodes the ICP27 protein (Larralde et al., 2006), and UL49/MDV062, which encodes the tegument glycoprotein VP22 (Trapp-Fragnet et al., 2014). Both of these genes encode proteins essential for viral replication, are well-characterized, discrete (*i.e.*, their ORFs do not overlap with other MDV genes in either direction) and have orthologues across the subfamily *Alphaherpesvirinae* (Amor et al., 2011; Blondeau et al., 2008). We first investigated the effect of deoptimization upon UL49 and UL54 expression *in vitro* using a novel dual-expression reporter system. Successfully deoptimized genes would produce less gene product *in vitro*. We then made MDV mutants harboring the successfully deoptimized genes and carried out *in vivo* experiments to determine the degree of attenuation, if any, upon viral pathogenicity in the resulting mutant viruses.

## 2. Materials and methods

### 2.1. Cell culture, animals and animal care

Titration of virus stocks was done on monolayers of secondary duck embryonic fibroblasts derived from 9-day specific pathogen free Khaki-Campbell ducks and maintained in LM medium, a custom medium composed of equal parts L-15 Leibovitz medium (catalog L4386–10 L, SIGMA, Saint Louis, MO, USA) and McCoy's 5 A medium (catalog M4892–10 L, SIGMA) supplemented with 4% FBS. Line $15I_5x7_1$ white leghorn chickens ($F_1$ hybrid progeny of susceptible line $15I_5$ males and line $7_1$ females) were used in all experiments involving single-gene deoptimized viruses and were housed Horsfall-Bauer isolators maintained at negative pressure. The breeding flock was routinely monitored for antibodies against MDV, avian leukosis virus and reticuloendotheliosis virus and was negative for these viruses during the period of these experiments. All experiments were approved by the Avian Disease and Oncology Laboratory Institutional Animal Care and Use Committee (IACUC).

The *in vitro* assessment of codon pair deoptimized gene expression was done in DF-1 cells, which were assayed for mycoplasma contamination (catalog G238, abm) before the commencement of the studies described herein and found to be negative.

### 2.2. Viruses and BAC manipulation

The BAC clone (GenBank sequence ID HQ149525.1) used for the genetic engineering and reconstitution of the codon pair deoptimized mutant viruses was the rMd5B40 BAC, a very virulent (vv) MDV cloned strain (Niikura et al., 2011). For the deoptimized genes, unique restriction sites introduced into each recoded gene were used to insert the kanamycin resistance gene (*nptII* from plasmid pEPkan-S2) with 50–60 bp flanking regions homologous to the viral gene destined for replacement. The entire recoded gene, now interrupted by the kanR/*nptII* insert, was amplified using Phusion High Fidelity PCR Master Mix with GC Buffer (catalog M0532S, New England Biolabs) using primers with flanking regions homologous to the MDV wild type genes destined for replacement. The recombinant gene was inserted into the rMd5B40 BAC using two-step red-mediated recombination as previously described (Tischer et al., 2006). The resulting rMd5B40-based constructs with a single deoptimized gene were transfected into DEF cells for viral reconstitution, amplification, recovery and titration to create viral stock solutions.

### 2.3. Deoptimization of UL49 and UL54

To deoptimize the MDV genes used in this study it was first necessary to determine the codon pair bias of the host. We used NCBI RefSeq release 41 as a source of sequence for this study. The sequences of 18,595 non-redundant chicken genes containing a total of 8,661,076 codon pairs were parsed into 3721 bins consisting of all possible codon pair combinations (excluding STOP codons). The codon pair score (CPS) for each possible codon combination in the gene set was calculated using the method of Coleman *et al.*, (Coleman et al., 2008). Briefly, the CPS for each possible in-frame codon pair was calculated as the natural logarithm of the number of observed codon pairs (*Obs*) divided by the expected number of codon pairs (*E*) within the subset of genes analyzed (equation a, below).

$$\ln(Obs/E)$$

The unbiased expected number of occurrences of each codon pair was calculated taking into account amino acid frequency differences (equation b, below).

$$E = \left(\frac{C1 \times C2}{A1 \times A2}\right) \times A1A2$$

where C1 and C2 code for the amino acids A1 and A2 and are the number of times C1 and C2 occur in the subset of genes analyzed. A1 and A2 are the number of times amino acids 1 and 2 appear in the dataset, and A1A2 is the number of times this pairing occurs. Using the CPS calculated for the genes of the chicken genome we were able to calculate each gene's codon pair bias score (CPB score, again after Coleman et al., 2008). The CPB score of a gene is therefore the arithmetic mean of the CPS of all codon pairs within the gene (equation c, below)

$$CPB = \frac{1}{n}\sum_{i=1}^{n} CPS_i$$

where $CPS_i$ is the calculated codon pair score for the $i_{th}$ codon pair in a gene and $n$ is the number of codon pairs in the gene. Using this methodology we calculated the CPB score for each of the 86 genes present in MDV strain Md5 and used an algorithm to replace frequently used codon pairs with infrequently used (but synonymous) codon pairs. MDV UL49 and UL54 genes were deoptimized by computer algorithm,