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Attenuate Newcastle disease virus by codon modification of the glycoproteins and phosphoprotein genes

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ABSTRACT

A codon modification strategy was used to attenuate the avian pathogenicity of an oncolytic mesogenic Newcastle disease virus (NDV) by targeting the three major virulence factors: the fusion (F) protein, hemagglutinin neuraminidase (HN) and phosphoprotein (P). Recoding the F and HN genes with rare codons greatly reduced expression of both F and HN proteins and resulted in their low incorporation into virions. The F and HN recoded virus was partially attenuated in chickens even when the F protein cleavage site was modified. Full attenuation was achieved when the 5′ portion of the P gene was recoded. The recoded P, F and HN triple gene mutant exhibited delayed cell death in human cancer cells with prolonged expression of a GFP transgene. While this engineered attenuated NDV strain has lower oncolytic potency, its capacity for prolonged transgene expression may allow its use as a vaccine or gene delivery vector.

1. Introduction

Newcastle Disease Virus (NDV) is a potent oncolytic virus that has been shown to be safe and exhibits activity as a cancer therapy in clinical studies (Lam et al., 2011; Wei et al., 2012; Zamarin and Palese, 2012). NDV mediates oncolysis of cancer cells through direct cell killing and indirectly by activating macrophages and NK cells, and generation of adaptive immune responses against cancer antigens. Based on its chicken virulence, NDV is classified as lentogenic (nonvirulent), mesogenic NDV (intermediate virulent) and velogenic NDV (highly virulent). In addition to velogenic NDV, mesogenic NDV was reclassified as a select agent in 2008, precluding its development as an oncolytic agent or as a vaccine vector.

NDV is a negative stranded RNA virus in the family *Paramyxoviridae*. Its genome consists of six transcription units encoding nucleoprotein (NP), phosphoprotein (P, and V/W via gene editing), matrix (M), fusion (F), hemagglutinin neuraminidase (HN) and large polymerase (L) proteins (Chang et al., 2001). The NP, P and L proteins constitute the viral RNA polymerase, while F and HN proteins are integral membrane proteins involved in viral entry and egress. The F protein is synthesized as a F0 precursor that is cleaved into F1 and F2 subunits by a cellular proteinase required for its fusion activity to mediate viral entry into cells. The F protein is a key virulence factor (Peeters et al., 1999). Both mesogenic and velogenic NDV strains have multiple basic amino acids at the F protein cleavage site that can be cleaved efficiently in infected cells, while lentogenic NDV with a single basic amino acid at its F0 cleavage site is not cleaved. In addition to the F protein, the V protein translated from the P gene via gene editing is an interferon antagonist, which is also an important virulence factor (Steward et al., 1993).

In addition to its application as an oncolytic virus, NDV has been explored as a vaccine vector (Kim et al., 2014; Xiao et al., 2011). Although both lentogenic and mesogenic strains have been evaluated preclinically as veterinary or human vaccine vectors, the mesogenic NDV such as the Beaudette C (BC) strain appeared to be more effective than the lentogenic LaSota strain. The modified BC vector expressing the HA of a highly pathogenic influenza H5N1 virus induced protective immunity in chickens (Kim et al., 2014). In order to use a mesogenic NDV as an effective vector for gene delivery or as an oncolytic agent, several methods have been used to genetically modify the NDV genome to reduce its virulence in birds, such as modification of the F, HN and P genes (Hao et al., 2016; Heiden et al., 2014; Huang et al., 2003; Kim et al., 2014). Previously, we generated a candidate oncolytic NDV based on a mesogenic NDV 73T strain by modifying the F protein cleavage site and inserting a 198-nucleotide sequence into the HN-L intergenic region, which led to reduced avian pathogenicity without compromising its oncolytic potency (Cheng et al., 2016).

Codon modification has been shown to be a successful way of

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attenuating a number of RNA viruses, such as poliovirus, influenza A virus, vesicular stomatitis virus, and respiratory syncytial virus (Broadbent et al., 2016; Cheng et al., 2017; Mueller et al., 2010, 2006; Song et al., 2012; Wang et al., 2015; Yang et al., 2013). Codon modification through codon pair deoptimization (CPD) or codon usage preference through synonymous mutations reduces protein translational efficiency of the target genes and results in viral attenuation. Here, we applied the re-coding strategy to three NDV virulence factors (F, HN and P) to achieve the goal of attenuating a mesogenic 73T strain.

2. Materials and methods

2.1. Cell lines

Chicken fibroblast cells (DF-1, ATCC, CRL-12203™) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS); African green monkey kidney cells (Vero, ATCC, CCL-81™), human fibrosarcoma cells (HT1080, ATCC, CCL-121™) and human cervical adenocarcinoma cells (HeLa, ATCC, CCL-2™) were cultured in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% FBS. Baby hamster kidney cell expressing T7 RNA polymerase (BSR-T7) was obtained from Dr. Conzelmann (Ludwig Maximilians University).

2.2. Generation of recombinant viruses

Rare codon- and human codon-incorporated HN, F, and P genes were designed using OptimumGeneTM algorithm (Sharp and Li, 1987) (GenScript USA Inc.). All codon changes were synonymous without causing any amino acid changes. The recoded genes were synthesized de novo (GenScript USA Inc.) and introduced into NDV 73T antigenomic cDNA to replace the respective wt counterpart sequences. Recombinant viruses were rescued by reverse genetics as described previously (Cheng et al., 2016) and confirmed by sequencing RT-PCR-amplified cDNA. For monitoring the viral replication under fluorescent microscope, wt or codon modified NDVs were engineered to insert a green fluorescent protein (GFP) transcriptional cassette between the P and M genes. The viruses were propagated in 9–11 days old embryonated SPF chicken eggs and titered by plaque assay in Vero cells. Viral genetic stability was examined by serial passage in HeLa cells at MOI of 0.1–0.01. Viral genome at passage 10 was sequenced using RT/PCR amplified cDNAs (Sequentech).

2.3. Virus plaque morphology and growth kinetics

Viral plaque morphology in HeLa or DF-1 cells were examined by infection with serially diluted virus and incubated under 1% methylcellulose overlay at 37 °C for 36 h. The plaques were immunostained with chicken anti-NDV polyclonal antibody followed by horseradish peroxidase (HRP)-conjugated anti-chicken antibody and visualized by AEC Substrate Chromogen

(DAKO). Viral growth kinetics were conducted in DF-1 and HeLa cells and titered by plaque assay in Vero cells.

2.4. Northern and Western blot analysis

Total intracellular RNA was either isolated from HeLa cells transfected with F and HN or Fr and HNr protein expression plasmids (2.0 μg) at 20 h posttransfection using an RNeasy minikit (Qiagen) (Cheng et al., 2016). Northern blot was hybridized with a biotin-labeled riboprobe (Sigma Aldrich) specific to the wt NDV F, HN, or to the rare codon coded HN and F genes. For Western blot analysis, the cell lysates were separated on 4–20% Novex Tris-glycine SDS-PAGE gels (Thermo Fisher Scientific) under denature condition and transferred to polyvinylidene difluoride (PVDF) membrane. Viral proteins were detected by specific antibodies as described previously (Cheng et al., 2016).

2.5. Confocal microscopy

HeLa or DF-1 cells were seeded at 10,000 cells/well in 96-well μ-plates (Ibidi) overnight. The cells were infected with each virus at MOI of 1 for 24 h, fixed in 4% paraformaldehyde in PBS at pH 7.4 for 10 min, and stained with anti-NDV-F or anti-NDV-HN polyclonal antibody followed by FITC- or TRITC-conjugated secondary antibody and DAPI (4′,6-diamidino-2-phenylindole) staining. Cell images were examined using a Zeiss Axio Observer. Z1 inverted microscope with 40 × /1.2NA LCIPlan Apo or 100 × /1.4NA Plan Apo objectives (Carl Zeiss Microscopy). Images were acquired using a Yokogawa CSU-X1 Spinning Disk Unit (Yokogawa Electric Corporation) with Evolve 512 EMCCD (Photometrics), processed using ZEN 2.3 (Carl Zeiss Microscopy) and analyzed using Columbus software (PerkinElmer).

2.6. Transmission electron microscopy

Viruses were amplified in embryonated chicken eggs, clarified at 4000 rpm for 20 min followed by ultracentrifugation at 25,000 rpm for 2 h through a 20% (wt/vol) sucrose cushion. Virus pellet was resuspended in PBS, fixed with 2.5% (vol/vol) glutaraldehyde at room temperature, negatively stained with phosphotungstic acid and imaged by transmission electron microscopy (JFE enterprises).

2.7. In vitro cell killing assay

HT1080 cells were seeded at density of 10,000 cells/well (96 well plate) and infected at MOI of 0.1 and 1. Cell viability was analyzed at 3, 7 and 14 days post-infection using CellTiter Glo kit (Promega). The relative percentage of live cells was determined by comparing the ATP level of each testing sample to the level of the mock-infected cells (set at 100% viability).

2.8. Chicken pathogenicity test

The pathogenicity of the recombinant viruses in chickens was determined by the intracerebral pathogenicity index (ICPI) test in 1-day-old SPF chicks. Each virus was grown in embryonated chicken eggs and supplied at a hemagglutination (HA) titer of 32 for ICPI test at the USDA's National Veterinary Service Laboratory (NVSL; Ames, IA) (OIE, 2009).

3. Results

3.1. Construction of P, F and HN gene re-coded NDV variants

By altering gene codon usage to either rare codons or codons preferentially utilized in humans, it might be possible to engineer NDV variants that would not replicate in chickens as efficiently as the parental mesogenic strain but retain their ability to replicate effectively in human cells. The P, F and HN genes were chosen for recoding because they each contribute to a critical step in NDV infection or replication and to avian pathogenicity. The P gene encodes not only the phosphoprotein involved in RNA transcription and replication but also two additional proteins, V and W via P gene mRNA editing. Thus, only the N terminal portion of the P gene coding sequence was recoded to avoid changing the amino acid sequence of the V and W proteins. However, codon modification of the 5′ part of the P gene could still potentially affect the P, V and W protein level. Because virus entry and egress require balanced fusion and neuraminidase activities, the F and HN proteins were recoded simultaneously (Fig. 1A). The codon adaptation index (CAI) parameter is an indicator showing distance to the human codon usage preference. The three NDV genes (P, F and HN) have CAI ranging from 0.68 to 0.76. Rare codon incorporation altered 35–36% of the nucleotides of the target genes reducing the CAI of P, F and HN genes to 0.41, 0.48 and 0.5, respectively. Humanized codon

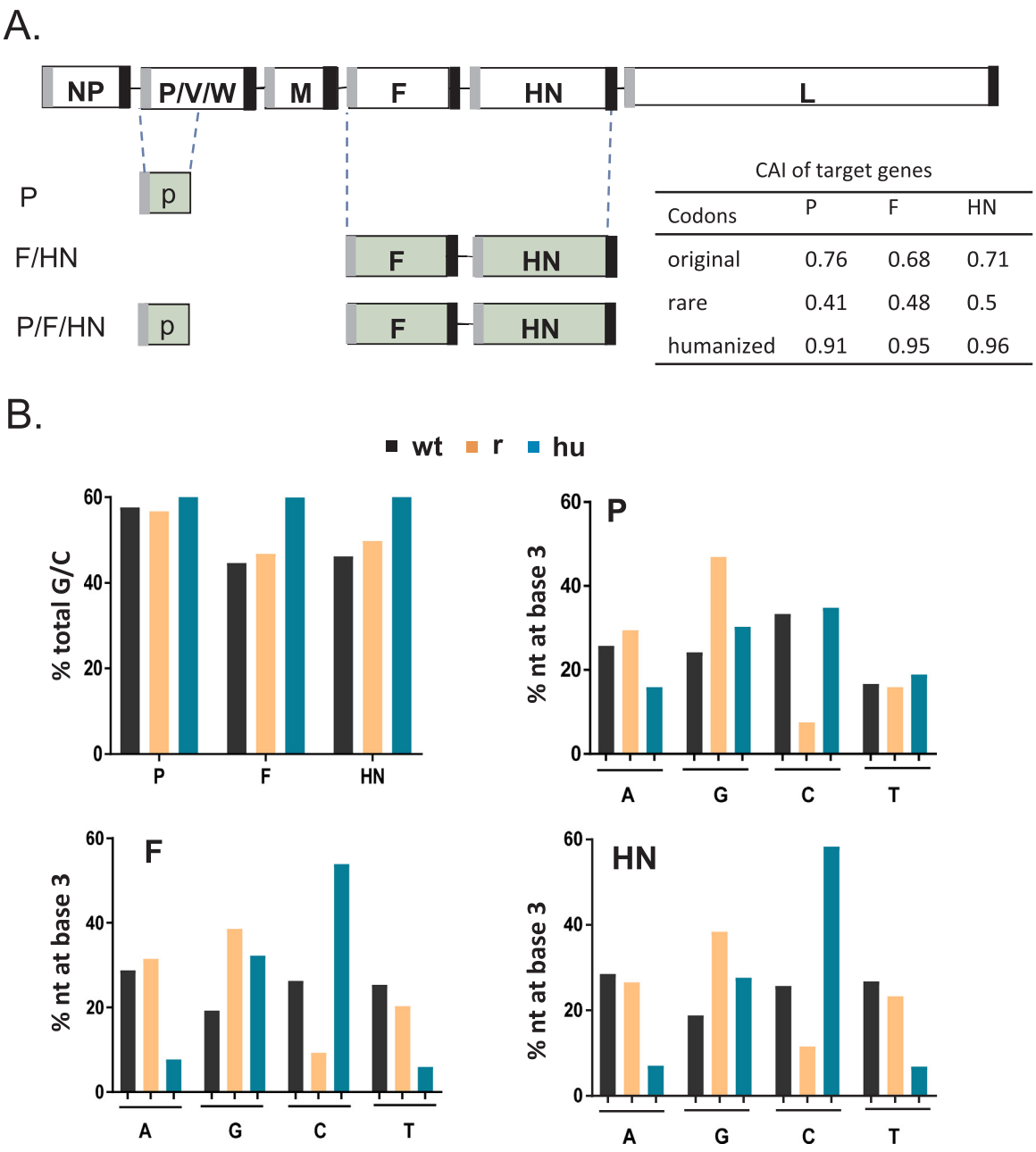

Fig. 1. Construction of recombinant NDV mutants. A. Schematic diagram of the genomic structure of NDV with complete or partial codon modification in P, F and HN genes. Codon modified ORFs are in blue and original ORFs are in open box. Codon adaptation index (CAI) of the P, F and HN genes with original, rare codon or humanized codons are presented in the table. B. Total G/C content of the wt, random (r) and human (hu) codon recoded P, HN, and F genes and frequency of A, G, C or T base at the third nucleotide position of the codons for the recoded P, F and HN genes. Rare codons did not change G/C content, enriched G and suppressed C without affecting A/T usage for all three genes. Humanized codons increased total G/C content, enriched G/C and suppressed A/T.

incorporation changed 25–26% of nucleotide sequences and increased their CAI values to 0.91–0.96.

Codon sequence change on the G/C content of each recoded gene was calculated and shown in Fig. 1B. The NDV P gene has a higher G/C content (57.73%) than the F (44.71%) and HN (46.33%) genes. The rare codons only slightly changed the G/C contents, 56.81%, 46.87% and 49.94% for P, F, and HN respectively. The humanized codons increased G/C contents for all three genes, 61.23% for P, 60% for F and 61% for HN. The frequency of nucleotide utilization at the third base position was calculated for the altered codons (Fig. 1B). Rare codon incorporation enriched guanine (G, 41%) over cytosine (C, 9.6%) without affecting adenine (A) and thymidine (T) usage. Yet, the overall G/C content at the 3rd base was 50.6%, which was similar to the wt NDV genome of 50.4%. Humanized codon incorporation resulted in more G/C, with the mean G/C content at the third base position of the 3 recoded genes increased to 79.9%.

3.2. *Effect of re-coding on NDV replication in vitro and chicken virulence*

Recombinant NDV variants with rare or humanized codons in P, F and HN were generated by reverse genetics (Table 1). The viruses were amplified in embryonated chicken eggs, titrated in chicken DF-1 and human HeLa cells and the representative plaque images are shown in Fig. 2A. Overall, the viruses with rare codons in the P gene (Pr) and F and HN genes (Fr/HNr) had smaller plaque sizes in both chicken DF-1 cells and human HeLa cells. Viruses with humanized codons in the P and F/HN (Ph and Fh/HNh) genes exhibited plaque sizes comparable to the parental wt NDV in both chicken and human cell lines. The viruses

Table 1
Effect of codon modification on chicken virulence.

Virus	Gene codon modification	Viral titer[a] (PFU/ml)	ICPI[b]
73T wt	Wild type	8.7	1.7
Pr	P with rare codons	7.7	0.91
Ph	P with humanized codons	7.5	1.23
F*h/HNh	F* and HN with humanized codons	8.6	1.39
F*r/HNr	F* and HN with rare codons	8.6	0.98
Fr/HNr[c]	F and HN with rare codons	8.6	0.83
Ph/Fr/HNr[c]	P with humanized codons, F and HN with rare codons	7.5	0.13

[a] Viral titer in eggs.
[b] Chicken intracerebral pathogenicity index; NA, not available.
* wt F protein cleavage site ^{111}GRRQKR-F^{117}.
[c] Modified F protein cleavage site ^{111}HNRTKR-S^{117}.

with rare codons incorporated into the single P, or double HN and F genes exhibited a greater reduction in chicken virulence than those with humanized codons (Table 1). However, the ICPI values for all the re-coded viruses were above 0.7 that is the select agent threshold established by the USDA and therefore all still categorized as select agents due to their chicken virulence. Incorporation of a previously described F protein cleavage site (12) into a virus encoding F and HN genes with rare codons resulted in a further reduction in chicken virulence from 0.98 (Fr*/HNr virus with a wild type F cleavage site) to 0.83 (Fr/HNr virus with a modified F cleavage site). To further reduce the pathogenicity of the Fr/HNr virus, Ph or Pr was incorporated. The resulting triple gene mutant, Pr/Fr/HNr did not replicate well in HeLa cells while Ph/Fr/HNr which contained the altered F protein cleavage site, exhibited reduced viral titer in emryonated chicken eggs by 10-fold and a low ICPI value of 0.13 (Table 1) that was fully attenuated. This reduced titer in eggs likely relates to the recoded P gene, as all viruses with recoded P grew to lower titers than viruses with wild type P codons.

The Ph/Fr/HNr triple gene mutant with the F protein cleavage site modification was further characterized for its growth kinetics in chicken (DF-1) and human (HeLa) cells. The Ph single gene mutant, Fr/HNr double gene mutant containing the F protein cleavage site modification, and wt 73T viruses were evaluated for comparison (Fig. 2B). In DF-1 cells, the titer of Ph/Fr/HNr was 3-fold reduced on day 2 and reached peak titer on day 3, similar to Ph, Fr/HNr and wt viruses. In HeLa cells, Ph/Fr/HNr showed decreased growth kinetics and lower titers compared to wt virus on day 1 and 2, but reached a peak titer of approximately 1.0E+ 7 PFU/ml on day 3, which was lower than the peak titer of wt NDV on day 2. The titers of Ph and Fr/HNr were also lower than wt NDV on day 2 and peaked on day 3. However, the peak titer differences of these viruses were not statistically significant. Genetic stability of the Ph/Fr/HNr virus was confirmed by serial passage in HeLa cells for 10 times and passage 10 samples were sequenced. No mutations were found in the recoded P, F and HN genes (data not shown).

3.3. Viral RNA and protein synthesis of NDV with P, F and HN gene codon modification

To determine the impact of gene re-coding on protein synthesis of the targeted genes, viral protein levels in the Ph/Fr/HNr-infected DF-1and HeLa cells at 20 h post-infection were compared to the wt, Ph, and Fr/HNr viruses by Western blotting (Fig. 3A). In both the DF-1 and

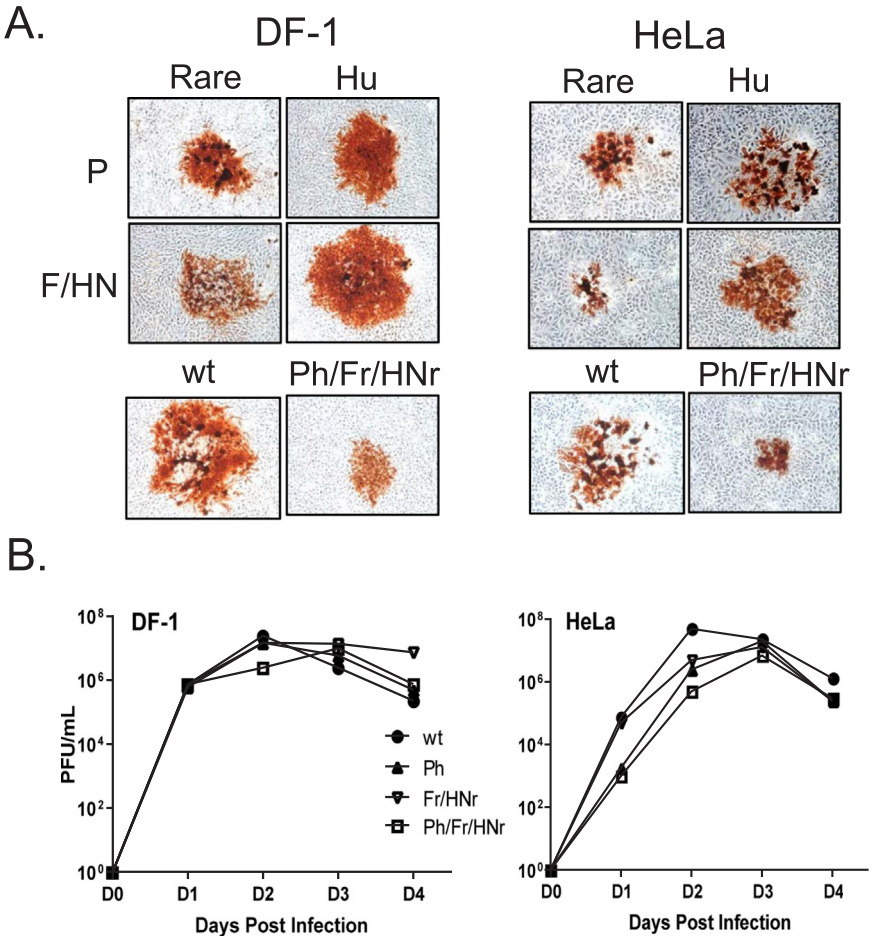

Fig. 2. Replication of recovered viruses in DF-1 and HeLa cells. A. Plaque phenotypes of wt and codon modified viruses on DF-1 and HeLa cells enumerated by immunostaining and representative images are presented. B. Multicycle growth kinetics of the indicated viruses at MOI 0.01 for 4 days. Viral titers from the infected cell culture supernatants were plotted.

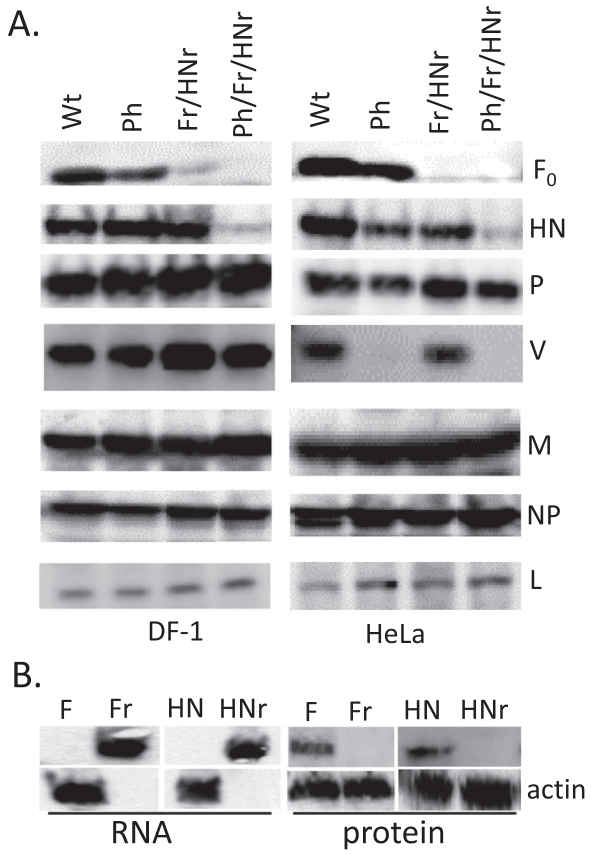

Fig. 3. Viral mRNA and protein synthesis in infected cells. A. Western blot analysis of infected DF-1 or HeLa cells. Cells were infected with wt or codon modified viruses at MOI of 1 PFU/cell and the cell lysates were subjected to Western blot 24 h post-infection using viral protein-specific antibodies; the data are representative of the results of three independent experiments. B. Transient expression of F and HN proteins. Plasmids encoding Fwt, Fr, HNwt or HNr under the CMV promoter were transfected into HeLa cells, total cellular RNA was blotted with F, Fr, HN, or HNr-specific probes, the F and HN proteins in transfected cells were examined by Western blot using actin as a loading control.

HeLa cells, the Fr/HNr encoding virus exhibited greatly reduced levels of F protein expression while with the Ph/Fr/HNr virus, both F and HN protein levels were greatly reduced. In addition, the viruses with the Ph gene, Ph and Ph/Fr/HNr, had significantly reduced V protein synthesis in HeLa cells. The Ph/Fr/HNr virus had greatly reduced expression of the three targeted F, HN and V proteins in HeLa cells. To confirm that reduced F and HN protein synthesis was due to poor protein translation efficiency not due to reduction in mRNA transcription, the wild type F and HN genes, and rare codon coded F and HN genes were each cloned under the CMV promoter and examined by transient expression in HeLa cells (Fig. 3B). The wt and recoded F and HN genes had similar levels of RNA detected by Northern blots. The wt F and HN proteins were expressed in comparable levels; however, the Fr and HNr proteins were barely detectable. Therefore, the rare codons reduced the HN and F protein syntheses equally based on the transient expression study, which was different from the data obtained for the Fr/HNr virus with a great reduction of the F protein than HN in infected cells. The reduction of the HN (as well as F) protein expression in Ph/HNr/Fr infected cells was the consequence of the introduction of the human codon coded P protein that was found to reduce the V protein synthesis in HeLa cells.

3.4. Reduced glycoprotein incorporation into virions

Cell surface expression of the F and HN glycoproteins of the Ph/Fr/HNr mutant was compared to Ph, Fr/HNr and wt viruses by immunofluorescence (Fig. 4). The fluorescence intensity of the stained F protein (green) and HN protein (red) was greatly reduced in Ph/Fr/HNr and Fr/HNr infected cells compared to wt virus in both DF-1 and HeLa cells. The budding of the P/F/HN recoded virus (Ph/Fr/HNr) from the infected HeLa cells was compared with Ph, Fr/HNr and wt NDV by transmission electron microscopy (TEM). There was no obvious difference observed in the number of budding virions from the infected cell membrane for these viruses (Fig. 5A). The glycoprotein incorporation into virions produced in embryonated chicken eggs by TEM showed that the Ph/Fr/HNr and Fr/HNr viruses had no visible spikes observed on their virion surface, whereas wt and Ph viruses had clear spikes on the virion surface. Lower incorporation of viral glycoproteins did not appear to affect virion morphology (Fig. 5B).

3.5. Expression of the GFP gene by the Ph/Fr/HNr NDV in HeLa cells

To monitor foreign gene expression by the Ph/Fr/HNr virus, a GFP transgene cassette was inserted into Ph/Fr/HNr virus between the P and M genes. The GFP cassette was also inserted at the same position in the wt NDV backbone (Rangaswamy et al., 2017). Human fibrosarcoma cells (HT1080) were infected and cell viability and GP expression were monitored (Fig. 6A). Wt NDV-GFP infection caused rapid killing of these cancer cells, as indicated by cell detachment from the tissue culture plates starting at 1 day post-infection. More than 90% of cells were killed at 3 days post-infection even when the fibrosarcoma cells were infected at a low MOI (0.1 PFU/cell). Therefore, wt NDV-GFP infected cells at Day 7 and 14 were not shown. In contrast, the oncolytic activity of the Ph/Fr/HNr-GFP virus was greatly reduced, with the majority of infected cells remaining viable even at 14 days post-infection at both high and low MOI (Fig. 6B) with sustained GFP expression.

4. Discussion

Virus codon usage patterns reflect the evolutionary changes allowing viruses to adapt their fitness to the host cells they infect. Synonymous codon usage is not random; different organisms and

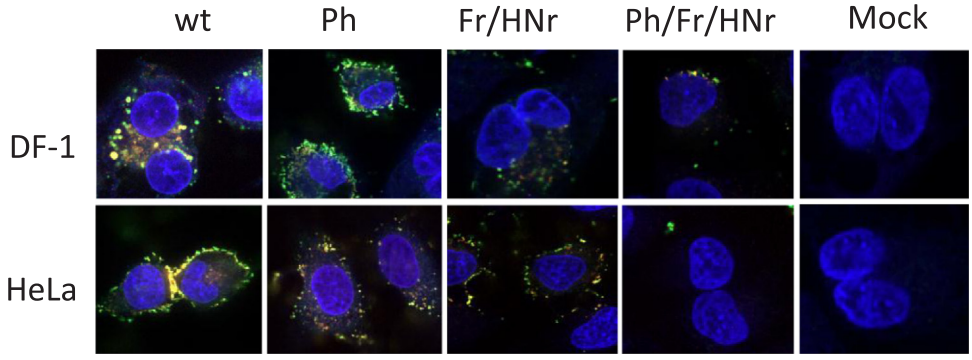

Fig. 4. Expression of the F and HN glycoproteins in NDV-infected cells by immunofluorescence. DF-1 and HeLa cells were mock infected or infected with wt, Ph, Fr/HNr or Ph/Fr/HNr at MOI of 1 for 24 h and stained with anti-NDV-F antibody (green) and anti-NDV-HN antibody (red). DAPI (4′,6-diamidino-2-phenylindole) was used to stain cell nucleus (blue).

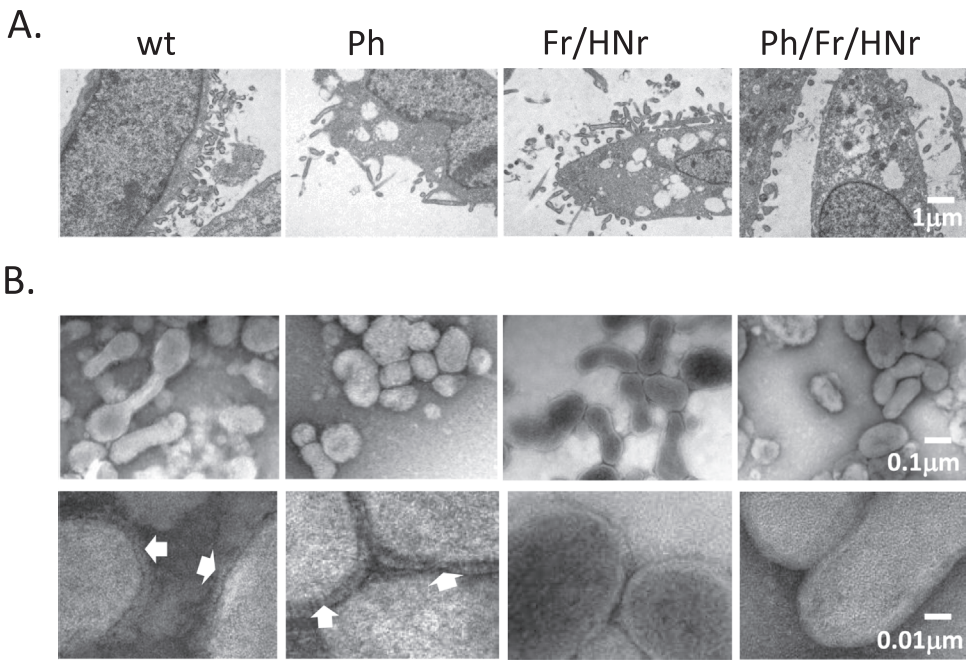

Fig. 5. Virus budding from infected cells and virion morphology. A. wt, Ph, Fr/HNr and Ph/Fr/HNr infected HeLa cells at MOI of 1 were viewed by negative staining TEM. B. Morphology of wt and different codon modified virions were viewed by TEM at low and high magnification. Glycoprotein spikes of wt and Ph were pointed by white arrows. No glycoprotein spikes were observed on the virion surface of Fr/HNr and Ph/Fr/HNr.

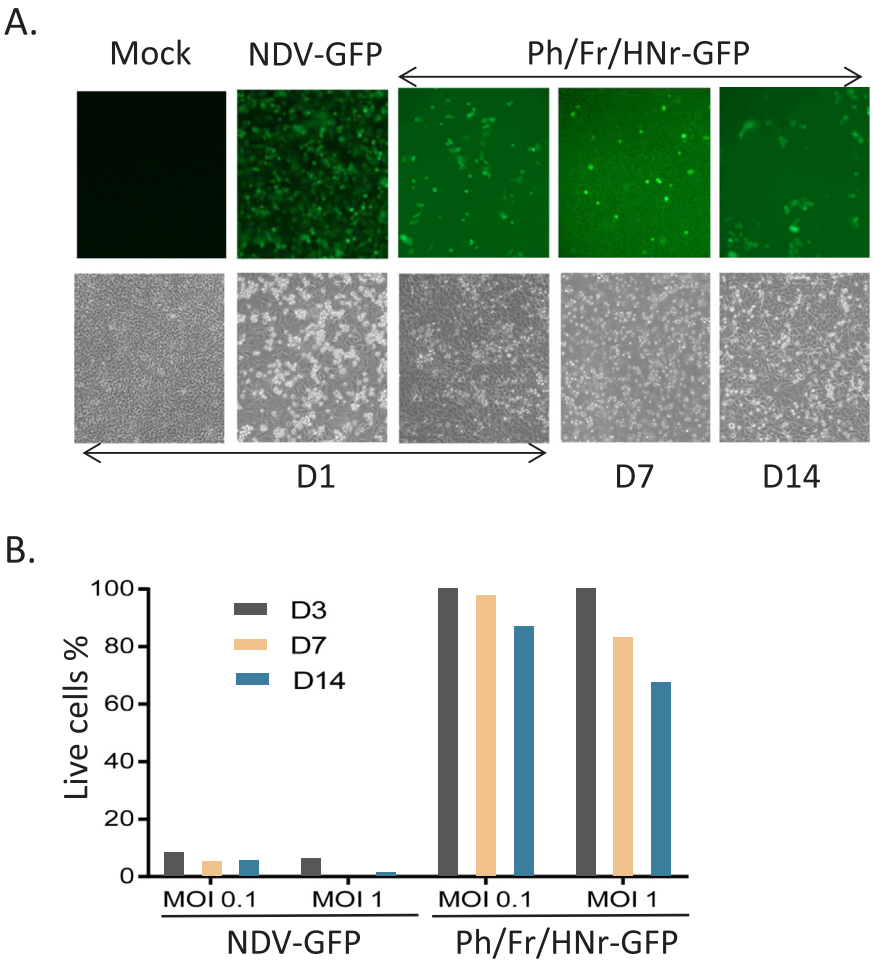

Fig. 6. GFP transgene expression and viral replication in tumor cells. A. GFP transgene expression in NDV-GFP and Ph/Fr/HNr-GFP infected HeLa cells (upper) and respective phase contrast image (bottom) at moi of 0.1. NDV-GFP infected HeLa cells showed cytopathic effect (CPE) on day 1 while Ph/Fr/HNr-GFP infected HeLa cells expressed GFP until day 14. B. Cell viability of infected HT1080 cells. Cell viability of Ph/Fr/HNr-GFP and wt infected cells at MOIs of 1 and 0.1 was determined at days 3, 7 and 14 post-infection and expressed as the percentage of viable cells relative to uninfected cells.

different genes from the same organism encode specific codons at different frequencies. Here, by recoding the F, HN and the N-terminal P protein genes, and including a previously described protein cleavage site modification in F, we were able to successfully generate a fully attenuated NDV 73T virus. It has been hypothesized that codon bias contributes to protein translation efficiency by tuning translation initiation, elongation, and differential protein production. Translation of multiple synonymous codons by a single tRNA is through wobble base-pairing. The tRNA has a different affinity for A, G, C and U at the third base position of the codon (Crick, 1966; Soll et al., 1966). In addition, codon bias correlates with tRNA pools, codon frequency and tRNA gene copy numbers for specific host cells (Crick, 1966; Zhou et al., 2016). Recoding virus genes or genomes by synonymous mutation represents an effective way of generating novel live attenuated candidate viral vaccines. The introduction of a large number of nucleotide substitutions at mainly the third base of codons makes it impossible for virus to revert to the wt phenotype. Indeed, the recombinant NDV with synonymous codons in P, F and HN (Ph/Fr/HNr) generated here remained attenuated and was genetically stable after serial passages in human cells.

The rare codons we introduced into NDV increased G but reduced C content at the third base, while humanized codons increased G/C and reduced A/T content. The rare codon usage impacted viral phenotype to a greater extent than humanized codons. The NDV natural host is an avian species, which has a mean CAI of approximately 0.72. Rare codon usage lowered the CAI to ~0.48, while humanized codons raised the CAI to approximately 0.94. This may explain why the viruses engineered with rare codons in the F and HN genes replicated relatively better in chicken DF-1 cells than human HeLa cells as indicated by their reduced plaque size and delayed growth kinetics in HeLa cells.

Although both the F and HN genes were engineered with rare codons, the HN protein synthesis in Fr/HNr virus-infected avian and human cells was less impacted than F. A similar phenomenon was observed in the codon deoptimized influenza virus with the normal NA protein expression level and greatly reduced HA protein (Broadbent et al., 2016). However, expression of the HN protein was also reduced for the Ph/Fr/HNr virus in DF-1 and HeLa cells, thus Ph reduced the HN protein expression for the triple mutant. How the human codon coded P gene resulted in the reduced HN protein level is yet to be determined. The Ph gene reduced viral replication by 10-fold for Ph and Ph/Fr/HN viruses in chicken embryonated eggs (Table 1), yet only the triple gene recoded Ph/Fr/HN virus was fully attenuated in chickens. It is intriguing to see that such low levels of the F and HN protein expressed in the Fr/HNr and Ph/Fr/HN viruses were still capable of triggering fusion between the virion envelope and the cell plasma membrane to mediate viral entry into the cells. Although the reduction of the viral glycoprotein levels through codon bias (mostly codon pair bias) has been shown to be an effective means to attenuate polio virus (Coleman et al., 2008), influenza virus (Mueller et al., 2010; Yang et al., 2013) and RSV (Le Nouen et al., 2014), it is not sufficient for influenza H1N1 strain (Broadbent et al., 2016), Rabies virus (Wirblich and Schnell, 2011) and NDV reported here.

The NDV F protein is the key determinant for chicken virulence. The F0 form of the protein is efficiently cleaved into the mature F1 and F2 subunits in chicken cells infected with both the mesogenic and velogenic NDV strains. The modification of the wt F protein cleavage site (^{111}GRRQKR-F^{117}) to the HNRTKR-S sequence we reported earlier (Cheng et al., 2016), only reduced the ICPI value by 0.15 from 0.98 to 0.83. The fully attenuated phenotype of NDV-Ph/Fr/HNr virus was attributed to the addition of the humanized codon biased P gene to the virus expressing Fr/HNr with the modified F protein cleavage site. The P gene encodes not only the P protein, but also the V and W proteins through mRNA editing. The V protein was barely detectable in Ph and Ph/Fr/HNr infected HeLa cells but expressed well in DF-1 cells. These data suggest that the P gene mRNA editing function tolerates sequence changes in avian cells better than in human cells, perhaps due to an evolutionary adaptation of avian virus allowing better grown in chicken cells than in human cells and different host factors involved in the gene editing process. The NDV V protein is an interferon antagonist protein that has been shown to be a host specific virulence factor (Alamares et al., 2010; Huang et al., 2003). Although the P and V protein levels in the chicken cell line were not greatly affected in vitro by codon modification, viral pathogenicity in chickens was significantly decreased.

The attenuated Ph/Fr/HNr exhibited greatly reduced oncolytic activity as shown by reduced cancer cell killing, making it unlikely to be developed as an oncolytic viral agent. However, it may be possible to explore its application as a viral vector as this virus can be cleaved much more efficiently than lentogenic NDV (Dey et al., 2017; Xiao et al., 2011). Viral vectors offer an efficient means to deliver genes for vaccine and gene therapy applications (Ajith, 2017; Nayerossadat et al., 2012; Souza et al., 2005). NDV replicates in the cytoplasm of the infected cell and there is a very low risk for gene integration (Schirrmacher, 2016). A similar paramyxovirus, Sendai virus, has been evaluated as a gene delivery vehicle for gene therapy (Nakanishi and Otsu, 2012; Nayerossadat et al., 2012). The Ph/Fr/HNr-GFP virus did not kill infected cells efficiently and therefore may not serve as an effective oncolytic virus. However, its capacity for prolonged expression of a transgene makes it attractive as a potential vaccine vector or as a vector for gene delivery (Peeples et al., 1992; Rawlinson and Moseley, 2015). The protein expression level and immunogenicity of the potential vaccine candidates delivered by this recombinant NDV require further in vitro and in vivo studies to validate the utility of this codon modified NDV virus for prophylactic or therapeutic applications.

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