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Delayed Newcastle disease virus replication using RNA interference to target the nucleoprotein

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ABSTRACT

Each year millions of chickens die from Newcastle disease virus (NDV) worldwide leading to severe economic and food losses. Current vaccination campaigns have limitations especially in developing countries, due to elevated costs, need of trained personnel for effective vaccine administration, and functional cold chain network to maintain vaccine viability. These problems have led to heightened interest in producing new antiviral strategies, such as RNA interference (RNAi). RNAi methodology is capable of substantially decreasing viral replication at a cellular level, both in vitro and *in vivo*. In this study, we utilize microRNA (miRNA)-expressing constructs (a type of RNA interference) in an attempt to target and knockdown five NDV structural RNAs for nucleoprotein (NP), phosphoprotein (P), matrix (M), fusion (F), and large (L) protein genes. Immortalized chicken embryo fibroblast cells (DF-1) that transiently expressed miRNA targeting NP mRNA, showed increased resistance to NDV-induced cytopathic effects, as determined by cell count, relative to the same cells expressing miRNA against alternative NDV proteins. Upon infection with NDV, DF-1 cells constitutively expressing the NP miRNA construct had improved cell survival up to 48 h post infection (h.p.i) and decreased viral yield up to 24 h.p.i. These results suggest that overexpression of the NP miRNA in cells and perhaps live animal may provide resistance to NDV.

1. Introduction

Virulent strains of Newcastle disease virus (NDV) are the causative agent of Newcastle disease (ND), a devastating disease of poultry worldwide [1]. Since highly virulent NDV strains cause up to 100% mortality in infected flocks, adequate control of ND is vital to ensure healthy, productive poultry populations [2]. While vaccine campaigns are routinely practiced, these are severely limited by elevated costs, need of trained personnel for adequate administration, and long term thermostability when transporting the vaccine [3]. These problems are heightened in developing countries, where vaccine costs become excessive in subsistence farming settings [3–5]. Such limitations have led researchers to explore new avenues to control problematic pathogens, such as NDV, to generate new, sustainable antiviral strategies [3,5,6].

Ribonucleic acid interference (RNAi) is a naturally occurring intracellular process found in most organisms in which gene expression is controlled through silencing of specific messenger RNAs (mRNA) [7]. RNAi pathways are capable of being activated by several avenues including micro RNA (miRNA), small interfering RNA duplexes (siRNA) and short hairpin RNA (shRNA). These mechanisms of gene silencing are evolutionarily conserved and can silence mRNA at multiple stages of expression including transcription, post-transcription, and translation [8,9]. In the miRNA pathway, double stranded miRNAs are processed by the protein Drosha, further modified by Dicer, and the product is then integrated into the RNA-induced silencing complex (RISC) [10]. This multi-protein complex

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will then unravel the double stranded RNA, and retain a single guide strand which will direct the knockdown of the target sequences bases on Watson-Crick base paring [8,11]. In the laboratory, synthetic vectors can be used to express miRNA, siRNA or shRNA, and have been widely used in the scientific community as a means to decrease viral yields by lowering the amount of viable mRNA that encodes for viral proteins, or other RNA intermediates that are necessary for viral replication [12–14]. RNAi approaches have been successfully used in studies of respiratory syncytial virus (RSV), avian influenza virus (AIV), parainfluenza virus, and coronavirus (including severe acute respiratory syndrome virus) [12–14]. Alvarez et al. reported reduction in RSV viral concentrations induced by siRNA without inducing off target pro-inflammatory effects, a potential problem [15]. In similar research, suppression of AIV replication was achieved by using shRNA to knockdown expression of the viral polymerase leading to reduced bird to bird transmission of the virus [16]. These studies suggest RNAi may be highly effective in disrupting viral replication and decreasing expression of virus genes [17]. These approaches may now be applied to NDV.

NDV is classified as Avian Paramyxovirus serotype 1 (APMV-1), and is an enveloped virus with a negative sense, single stranded RNA genome of approximately 15 kb, which encodes six structural proteins, from 3′ to 5': nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN), and the large polymerase protein (L) [18,19]. Using pre-miRNA to activate the cellular RNAi pathway, a miRNA can be used to target the messenger RNA of NDV structural proteins, leading to the degradation of the transcripts and inhibiting viral replication [11]. Furthermore, by coupling miRNA expression with a lentiviral (LV) delivery system, it is possible to create stable cell populations that constitutively express the miRNA sequence [8]. The use of LV vectors to incorporate exogenous genetic material into the host genome also lends to the possibility of creating transgenic animals capable of germline transmission of the transgene [20]. An LV delivering a miRNA that can induce resistance to NDV could be delivered to a donor bird at various stages resulting in an animal with an endogenous antiviral defense against NDV [6,20]. This approach could lead the basis for a functional, preventative antiviral strategy that does not require the use of additional prophylaxis in chickens.

In this study, we attempted to determine if constitutive expression of miRNA sequences targeting the mRNA of five of the structural NDV proteins in chicken embryo fibroblast cells (DF-1) would lead to decreased viral yield after infection, and/or resistance against NDV cytopathic effects.

2. Materials and methods

2.1. Cell lines

DF-1 cells (Chicken embryo fibroblast cell line; ATCC CRL-12203) were cultured with fibroblast medium [Dulbecco's modified Eagle's medium (DMEM) high glucose (Hyclone) with 10% fetal bovine serum (FBS; Hyclone), 4 mM L-glutamine (Gibco), and 1x Pen/strep (Gibco)] at 37 °C in a 5%CO_2 atmosphere. Human embryonic kidney 293FT cells (Invitrogen) were cultured at 37 °C in a 5%CO_2 atmosphere in complete medium [DMEM high glucose (Hyclone) with 10% FBS (Hyclone), 0.1 mM MEM Non-Essential amino acids (NEAA; Gibco), 6 mM L-glutamine (Gibco), 1 mM MEM Sodium Pyruvate (Gibco), and 1x Pen/strep (Gibco)] supplemented with 500 μg/mL Geneticin (Gibco CAT# 10131).

2.2. Virus

NDV strains LaSota-Virulent (LS-V) was obtained by the Southeast Poultry Research Laboratory (SEPRL) repository. LS-V is a virulent strain derived from the non-virulent LaSota wild-type by site directed mutagenesis of the F gene cleavage site, and it has an intracerebral pathogenicity index (ICPI) of 1.69 [21]. LS-V stock was produced as follows [22]. Briefly, virus (100 uL) was propagated in the chorioallantoic cavity of 9–10 day old embryonating specific pathogen free (SPF) eggs (SEPRL White Leghorn SPF flock). Dead eggs, or eggs surviving after 5 days of incubation were chilled at 4 °C for 24 h, and HA-positive allantoic fluid was extracted, pooled, clarified by centrifugation (5000 rpm for 10′), and divided into 1 ml aliquots in cryovials, which were stored at −80 °C. Virus stock was tittered in DF-1 cells in 96-well plates, and titer expressed as tissue culture infection dose 50% (TCID50)/ml. For the purpose for this study, multiplicity of infection (MOI) calculations were carried out using the viral titer expressed in TCID50.

2.3. miRNA design

Sequences were designed based on BLOCK-iT™ Pol II miR RNAi Expression Vector Kit (Invitrogen (Grand Island, CA) CAT# K4938-00) guidelines, and aimed against the transcribed sequences (mRNA) of five NDV genes: one each for NP, P, M, F, and three for the L gene (L1, L2, L3), for a total of seven miRNA sequences. Sequences were designed based on the consensus alignment of multiple NDV strains representing the most commonly circulating NDV genotype II. A genotype II strain representative (such as LS-V) was used since it had been characterized extensively by our group [21,23]. An additional sequence (scramble, SCR) with no identity to known chicken or NDV genes was used as a control in all downstream experiments. The complete sequences of the miRNAs are provided in Table 1. No miRNA was designed for the HN protein due to lack of highly conserved sequences for the protein.

2.4. Production of expression constructs

For each of the eight sequences, double-stranded oligonucleotides (ds oligo) containing the engineered pre-miRNA cassettes were reconstituted by annealing two HPLC-purified single-stranded oligos (custom made, Integrated DNA Technologies). The oligonucleotides were designed according to the Invitrogen manual guidelines (BLOCK-iT™ Pol II miR RNAi Expression Vector Kit): from 5′ to 3′, the top oligo contained a 5 - nucleotide (nt) overhang for ligation in the vector, 21- nt reverse target sequence (Table 1), a 19 - nt spacer (terminal loop), and a 19 - nt sense target sequence, with an internal 2 - nt deletion (inner loop). The bottom oligo consisted of the reverse complement to the top sequence, with 5′ overhang and no 3′ overhang. Annealing of ds oligos was verified by Ethidium Bromide gel electrophoresis. Each resulting ds oligo was ligated into pcDNA™ 6.2-GW/± EmGFP-miR vector. Ligated vectors were used to transform chemically competent *E. coli* cells (TOPO10, Invitrogen (Grand Island, CA) CAT# C4040-03) following manufacturer's protocol. Transformed cells were grown in LB agar plates with 100 μg/mL blasticidin (Gibco CAT# A11139-03) for selection. DNA was extracted from transformants using Qiagen Plasmid MINI prep (Qiagen (Valencia, CA) CAT# 12123) and insertion was verified by DNA sequencing (AB-3730 automated DNA sequencer). miRNA expression constructs were designated as miR-NP, miR-P, miR-F, miR-L1, miR-L2, miR-L3, miR-M, and miR-SCR.

2.5. Production of lentivirus

Previously verified expression constructs were subjected to Rapid BP/LR recombination reaction per manufacturer's protocol (Invitrogen's Gateway® Technology; CAT# K4938-00) in order to transfer the pre-miRNA cassette to the pLenti6/V5-DEST destination vector (Invitrogen (Grand Island, CA) CAT# V496-10), which is

Table 1
List of sequences in miRNA design.

Gene Target	Sequence of miRNA
NP (F)	5′-**TGCTG**TAAAGTTGTGCATACTCGGCAGTTTTGGCCACTGACTGACTGCCGAGTGCACAACTTTA-3′
NP (R)	5′-**CCTG**TAAAGTTGTGCACTCGGCAGTCAGTCAGTGGCCAAAACTGCCGAGTATGCACAACTTTAc-3′
P (F)	5′-**TGCTG**TTGTCCATGATATGCTGTGTTGTTTTGGCCACTGACTGACAACACAGCATCATGGACAA-3′
P (R)	5′-**CCTG**TTGTCCATGATGCTGTGTTGTCAGTCAGTGGCCAAAACAACACAGCATATCATGGACAAc-3′
F (F)	5′-**TGCTG**TAGGTGGCACGCATATTATTTGTTTTGGCCACTGACTGACAAATAATACGTGCCACCTA-3′
F (R)	5′-**CCTG**TAGGTGGCACGTATTATTTGTCAGTCAGTGGCCAAAACAAATAATATGCGTGCCACCTAc-3′
L1 (F)	5′-**TGCTG**CACAACATCTCAGCTGCTTGAGTTTTGGCCACTGACTGACTCAAGCAGGAGATGTTGTG-3′
L1 (R)	5′-**CCTG**CACAACATCTCCTGCTTGAGTCAGTCAGTGGCCAAAACTCAAGCAGCTGAGATGTTGTGc-3′
L2 (F)	5′-**TGCTG**GATACGTTTCTTATTGCTGTTGTTTTGGCCACTGACTGACAACAGCAAAGAAACGTATC-3′
L2 (R)	5′-**CCTG**GATACGTTTCTTTGCTGTTGTCAGTCAGTGGCCAAAACAACAGCAATAAGAAACGTATCc-3′
L3 (F)	5′-**TGCTG**GTGTAGAGCCTTGAGTATTGAGTTTTGGCCACTGACTGACTCAATACTAGGCTCTACAC-3′
L3 (R)	5′-**CCTG**GTGTAGAGCCTAGTATTGAGTCAGTCAGTGGCCAAAACTCAATACTCAAGGCTCTACACc-3′
M (F)	5′-**TGCTG**GATTTGCTTCTTCCCATCTCCGTTTTGGCCACTGACTGACGGAGATGGAGAAGCAAATC-3′
M (R)	5′-**CCTG**GATTTGCTTCTCCATCTCCGTCAGTCAGTGGCCAAAACGGAGATGGGAAGAAGCAAATCc-3′

Bold indicates directional overhangs for ligation into the expression construct.

used for lentiviral packaging. Plasmids were transformed into One Shot® Stbl3™ Chemically Competent *E. coli* (Invitrogen (Grand Island, CA) CAT# C7373-03) according to manufacturer's protocol. Transformed cells were selected in LB agar plates supplemented with 100 μg/mL ampicillin (Sigma CAT# A0166). Transformant cells were grown in LB broth supplemented with 100ug/ml ampicillin for DNA extraction using MINI prep (Qiagen (Valencia, CA) CAT# 12123), or MIDI prep (Qiagen (Valencia, CA) CAT# 12143). Ampicillin-resistant colonies were screened by electrophoretic banding upon double enzymatic restriction digestion with XhoI and AFLII nucleases, to assess correct insertion of the pre-miRNA cassette (New England BioLabs (Ipswich, MA) CAT# R0156; CAT# R0520S). DNA sequencing was then used to confirm the restriction digestion results. Confirmed lentiviral vectors were used to package lentiviruses, and to produce virus stocks. Production of lentiviruses was conducted following ViraPower™ Lentiviral Expression System (Invitrogen (Grand Island, CA) CAT# 1165651) protocol using 293T producer cell line (Invitrogen (Grand Island, CA) CAT# R700-07). Produced lentiviral populations were concentrated using PEG-*it*™ Virus Precipitation Solution (5X) following manufacturers protocol (System Biosciences (Mountain View, CA) CAT# LV810A-1). Lentivirus stock was aliquoted in 1 ml cryovials and stored at −80 C.

2.6. Efficacy of lentiviral vectors (transient expression) against NDV cytolytic challenge

In order to evaluate the efficacy of designed miRNA cassettes to protect against NDV cytolytic effect, DF-1 cells were transfected with lentiviral plasmids (able to express the miRNA cassette) and subsequently infected with LS-V to assess the amount of cellular death. DF1- cells were plated at 5×10^5 cells/well in 6-well-plates, in technical replicates for each transfection group (NP, P, M, F, L1-3, SCR, and non-transfected). 24 h post plating, cells in each well were transfected with a mixture of 2 μL of Lipofectamine™ 2000 and 2 μg of lentivector plasmid DNA (Invitrogen CAT#11 668) in 1 ml of Opti-MEM® I Reduced Serum medium (Invitrogen CAT# 31985) for 5 h. After transfections, cells were maintained in fibroblast medium. 24 h following transfection, cells were infected with LS-V NDV at a multiplicity of infection (MOI) of 0.01 (MOI was calculated based on counting extra wells of SCR-transfected DF-1 cells at the time of infection). Briefly, for each well, virus was diluted in 1 ml of low serum (1%) fibroblast media and absorbed with DF-1 cells for 1 h at 37 C with 5%CO2; after absorption cells were washed with PBS and added fibroblast media. At 72 h post-infection (hpi), DF-1 cells were washed and number of viable cells counted with Nexcelom Bioscience Cellometer Auto T4 using Trypan Blue (Sigma CAT# T8154) dye exclusion.

2.7. Transduction of DF-1 cells

2×10^5 DF-1 cells were plated into 6 well plates, 24 h later cells were exposed to 500 μL of 1X Polybrene (Sigma CAT# H9268) solution in fibroblast medium. Previously frozen lentiviral stocks for the NP and SCR targets were thawed and ice and gently mixed. Lentiviruses were diluted 1:10 in 500 μL fibroblast medium, gently mixed by pipetting, then added to each well. Plates were incubated for 72 h at 37 °C in a humidified 5% CO_2 incubator. After 72 h, medium was changed to fibroblast medium containing blasticidin (10 μg/mL).

2.8. FACS sorting

Since in the lentiviral constructs the EmGFP gene is co-cistronic with the miRNA cassette, transduced cells underwent two rounds of clonal sorting for GFP in order to produce cell populations expressing high level of miRNAs. Briefly, transduced DF-1 cells were clonally sorted using Beckman Coulter MoFlo XDP based on the highest level of expression of the EmGFP reporter system (530/40 BP filter) into 1 well of a 96-well plate, and expanded in fibroblast medium. Upon expansion, cells were sorted a second time using the same criteria and culture method. After 2 passages, blasticidin (10 μg/mL) was added to fibroblast medium for selection. In this way, two stably transduced, highly fluorescent DF-1 expressing miRNA for NP, and SCR were produced.

2.9. Viral challenge of transduced cells

Stably transduced DF-1 cells containing miRNA for NP and SCR targets and naïve DF-1 cells were plated at 8×10^5 cells/well into 6-well plates, with three technical replicates for each group. 24 h later, cells were infected with LS-V at MOI of 0.01 (MOI calculation was based on counting naïve DF-1 in extra wells). Cells were then infected with LS-Vir NDV for 1 h at 37 °C in modified fibroblast medium containing only 1% FBS, as previously described. Post infections, cells were washed twice with PBS, and then returned to fibroblast medium. To assess viral growth in transduced cells, 200ul of supernatant were collected at 1, 12, 24 and 72 h.p.i., and replaced with 200ul fresh media each time. At 72 h.p.i, phase and fluorescent images were collected, and viable cells counted by Nexcelom Bioscience Cellometer Auto T4 using Trypan Blue (Sigma CAT# T8154) dye exclusion. The amount of virus in the collected supernatant was assessed by limiting dilution in DF-1 cells in 96-well plates, and expressed as TCID50/ml according to the Spearman-Karber method.

2.10. Statistical analysis

Means from multiple groups in the experiment (both for cell count or virus titer per time point) were analyzed by ANOVA with Tukey *post hoc* test. When only two groups were compared, two-sample t-test was performed. For all tests, significance was reported at the level of $P \leq 0.05$.

3. Results

3.1. Transient expression of the NP miRNA construct leads to reduced cytopathic effects and increased cell survival in NDV challenged cells

To evaluate the ability of miRNA constructs to targeting and knockdown NP, P, F, L and M mRNA, miRNA constructs expressing miR-NP, miR-P, miR-F, miR-L1, miR-L2, miR-L3 and miR-M were individually transfected into DF-1 cells. To control for potential off target effects, a scramble miRNA (miR-SCR) was also transfected into DF-1 cells. All constructs contained an EmGFP reporter to determine if cells were successfully transfected. At 0 h, phase images showed an intact monolayer consisting of healthy DF-1 transfected cells (Fig. 1A–D, I–L). Expression of EmGFP in transfected cells showed DF-1 cells are capable of being transfected and can successfully express constructs (Fig. 1E–H, M-P). Flow cytometry analysis of transfected miR-NP cell population demonstrates a transfection efficiency of 96.3%. Transfected DF-1 cells were then challenged with LS-V NDV strain at an MOI of 0.01. By 72 h post infection (h.p.i), most of the transfected cells (including scramble control) had visible cytopathic effects (CPE) characterized by disrupted monolayers and the formation of syncytia (Fig. 2A–D, I–L). However, DF-1 cells transfected with miRNA targeting the NP mRNA (miR-NP) were able to maintain their monolayers up to 72 h.p.i before displaying CPE (Fig. 2A). Cell counts also confirmed that targeting the NP mRNA could attenuate cell death triggered from NDV infection indicated by the significant increase (up to a 15 fold increase) in cell survival at 72 h.p.i (Fig. 3). Considering these observations, subsequent experiments were conducted exclusively using the miR-NP construct as a potential viral knockdown target.

3.2. Enhancing and challenging transduced DF-1 cells

To determine the potential of the miR-NP construct to convey long term protection at the cellular level, DF-1 cells were transduced with miR-NP and miR-SCR constructs that had been packaged in LV. To isolate a homogeneous population of cells that highly express pLV-shNP and pLV-shSCR, transduced DF-1 cells underwent two rounds of FACS sorting to isolate cells that were highly GFP positive. Due to the EmGFP gene and miRNA cassette being cistronic, cells expressing the EmGFP should also express the miRNA product. After stable cultures of post-sorted cells were established, each of the DF-1 transduced cell populations (pLV-miR-NP and pLV-miR-SCR) and a naïve control cell line (a non-transduced cell line) were challenged with LS-V at MOI 0.01. Phase contrast images at 48 and 72 h.p.i showed similar results to transfection results (Fig. 4A–F). pLV-miR-NP cultures retain an intact monolayer at 48 h.p.i (Fig. 4A; as indicated by arrow) while the SCR control and naïve DF-1 cultures show substantial syncytia formation and destruction of the monolayer (Fig. 4B–C; as indicated by arrowheads). However, by 72 h.p.i additional CPE were apparent in all of the cultures including pLV-miR-NP as demonstrated by the overwhelming presence of syncytia and little to no visibly healthy cells (Fig. 4D). Further characterization of NDV resistance of pLV-miR-NP was evaluated by determining viral titers

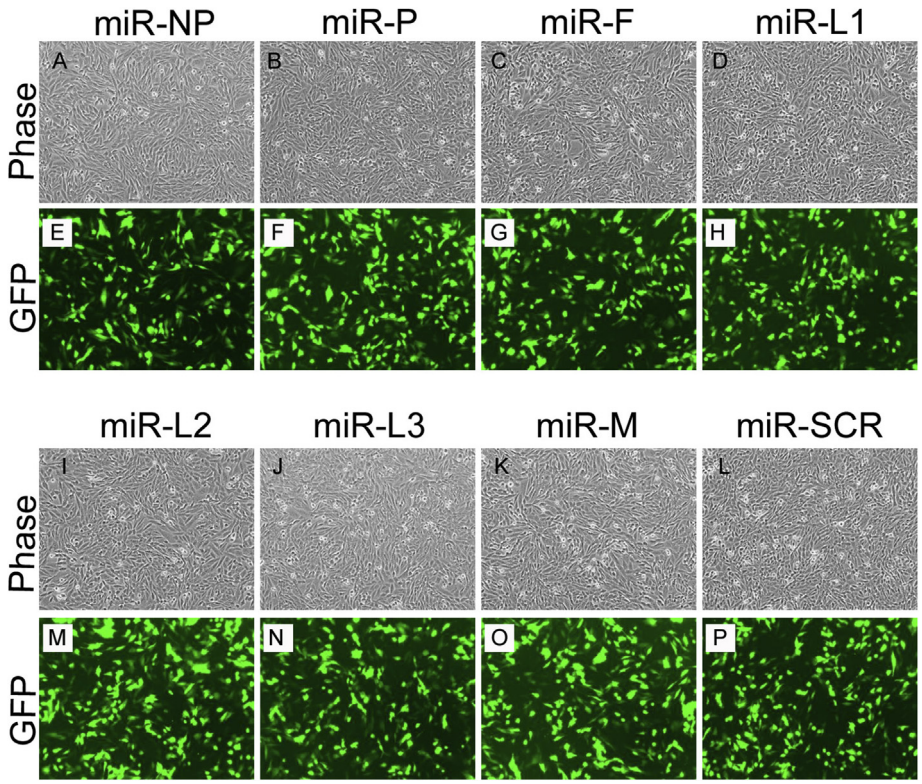

Fig. 1. miRNA transfected DF - 1 cells express high levels of GFP and poses healthy monolayers pre-NDV challenge. Phase contrast images of cultures transduced with miR-NP, miR-P, miR-F, miR-L1, miR-L2, miR-L3, mir-M and miR-SCR constructs have healthy and intact monolayers (A-D; I–L). EmGFP fluorescence expression indicated constructs successfully entered and were transcribed in DF-1 cells (E–H; M–P). All cultures had relatively similar levels of EmGFP expression. Phase and fluorescence images 10x.

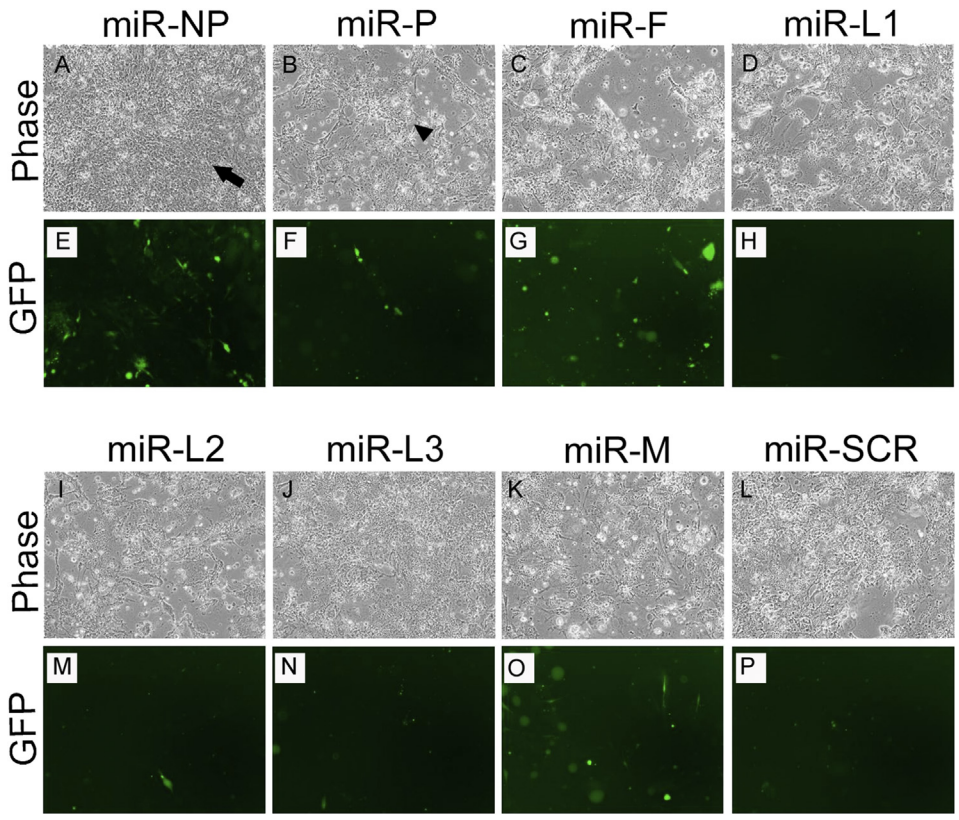

Fig. 2. miR-NP transfected DF-1s demonstrate higher levels of cell survival than other miRNA. At 72 h.p.i. most DF-1cultures show significant cell loss, high levels of cellular debris and syncytia formation (B–D; I–L) while miR-NP culture (A) maintains a monolayer and visually appears to have a higher number of cells. Along with destruction of the monolayer, EmGFP expression has been greatly decreased in most cultures (F–H, M–P) with the best retention in fluorescence in the miR-NP culture (E). Arrow indicates representative morphology of healthy DF-1 cells and arrowhead highlights examples of syncytia formation. Phase and fluorescence images 10x.

in supernatant collected after LS-V infection (MOI 0.01) at 0, 12, and 24 h.p.i. pLV-miR-NP resulted in significantly ($p < 0.05$) lower NDV viral titers at both 12 and 24 h time compared to pLV-miR-SCR (Fig. 4G). Taken together, these results suggest that DF-1 cells transduced with pLV-miR-NP are capably of decreasing the amount of NDV viral replication following in vitro viral challenge with a velogenic strain of NDV.

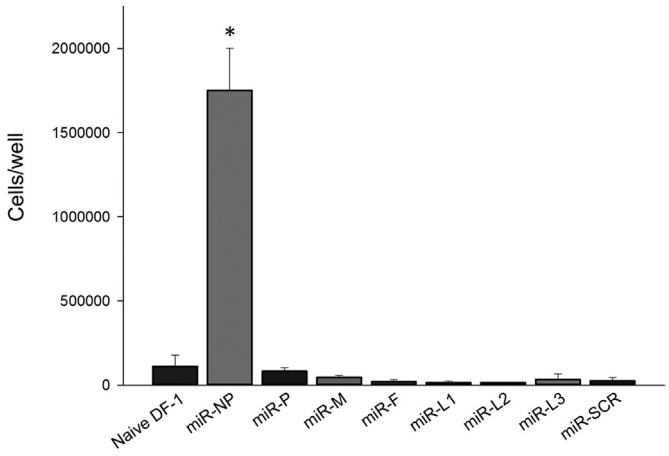

Fig. 3. miR-NP transfected DF-1 cells have greater viability 72 h after LS-V challenge. Cell counts at 72 h.p.i with LS-V (MOI 0.01) reveals DF-1 miR-NP cultures had a significantly higher cell population when compared to the other miRNA transfected cells. * indicates significance difference compared to Naïve DF-1, DF-1 cells transduced with other miRNA sequences and scramble control sequence at a $p < 0.05$. Data are the means of three biological replicates, and represented as mean + standard deviation of the mean (SDM).

4. Discussion

In this study we demonstrated that knockdown of the NP NDV viral mRNA in DF-1 cells could lead to decreased cell death and reduced titers (up to a 2-log decrease compared to pLV-miR-SCR control) during early stage infection with highly virulent LaSota NDV. Knockdown of the NP protein resulted in delayed viral replication when compared to the scramble control. In unsegmented, negative strand RNA viruses, such as NDV, the NP protein plays a significant role in the replication and transcription of NDV [24]. Specifically, NP, together with both the P and L proteins, interacts with the genomic RNA to form the ribonucleoprotein (RNP) which is the template for RNA synthesis. In this protein complex, NP encapsulates the RNA genome allowing proper function of the NDV polymerase [25,26]. NP knockdown lowers the amount/viability of RNP and, by disrupting this essential lifecycle step, it is reasonable to suspect that knocking down the NP transcripts within infected cells resulted in the observed delay of viral replication, as shown by another group working with NDV [17]. A similar study conducted with AIV (an orthomyxovirus) also used RNAi to target several of AIV's proteins and reported knockdown of NP was notably effective in limiting production of the virus compared to other targets [27]. Furthermore, other influenza studies have also targeted the NP protein using RNAi and observed a decrease in the amount of the reciprocal viral mRNA, virion RNA and its complementary RNA [28]. The NP protein has able been indicated as a target for administering antiviral drugs [29]. Results such as these

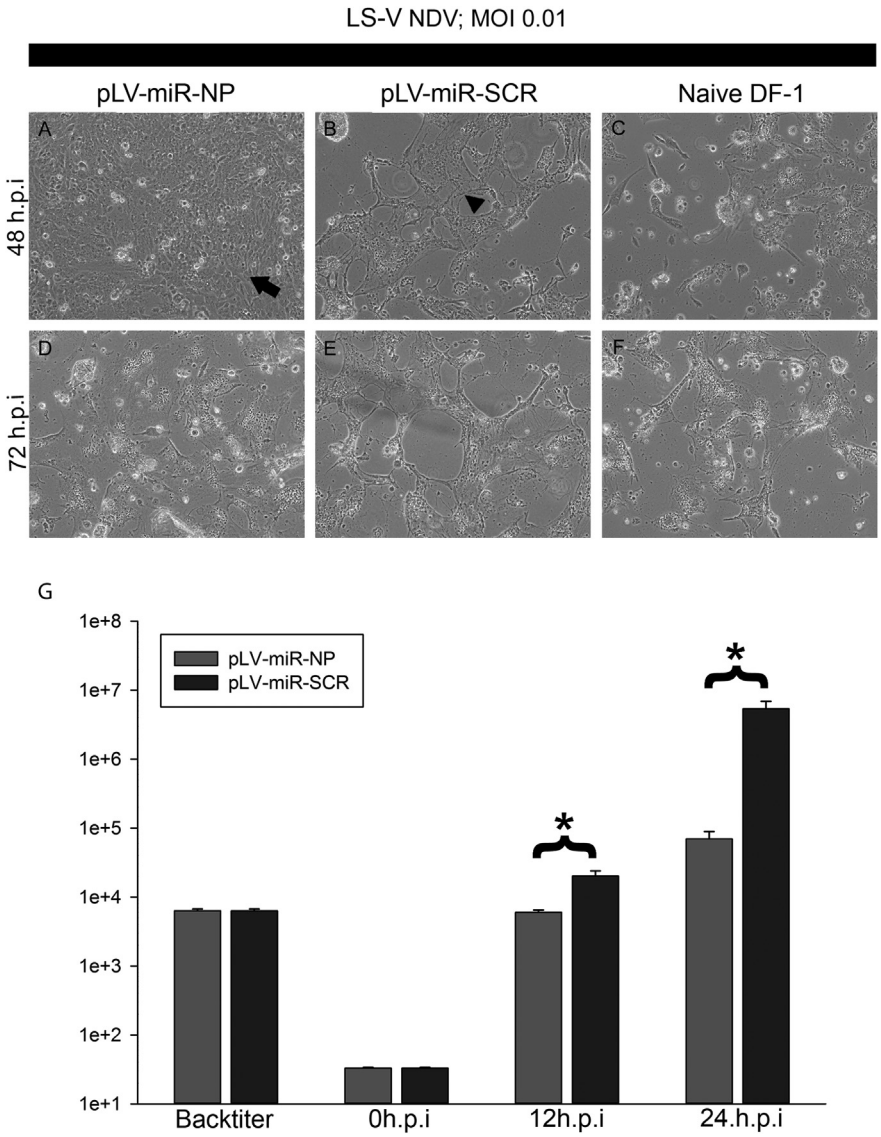

Fig. 4. DF-1s transduced with LV-miR-NP challenged with of LS-V MOI 0.01 have increased cell survival and decreased viral titers. Representative images 48 h.p.i. (A–C) and 72 h.p.i. (D–F) Phase images show DF-1 transduced with pLV-miR-NP have less CPE when compared to pLV-miR-SCR and Naïve cell controls at 0.01 MOI (LS-V NDV) at 48 h.p.i. Arrow indicates representative morphology of healthy DF-1 cells and arrowhead highlights examples of syncytia formation. Titrations of supernatant (log $TCID_{50}$/mL) collected at 0, 12, and 24 h.p.i demonstrate a pLV-miR-NP cultures have less viral presence in the supernatant when compared to SCR control at 12 and 24 h.p.i. (G). Backtiter is indicative of LS-V added to infect cultures. * indicates significance difference at a $p < 0.05$. Data are the means of three biological replicates, and represented as mean + standard deviation of the mean (SDM).

suggest that NP may be a prime target for controlling and limiting viral replication NDV and similar viruses.

pLV-miR-NP transduced DF-1 cells were observably healthier than control cells types up to 72 h after challenge with LS-V. However, these cells were unable to survive long term in culture with cells at 72 h.p.i., showing significant cell death and syncytia formation by at this time point. While long-term survival was not observed in vitro, it is possible that delaying the rate of infection can ultimately lead to improved animal survival. Commonly, a standard challenge dose between 10^5 and 10^6 EID50 is used experimentally to induce 100% infection and clinical signs in chickens [18]. Based on our observation that pLV-miR-NP led to a 2-fold decrease in viral titers in vitro, in theory, a transgenic bird containing our construct may require an increased amount of virus to generate an infection. It is also reasonable to consider that a decreased amount of viral titers detected could translate to a reduction in viral shedding of infected birds. Such is the case in a study completed by Lyall et al. exploring the amount of viral shedding in transgenic chickens expressing an shRNA against the polymerase of AIV [16]. Lyall describes that after viral challenge with a highly pathogenic strain of AIV, efficiency of transmission of the virus to other transgenic birds as well as non-transgenic animals was mitigated as assessed by histopathology and immunohistochemistry [16]. While some birds died in the study, the decrease of viral transmission can translate into a reduction of viral propagation to birds in close contact, decreasing the spreading of the disease and substantially contributing to outbreak control. With the success of infectivity studies with AIV, it is conceivable that similar results could be obtained with NDV challenge studies in transgenic birds.

Other researchers have had success using the NP protein as an anti-viral target utilizing RNAi methodology to inhibit NP expression and reduction of viral titers in culture, however this work was done in non-avian Vero cells [30]. It is more beneficial to conduct studies in an avian cell type as the NDV virus does not have to adapt to a non-native cell type, which may lead to mutations that are not

naturally found in chickens. In addition, the study of RNAi approaches in avian cultures is likely to be more representative with respect to the pathophysiology of NDV. Yue et al. also showed that shRNA targeting of NP in chicken embryonic fibroblasts lead to knockdown of viral NP mRNA [17]. However, this study failed to examine the effect of constitutive expression of the NP shRNA and only examine transient expression. Understanding the effect of continued long term expression is a key component if this technology is ever to be translated to use in live animals in a production setting. In this study, the miRNA sequences were designed based on conserved regions among genotype II representatives. This genotype was selected because of the extensive characterization of LS-V (a representative of genotype II), both *in vivo* and in vitro. In order to provide protection against the many NDV genotypes circulating worldwide (eighteen described so far [31]), other conserved genomic regions, in close proximity of the one used here, could be used to generate a broadly protective effect. For instance, this could be accomplished by deploying chaining pre-miRNAs that could deliver multiple miRNA species, therefore targeting multiple conserved regions at the same time.

5. Conclusion

pLV-miR-NP constructs constitutively expressed in DF-1 cells led to attenuated CPE and LS-V titers after NDV viral challenge. This study suggests that future transgenic animal studies are warranted. This would likely result in animals that are capable of having endogenous resistance to NDV infections and can have progressive benefits for small village farmers as well as large scale international poultry operations. With potential widespread adoption of resistant transgenic birds, one day the production of resistant animals maybe even more cost effective than the use of standard vaccine programs. Traditional vaccine programs have associated costs of transportation, logistics and infrastructure to maintain a cold chain, labor costs to administer vaccines and the cost of lost birds due to ineffective vaccines. In addition, the use of miRNA NDV resistant birds in economically impoverished countries and rural areas where vaccines are not readily available would be potentially paradigm changing by providing food and financial security.

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