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Executive Summary

Commercial production of MDV vaccines relies on the use of primary avian cells (Chicken Embryonic Fibroblasts - CEF) to amplify the vaccine. This process involving CEFs is both expensive and time consuming, and during an outbreak may limit the availability of vaccine. This project proposed the generation of a novel cell line for the amplification of MDV vaccine virus. We demonstrate here that removing immune defence mechanisms from DF-1 cells, an existing cell line, provides significant increases in viral titre and, possibly in conjunction with an additional gene deletion, may provide a new and convenient growth substrate for MDV vaccine to enhance the protection of Australian poultry flocks.

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Introduction

Background

Marek's disease virus (MDV) is an alpha herpes virus that is associated with losses to the worldwide poultry industry of $1–2 billion annually (Morrow and Fehler, 2004). Live vaccines have been widely used to control MD since their development in 1970 (Powell and Lombardini, 1987; Bublot and Sharma, 2004) and have shown great efficacy in reducing disease burden. Nevertheless, Australia has experienced several outbreaks of MDV and there is evidence that the virulence of this virus may be increasing (Witter, 1998; Morrow and Fehler, 2004). Although changes in MDV virulence and serotype pose a constant threat, vaccination remains an effective control measure and it is critical that the capacity and resources are available to meet any new challenges with regard to potential MDV outbreaks.

A key restriction for industry in the current production system for MDV vaccines is that the vaccine virus only replicates successful in primary avian cells (Denesvre 2013). At present, the gold standard for MDV vaccine production is through the use of Chicken Embryonic Fibroblast (CEF) primary cells which must be newly prepared from embryos (embryonic day 11). **This isolation is far from ideal for vaccine production as it is both expensive and time consuming** as well as the need to prepare these cells afresh each time. Moreover, should an MDV outbreak of any significance impact the Australian poultry industry, this method of vaccine production may **limit the availability of vaccine**. With this in mind, there is a need to develop new cost effective strategies for the increased production of MDV vaccines and a reduced reliance on the use of primary CEFs for vaccine production.

One novel approach to overcoming the dependence of MDV vaccine production on primary CEFs is the **use of immortalised cells lines to replace these inefficient and inconsistent primary cells**. Immortalised cells are superior as they can be stockpiled and expanded as required and have little variation in cell growth rate or in viral replication from batch to batch. Although immortalised cell lines are currently used for production of other vaccines, at present there is little evidence for cell lines that have shown efficacy in growing the MDV vaccine strains.

Vaccine producers have identified that the development of a novel cell line to generate MDV vaccine is of the highest priority. Furthermore, cell lines that are developed have the potential to be rapidly adopted and implemented into commercial vaccine production system to replace the less efficient CEFs.

***This project looks to improve viral vaccine production by providing a unique cell line tailored for enhanced vaccine production*.**

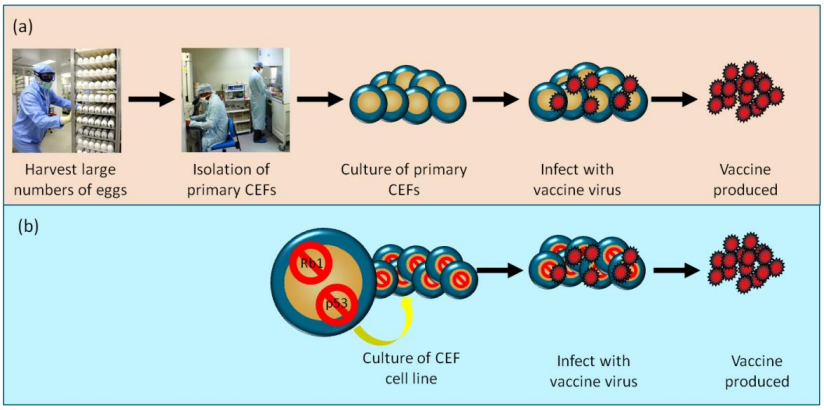
This project takes a multipronged approach to develop a novel avian cell line that has the capacity to grow MDV vaccine strains. Importantly, the development of a cell line that can replace primary CEFs would have

immediate commercial applicability to MDV and may be rapidly applicable to other avian vaccine production systems.

Initially, our aim was to undertake a comparative study to determine the potential for MDV vaccine production capacity in a range of currently available avian cell lines, such as DF-1 and HD11, compared to CEF. Previous studies have been performed using these cell lines with other avian viruses, however, no comparative studies are currently reported for MDV. This study provides an insight into the commercial potential for each cell line and their ability to grow MDV vaccine virus. This study may directly point to a cell line that has commercial advantages over CEFs.

As a second step, we sought to develop an immortalised cell line from CEFs (as they are known to grow MDV well) using a new molecular technology (CRSIPR) to delete (or knock-out) genes known to modulate cell proliferation. This approach has the potential to provide a cell that has unrestricted (immortalised) growth. In the production of other non-avian cell lines, there are two key gene that have proven efficacy in converting primary cells into cell lines, RB1 (Giacinti C, and Giordano A. 2006; Ji, P. And Zhu, L. 2005) and p53 (Odell, A. et al 2010). As both genes are regulators of cell cycle they promote cell proliferation and induce cells to grow indefinitely (Christman et al 2005; Christman et al 2006). Removal of these check points from CEFs would allow increased growth and eliminate the need to produce primary CEF preparations each time, thereby reducing the costs and time required to make vaccines (Figure 1).

Figure 1. Gene removal to enhance cell propagation.

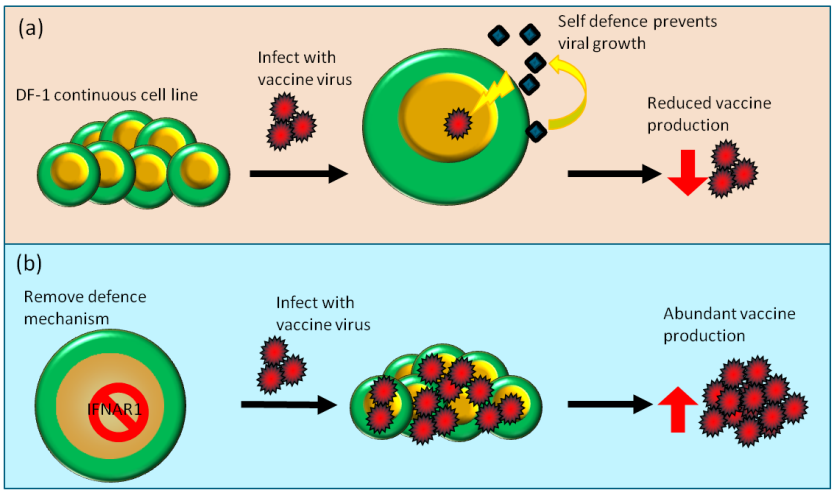


The schematic shows that although CEFs can be infected and produce vaccine virus, they cannot be propagated and require continuous egg harvesting and cell isolation steps each time vaccine is produced (a). If we remove either the Rb1 or p53 we will generate a continuous cell line that can grow vaccine virus and reduce cost and workload as well as being more consistent (b)

As a third approach to developing a continuous cell line for MDV vaccine growth we investigated the possibility of taking an existing cell line that can be infected with MDV vaccine virus and modifying that cell

line to enhance the growth of vaccine virus. Moreover, the currently available avian cell lines (such as DF-1, a fibroblast cell line) are poor amplifiers of vaccine virus replication, this may be in part due to the ability of these cells to 'defend themselves' against vaccine replication. The key mediators of this defence are the cytokines, such as the interferons (IFNs), which are produced rapidly in response to viral infection and interfere with, and restrict, virus growth (Figure 2).

Figure 2. *IFN activity decreases virus production.*



The schematic shows that the currently available avian cell line DF-1 is not good a producer of MDV vaccine as the cell defence mechanisms (such as IFN) can kill the virus (a). If we remove IFNAR1 (IFN receptor) we will reduce the ability of the cells to kill virus and hence generate a new cell line that can grow MDV vaccine successfully (b).

It has been known for some time that MDV has an intrinsic sensitivity to the antiviral actions of cytokines, particularly IFN (Haq *et al*., 2013). As such, there is strong evidence to suggest that in the absence of IFN MDV replication would increase significantly. Examples of this observation that impacting anti-viral activity leads to increased viral growth include the administration of influenza virus together with anti-IFN antibodies, which resulted in higher virus load, and in another example where IFN gene knock out mice show increased viral growth during infection. These data illustrate the importance of this cytokine in restraining viral growth. Similarly, this and other research supports the idea that, when vaccine strains are grown within cells the IFN response has the potential to produce a variety of immune molecules that may have a significant impact on the yield of virus. Therefore, strategies aimed at reducing IFN production in the cell will increase vaccine virus output. Furthermore, our laboratory has identified a number of targets, including the receptor for IFN Type I (IFNAR1), which when knocked down (reduction in gene expression by 60%) allowed greater than 10 fold increase in virus growth. This observation supports the argument that removal of the 'defence' system from the cell will allow improved viral titres. Therefore, strategies aimed at reducing IFN in the cell may lead to increase vaccine virus output.

Figure 3. Reduced IFN activity leads to increased virus production.

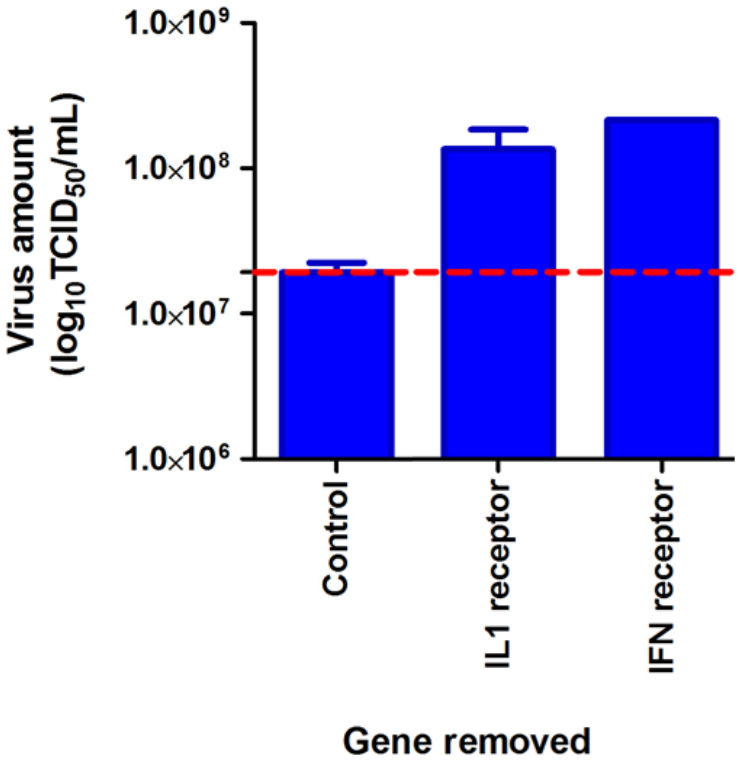


Figure 3: Our laboratory has discovered a number of genes whose expression relates to significantly increase viral growth. In the graph we see almost a full log increase in virus growth with the genes for IL-1 or IFN receptor are knock down (reduced expression) using siRNA.

Objectives

Aim 1: *Test a range of immortalised avian cell lines for their applicability for growth of MDV vaccine;*

Aim 2: *Using novel gene knock out (CRISPR) technology to remove the p53 and/or RB1 genes from CEFs and develop and immortalised CEF cells line for MDV vaccine growth;*

Aim 3: *Removal of antiviral genes (IFNAR1) from DF-1 cells to enhance their ability to grow MDV virus.*

Methodology

Cell Lines

Cells were cultured in 75 cm2 flasks (Corning, Lowell, USA) in a 37°C humidified incubator supplemented with 5% CO2. The chicken embryonic fibroblast cell line DF-1 from the American Type Culture Collection (ATCC) (CRL-12203) was maintained in Dulbecco's modified Eagles media (DMEM) (Thermo Scientific, Waltham, USA) supplemented with 10% (v/v) foetal calf serum (FCS), 2 mM glutamine, 1.5% (w/v) sodium bicarbonate, penicillin (100 U/mL), streptomycin (100 U/mL) and 10 mM N-2-hydroxyethyalpiperazine-N'-2-ethanesulfonic acid (HEPES). The chicken macrophage cell line HD11 (ATCC) (Beug et al.,, 1979) was maintained in DMEM supplemented with 10% (v/v) FCS, 2 mM glutamine, penicillin (100 U/mL), streptomycin (100 U/mL), and 10 mM HEPES. Madin-Darby Canine Kidney (MDCK) cells (ATCC CCL-34) were maintained in Eagles minimal essential media (EMEM) (Thermo Scientific, Waltham, USA) supplemented with 10% (v/v) FCS, 2 mM glutamine, penicillin (100 U/mL), streptomycin (100 U/mL), and 10 mM HEPES. Cells were routinely passaged as required (approximately every 3-4 days) to maintain optimal growth conditions. This involved harvesting by washing twice with phosphate buffered saline (PBS)-A and then incubating for 5 min at 37°C with 5 mL of trypsin (Gibco Invitrogen, Carlsbad, USA) and collected into approximately 10 mL of appropriate media. Cells were centrifuged at 500 g then resuspended in 5 mL of fresh media. New flasks were seeded with 1:5 or 1:10 dilutions for future use. Viable nuclei were quantified using the CellInsight Personal Cell Imager (Thermo Scientific) at a magnification of 10 ×, with images captured of all areas of each well.

Preparation of CEF's

CEF's were removed from frozen storage and washed in DMEM and resuspended in 20 mL DMEM with 2% FCS and counted cells using a Countess. Cell concentration was adjusted to 1.5 x 10^6 cells/mL and seeded into either T150 or T75 flasks.

Viruses

Influenza A/WSN/33 (H1N1) and A/Puerto Rico/8/1934 (H1N1) were passaged in 10-day old embryonated specific pathogen-free (SPF) chicken eggs (Australian SPF Services, Woodend, Vic, Australia). Allantoic fluid was harvested, aliquoted, and stored at -80˚C for inoculations.

MDV (MD-Vac) was obtained from Zoetis. A glass ampoule of MD-Vac live vaccine was removed from frozen storage, thawed rapidly in a warm water bath and diluted to 5 mL in DMEM. The cells were centrifuged at 400g for 5 minutes, s/n decanted and cells resuspended in 1 mL DMEM with 2% FCS. Ampoule contained 2000 doses of virus, at 1000 pfu/dose. Therefore we have 2 x 10^6 pfu in 1.2 mL, which is 1.6 x 10^6 pfu/mL. Approximately 1.5 x 10^7 viable cells were transferred along with either 1.1 mL or 0.1 mL of MDV infected cells into separate flasks in a total volume of 15mL along with an uninfected control. If all cells were infected with HVT, then the flask that received 1.1 mL of vaccine has an MOI of 0.2 and the flask that received 100 µL of vaccine an MOI of 0.02. We then proceeded with the cells infected with an MOI of 0.02 as those infected with 0.2 died too quickly. Subsequent passages of the virus in the different cell lines were performed with either a 1/1000 (low dose) or 1/6 (high dose) dilution of the 0.02 MOI pass 1 culture. Cell suspensions from the 2nd or 3rd passage infectious stocks were counted, and a similar number of cells from each sample was serially titrated for infection of CEF's in a 6-well plate plaque assay. The MOI for the Rispens strain was 0.015 for the generation of stock culture, which was then passed once in the different cell lines at a 1/25 dilution.

Quantitative real-time polymerase chain reaction

Cellular RNA was purified and cDNA synthesized as described [13]. Quantitative real-time polymerase chain reaction (qRT-PCR) experiments were conducted using an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The comparative threshold cycle (ΔΔCt) method was used to derive fold change gene expression. All results were normalized to chicken glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Primers and probes were from Applied Biosystems (refer Table 2 for list). PCR

cycling was performed using Applied Biosystems Taqman reagents as follows: 95˚C for 10 minutes, followed by 40 cycles of 95˚C for 15 seconds and 60˚C for 1 minute.

Tissue culture infectious dosage$_{50}$ (TCID50) analysis

Tissue culture supernatants and allantoic fluids containing virus were serially diluted 10-fold in PBS and 100 µL added to 96-well plates containing confluent MDCK cells. The plates were incubated for 5 days at 37˚C with 5 % CO_2. Following incubation, wells were inspected and scored for cytopathic effect. The infectious titer was calculated by the method of Reed and Muench [15].

Plaque assay

We seeded 6-well plates with 3mL of cell suspension/well (4.2 x 10^5/mL; 1.3 x 10^6 cells/well) and allowed the cells to adhere. Infectious stock was removed from frozen storage and thawed rapidly and a 1/10 dilution was prepared for counting. We calculated the volume of infectious cell suspension to deliver approximately 8.3 x 10^5 cells in 100 µL to the first well. From the initial dilution (in 150 µL), 10-fold serial dilutions were prepared up to 10^{-5}. A 100 µL aliquot of each sample was added to corresponding wells and incubated O/N at 39˚C. After 24 hours of infection, the 6 well plates of CEF's show ~80% confluence. The infectious media was removed and an agar layer added to each well and returned to incubator at 39˚C for a further 5 days (a total of 6 days of infection). Working in a class II cabinet, we fixed the cell sheet by adding 1.5 mL of 4% PFA and incubated at RT for 1 hour. Once the cells were fixed we removed and discarded the agar and rinsed the wells with water. The plates were allowed to air dry and 2mL of 0.05% neutral red stain was added to each well. The plates were incubated at RT until satisfactory staining is achieved (around 30 minutes to 1 h). We then gently poured off stain and rinse wells twice with water. The plates were allowed to dry and the plates were examined for focus forming units (typically white and approximately 0.5 mm in diameter). The well was excluded if it contained less than 5 plaques. The titre is calculated using the following equation, as is expressed as ffu/mL (or pfu/mL)

$$\text{pfu/mL} = \frac{\text{number of plaques}}{(\text{dilution})(\text{volume})}$$

Immunofluorescence

Cells were fixed with 4 % paraformaldehyde for 1 h, permeabilised with 0.1% Triton X-100 (Sigma) in PBS for 10 min, then blocked with 0.5% bovine serum albumin (BSA) (Sigma) in PBS for 30 min. Cells were stained to detect influenza virus A/WSN (mouse monoclonal (AA5H). Cells were stained with 1/200 dilution of the appropriate secondary antibody purchased from Invitrogen (anti-mouse AF488). Nuclei were labelled with DAPI 1:4000 in distilled water for 15 min. The number of viable nuclei per treatment group and the level of influenza virus antigen staining was quantitated using the CellInsight Personal Cell Imager (Thermo Scientific, Waltham, MA) using the Target Activation bioapplication of the Cellomics Scan software (iDev workflow).

Generation of CRISPR Cas9 mediated IFNAR1 KO cell lines

We used the RNA-guided Cas9 nuclease from the microbial clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) system, to produce a dual double-strand break (DSB) by duplexing constructs encoding two guides RNA (sgRNA) as it was previously reported [18]. Briefly, two sgRNA (GCCGCGTGCGCAGTCGTCAGAGG, left hand and AGCACCGGGACACCACGACCAGG, right hand); were cloned into to the pSpCas9(BB)-2A-GFP (Plasmid ID: 48138; ADDGENE) and transfected into the continuous chicken embryo fibroblast cell line (DF-1). The two sgRNA acted together in order to produce a deletion in the chicken interferon (alpha, beta and omega) receptor 1, IFNAR1 gene (Gene ID: 395665). The expected deletion was around 100bp in (Figure 5A). Transfected cells by Lipofectamine™ 2000, were sorted using a BD FACS Aria II cell sorter based on their GFP expression. A second round of sorting was performed to obtain single clones for further expansion and genomic DNA (gDNA) PCR screening. The identification of targeted cells was simplified to a quick gDNA PCR screening on clonogenic isolations of the cell lines after sorting. Primers for screening were: Forward 5'-CGGCCACCCAAACCTTAGAA-3' and reverse 5'-CCATCTCGCAGCAGTTGTCT-3'. gDNA and PCR was performed using DNeasy Blood & Tissue Kit (Quiagen) and GoTaq Flexi DNA Polymerase (Promega) respectively; following manufacturer's instructions. In order to confirm the identity and extent of the deletion, amplicons were excised, purified from the gel (The Wizard® SV Gel and PCR Clean-Up System)

and cloned into the pGEMt-Easy vector (Promega) for sequencing and analysis at Micromon, Department of Microbiology, Monash University, Clayton.

Table 1: Sequences for generating IFNAR1 KO cells

Target Gene	**Interrogated Sequence Accession no. (GenBank mRNA)**	**Exon Boundary**	**Assay Location**	**Amplicon Length**
GAPDH	AF047874.1	7-8	805	83
IFNAR1	AF082664.1	5-6	775	146

Table 2: Guide Sequences for Generating p53 KO Cells

Guide #1	ATGGGGGGGACTTCGACTTC**CGG**
Guide #2	CCCCATAATCCTCCGTGGAT**GGG**
Guide #3	GAATAAGGTCTATTGCCGCC**TGG**
Guide #4	CGACTTCCGGGTGGGGTTCG**TGG**
Guide #5	ATAGACGGCCACGGCGCGGA**GGG**
Guide #6	GGGACTTCGACTTCCGGGTG**GGG**
Guide #7	TCTTATAGACGGCCACGGCG**CGG**
Guide #8	GGGGGACTTCGACTTCCGGG**TGG**
Guide #9	TATAGACGGCCACGGCGCGG**AGG**
Guide #10	TGGGGGGGACTTCGACTTCC**GGG**

Table 3: Generation of CRISPCas9 mediated RB1 KO cell lines

Guide number	Guide sequence
Guide #16	AGTGGGGGAAGCGGAGTTCG**TGG**
Guide #27	GCCTTCTCCCTCACGCTGTC**CGG (RC)**
Guide #6	GAC**GG**CAGAGCGCCGCAACG**GGG**
Guide #10	CCGAGAACTGCTTCGGGTGAC**GG**

To obtain a 250bp sequence for designing guide RNA we included 130 bp at the 3’ end of Exon2

(168747881-168748128) of the RB1 gene. We designed four separate guides for the deletion of Exon 2.

RB1 exon 2 sequence including 3’ non-coding sequence

GCTGGAAGTGGGGGAAGCGGAGTTCGTGGCTCTGTGCGATGCGCTGAAGGCGCCGGACAGCGTGAGGGAGAAGGC
CTGGATGACGTACGAGAGCTTGGCGGCCGCCGACGGGGCTTCGGTGAGTGCGGTGCCCGCCGCGGGGCTGCGGTCC
GCTCGGAGCGGTGTCCGGCGGCCCCGAGAACTGCTTCGGGTGACGGCAGAGCGCCGCAACGGGGCGCAGGGGGAG
GCTGCGGGGTCTCCTTCTTTGGAG

Resulting deletion

Guide 16 and 6 = 168bp
Guide 16 and 10 = 188bp

Table 4: Primers designed for screening RB1 KO cells

Primer Set 1	Sequence (5'-3')	Template Strand	Length
Forward Primer	CAGGAGTCAGCGCACCAG	Plus	18
Reverse Primer	CTCCAAAGAAGGAGACCCCG	Minus	20
Product Length	453		

Primer Set 2	Sequence (5'-3')	Template Strand	Length
Forward Primer	GGTAAGCGCAACGCAACG	Plus	18
Reverse Primer	CCCGAACATCTCCAAAGAAGGA	Minus	22
Product Length	400		

Primer Set 3	Sequence (5'-3')	Template Strand	Length
Forward Primer	GGTAAGCGCAACGCAACG	Plus	18
Reverse Primer	CCCGAACATCTCCAAAGAAGGA	Minus	21
Product Length	401		

Primer Set 4	Sequence (5'-3')	Template Strand	Length
Forward Primer	GCACCCGGTAAGCGCAA	Plus	17
Reverse Primer	CATCTCCAAAGAAGGAGACCCC	Minus	22
Product Length	400		

Primer Set 5	Sequence (5'-3')	Template Strand	Length
Forward Primer	GGCGGCACCCGGTAAG	Plus	16
Reverse Primer	TCTCCAAAGAAGGAGACCCCG	Minus	21
Product Length	402		

Statistics

The difference between two groups was statistically analysed by a two-tailed Student's *t*-test. A *p*-value of <0.05 was considered significant. $**p<0.01$, $*p<0.05$, N.S. not significant. All data points are the average of triplicates, with error bars representing standard deviation. All data is representative of at least 2 separate experiments.

Results & Discussion

MDV is well known to cause significant losses to the poultry industry and the most effective control of the disease is vaccination. The propagation of MDV vaccine is not straight forward and typically requires primary CEFs and/or chicken kidney cells. The preparation of these cells is both time consuming and costly. Ideally, the identification of, or generation of, a continuous cell line that is capable of generating large quantities of virus would alleviate these problems. This cell line would also reduce batch to batch variation and possibly allow more stable production of MDV vaccines.

In our first aim we tested the commercial feasibility of available chicken cell lines to grow MDV. One of the proposed cell lines, LMH cells, which is a hepatocellular carcinoma cell line, was discounted early in our studies due to lack of commercial applicability. The LMH cells could not be cultured in significant numbers without the use of a gelatin layer (data not shown) making their use in large scale production not cost effective by comparison to other cell lines. Both DF1 cells and HD11 cells grew well in culture and were able to be tested for growth of MDV, and as such, we compared the growth of MDV in these two cell lines to the gold standard CEF cells. We also compared two different concentrations of the HVT strain of MDV as well as a single concentration of the Rispens strain in all three cells lines (Table 5). We demonstrated that the HVT strain was capable of growing in both the CEFs and the DF1 cells, however, the HD11 cells were not permissible. Although both the CEF and DF1 cells produced virus, the CEF cells produced >12 fold more virus that the DF1 cells. Intriguingly, the Rispens strain of virus was only able to propagate in the CEFs. Neither the DF1 nor HD11 cells could effectively grow this virus. The titres from the CEFs for both strains did appear somewhat reduced to what we might expect from MDV growth in these cells, suggesting our culture conditions may not have been optimal. The assay, however, still provided a point of comparison between the different cell lines. The fact that both DF1 and HD11 cells were incapable of propagating the Rispens virus and the DF1 cells produced >12 fold lower levels of the virus compared to CEFs would suggest they would not be a suitable direct replacement for CEFs in a commercial setting.

We then sort to generate a new cell line derived from CEFs using cutting edge genomic engineering techniques. We aimed to remove either the RB1 or p53 genes to allow uncontrolled and immortal cell growth

Table 5: MDV Growth in Cell lines

a) HVT (MOI 0.02 - 1/6)

Host Cell Line	Serial dilution						
	-1	-2	-3	-4	-5	Ctrl	Pfu/mL
CEF	>100	>100	40	15	2	0	1.5×10^6
DF-1	>100	100	12	0	0	0	1.2×10^5
HD11	0	0	0	0	0	0	0

b) Rispens

Host Cell Line	Serial dilution						
	-1	-2	-3	-4	-5	Ctrl	Pfu/mL
CEF	0	1	1	0	0	0	1.0×10^4
DF-1 WT	0	0	0	0	0	0	0
HD11	0	0	0	0	0	0	0

in CEF cells. We first designed sgRNAs for each of the genes to cause a change in the genomic DNA resulting in a non-functional transcript. We took two separate approaches towards the two genes. The p53 gene is not annotated in the chicken genome and, therefore, we were unable to design a 'screenable deletion'. Typically when designing a deletion of a gene, a portion of an exon is targeted giving a deletion of >100bp so that the deletion can be 'screened' by PCR. Therefore, our approach to generate a p53 gene deletion was to design a number of sgRNAs (table 2) in known coding regions derived from an expressed gene (EST) found on NCBI. The guides targeting the p53 gene were designed to cause an indel (insertion/deletion). These indels may or may not result in a non-functional transcript. After inserting these guides into a CRISPR-Cas9 vector we transfected DF1 and using a GFP marker derived from the vector FACS sorted GFP positive (transfected) cells. We then passaged both the transfected and non-transfected cells for a number of passages and observed their growth rate and morphology over time. We were unable to see any difference in growth rate (data not shown) or longevity of passage between the two populations. Both cell populations significantly changed morphology (Figure 4) at ~15 passages and would no longer proliferate. As we were unable to screen to see if any p53 indels had occurred we are unable to determine if the gene was not functional but the lack of p53 did not confer increased proliferation potential or if we were unsuccessful in generating a p53 KO. The approach to generating an RB1 KO cell line used a different approach, whereby we designed a number of sgRNAs (table 3) that would give a known deletion of >150bp which could then be detected using a combination of primers in table 4. Following transfection of the cells using two different combinations of sgRNA and long term culture we again saw the slowing of growth rate in both the transfected and non-transfected populations. Additionally the morphology changed to a heterogenous population based on size and shape (Figure 5). This suggested that both populations had become senescent. We then screened for the gene deletion (Figure 6) using the primer pairs from table 4. Only Primer pair 1 was able to detect the WT gene and neither of the sgRNA combination showed the presence of the reduced sized band indicating the cut was not present. This may have been for one of two possible reasons. Either the sgRNAs were not effective in cutting the gene and hence no cell had a proliferation advantage, or alternatively, it is possible that in the gene deletion was achieved but only to a very low extent and we are unable to detect the KO amongst the WT. In either circumstance the use of CRISPR was not able to confer unlimited growth potential

Figure 4: p53 knock out cells do not form a continuous cell line

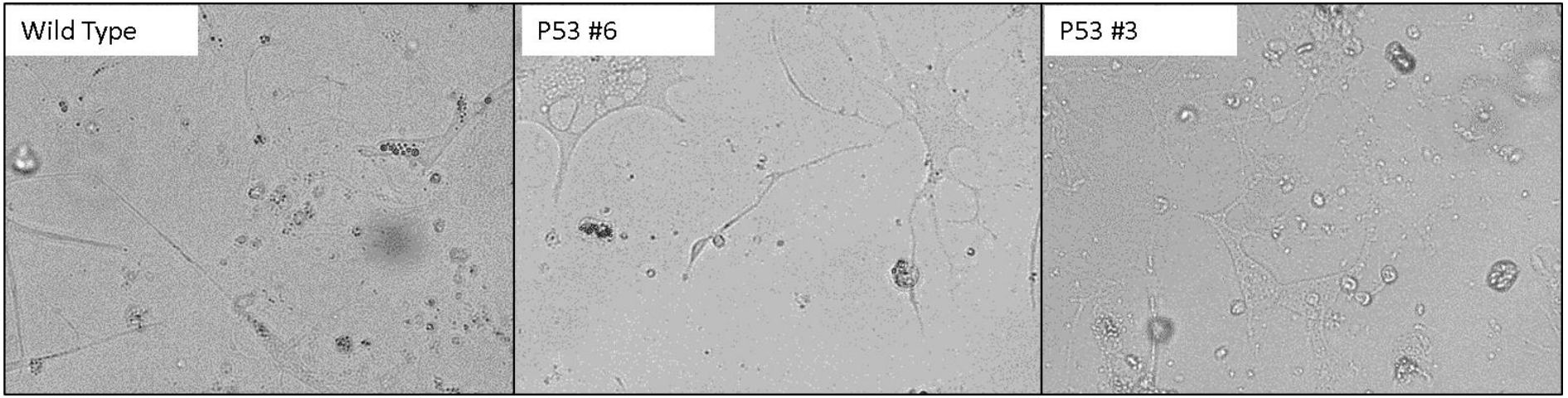


Figure 5: RB1 deficient cells do not form continuous cell lines

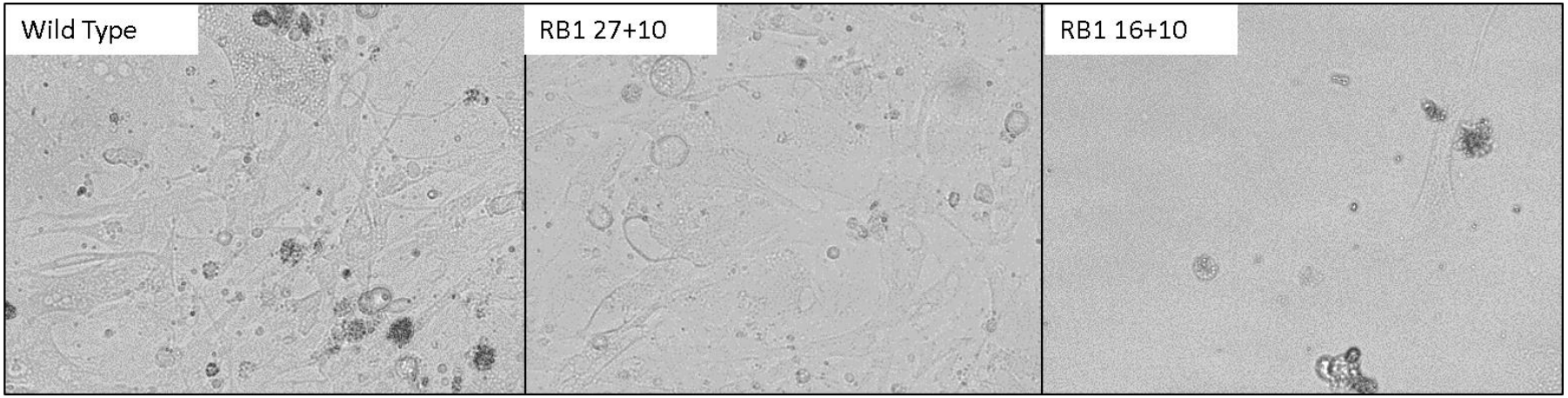
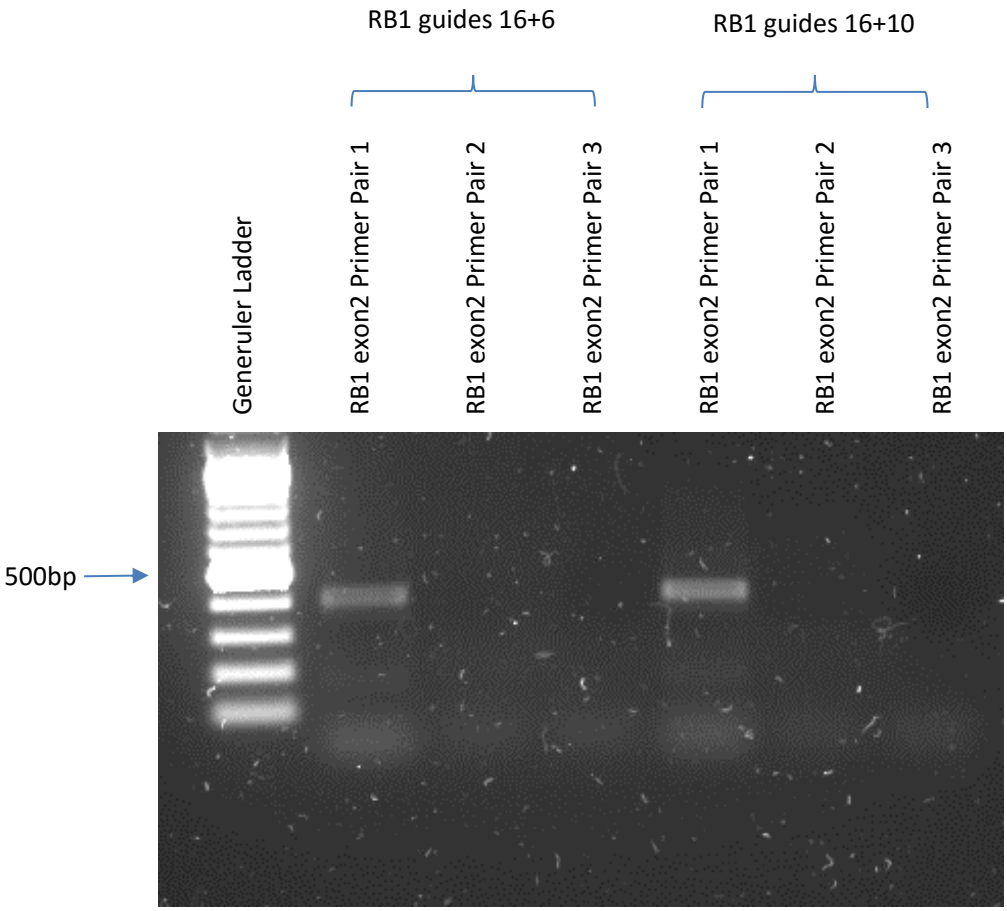


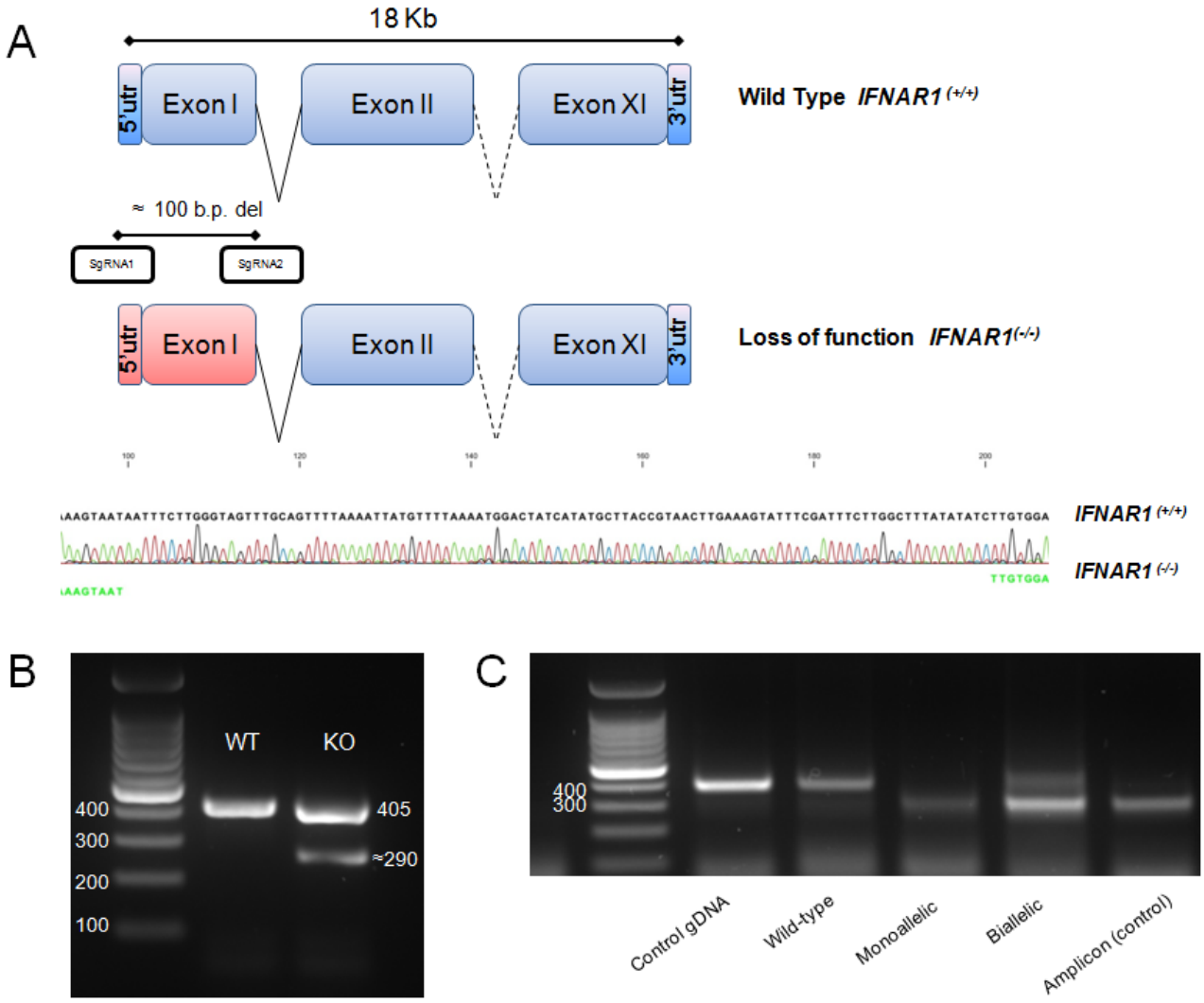
Figure 6: Detection of RB1 knock out cells



in this experiment. In future studies the generation of a homologous RB1 KO with confirmed deletion would be advantageous.

The third aim of this project was to remove an immune defence mechanism in an already established cell line in order to generate a new more productive cell line. Using data previously generated in our laboratory we chose to remove the interferon receptor, IFNAR1. The interferon system is a potent controller of viral infection and removal of this system is likely to have a significant impact on the production of MDV in cells. Based on the results from the first aim we chose to remove the IFNAR1 gene from DF1 cells as they had already been shown to be superior to HD11 cells in their MDV growth capabilities, in addition to a faster growth rate (data not shown). In order to achieve this we designed sgRNAs to flank the first exon of the IFNAR1 gene in the chicken (Figure 7a). These two guides were designed to cause a 100bp deletion which could be detected by PCR of a region including exon 1. This deletion is illustrated by the comparison of the $IFNAR1^{(+/+)}$ vs $IFNAR1^{(-/-)}$ sequence (Figure 7a). Following transfection of the cells we performed the PCR on both the WT cells as well as the KO and we were able to detect a band approximately 100bp smaller than the WT (Figure 7b). We can see from this image, however, that both the WT sized band as well as the reduced sized band (KO) are both present. This would suggest that there is a mixed population of WT and KO following the transfection. This is to be expected as the transfection efficiency and the CRISPR-Cas9 cutting are not 100% efficient. We then used flow cytometric sorting to generate single cell colonies from the mixed population. Following PCR of many of these single cell colonies we were able to identify cells that were WT (no cut), monoallelic (cut on a single copy of the gene) and biallelic (cut on both copies of the gene). We then aimed to further validate the removal of the gene by qPCR. We demonstrated that the basal level (resting unstimulated) of the IFNAR1 mRNA was significantly reduced in both the monoallelic and the biallelic cell lines we had generated (Figure 8a). As might be expected, the biallelic was further reduced when compared to the monoallelic as a copy of the WT gene still exists in the monoallelic. The IFNAR1 receptor is known to increase in expression when stimulated by virus or PolyI:C (a viral analogue). We therefore sort to determine the impact of stimulation using WSN influenza strain and PolyI:C. We chose WSN to validate this as it does not need to be adapted to the cells and is therefore easier to work with. We showed that stimulation with neither virus nor PolyI:C could recover the mRNA levels of to the same as WT expression (Figure 8a). We

Figure 7: Generation of IFNAR knockout cell lines.



therefore concluded that the IFNAR1 gene had been successfully removed from DF1 cells. Prior to undertaking any adaptation of MDV into our IFNAR1 KO cell line we needed to functionally characterise this KO with regards to viral growth. Again we chose WSN for ease of use and tested the viral growth in a plaque assay at 10 and 48 hour post infection and compared the WT cells to both monoallelic and biallelic (Figure 8b). The biallelic KO grew virus to a significantly higher titre at both time points when compared to the WT, however there was not significant difference when WT was compared to the monoallelic KO. It is not unexpected that a single remaining functional IFNAR1 gene is able to achieve viral control to the same levels as WT, however, this was strong evidence that the biallelic KO cell line had enhanced viral growth properties. Following the validation of the removal and IFNAR1 gene transcript and functional validation of increased viral growth using a model influenza virus, we tested the ability for the biallelic IFNAR1 KO cells (referred to as DF1 KO) to grow increased titres of MDV. We compared the DF1 KO cells with the gold standard CEF as well as DF1 WT cells. We did this using two different doses of the HVT strain of MDV (high = 0.2 and low = 0.02) as well as a single dose of the Rispens strain. Importantly we were able demonstrate an increase ability of the DF1 KO cells to grow the HVT, >12 fold and >4 fold, at both the low (Table 6a, Figure 11a) and high (Table 6b, Figure 11b) inoculum, respectively, when compared to the DF1 WT cells. Interestingly, the DF1 WT cells were unable to produce the Rispens strain of MDV and the KO DF1 cells had a productive titre. Neither the DF1 WT nor the DF1 KO produced an equivalent titre when compared to CEFs. However, when using the low dose inoculum the DF1 WT cells had >720 fold less MDV than CEFs, where DF1 KO had only >55 fold reduced capacity to produce the HVT strain of MDV. When the higher starting inoculum was used the CEF generated >12 fold more HVT than CEF whereas the DF1 KO were only 2.5 fold lower titre. Interestingly, the DF1 WT cells could not propagate the Rispens strain of MDV, whereas both the CEFs and DF1 KO could (Table 6c, Figure 11c). The titres in both CEFs and DF1 KO cells were quite low and suggest a higher starting MOI should be used for future experiments.

Taken together it is clear to see that the removal of the IFNAR1 gene has had a significant impact in increasing the MDV productivity of DF1 cells.

Figure 8: Demonstration of increased viral growth using influenza

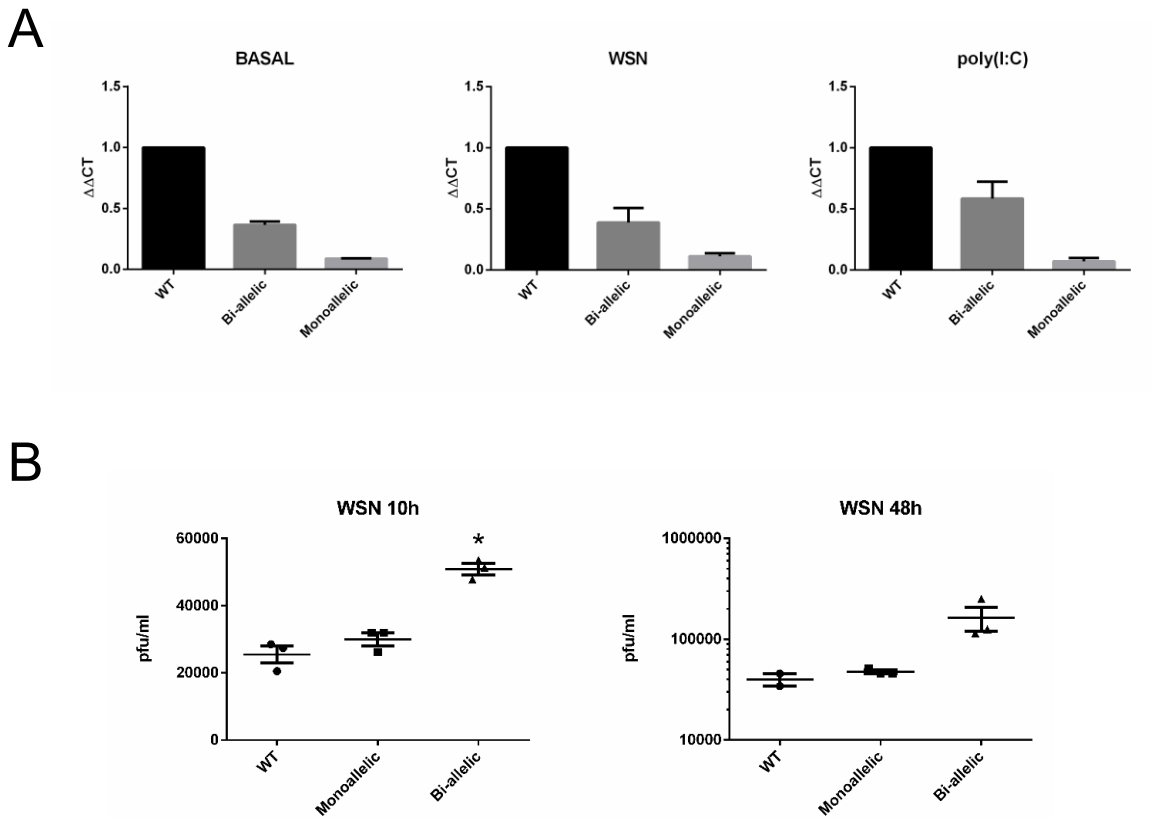


Table 6: Raw plaque assay data for MDV growth in KO cell lines

a) HVT (Low)

Host Cell Line	Serial dilution						
	-1	-2	-3	-4	-5	Ctrl	Pfu/mL
CEF	>100	>100	30	16	3	0	1.6×10^6
DF-1 WT	22	1	0	0	0	0	2.2×10^3
DF-1 KO4 (a)	70	20	5	0	0	0	5.0×10^4
DF-1 KO4 (b)	40	9	1	0	0	0	0.9×10^4
DF-1 KO4 (c)	>100	26	3	2	0	0	2.6×10^4

b) HVT (High)

Host Cell Line	Serial dilution						
	-1	-2	-3	-4	-5	Ctrl	Pfu/mL
CEF	>100	>100	40	15	2	0	1.5×10^6
DF-1 WT	>100	100	12	0	0	0	1.2×10^5
DF-1 KO4 (a)	>100	>100	50	7	1	0	7.0×10^5
DF-1 KO4 (b)	>100	>100	37	2	2	0	3.7×10^5
DF-1 KO4 (c)	>100	>100	30	6	0	0	6.0×10^5

c) Rispens

Host Cell Line	Serial dilution						
	-1	-2	-3	-4	-5	Ctrl	Pfu/mL
CEF	0	1	1	0	0	0	1.0×10^4
DF-1 WT	0	0	0	0	0	0	0
DF-1 KO4 (a)	30	0	0	0	0	0	3.0×10^3
DF-1 KO4 (b)	24	0	0	0	0	0	2.4×10^3
DF-1 KO4 (c)	22	1	0	0	0	0	2.2×10^3

Figure 11: Removal of IFNAR1 increases MDV production compared to WT DF1 cells

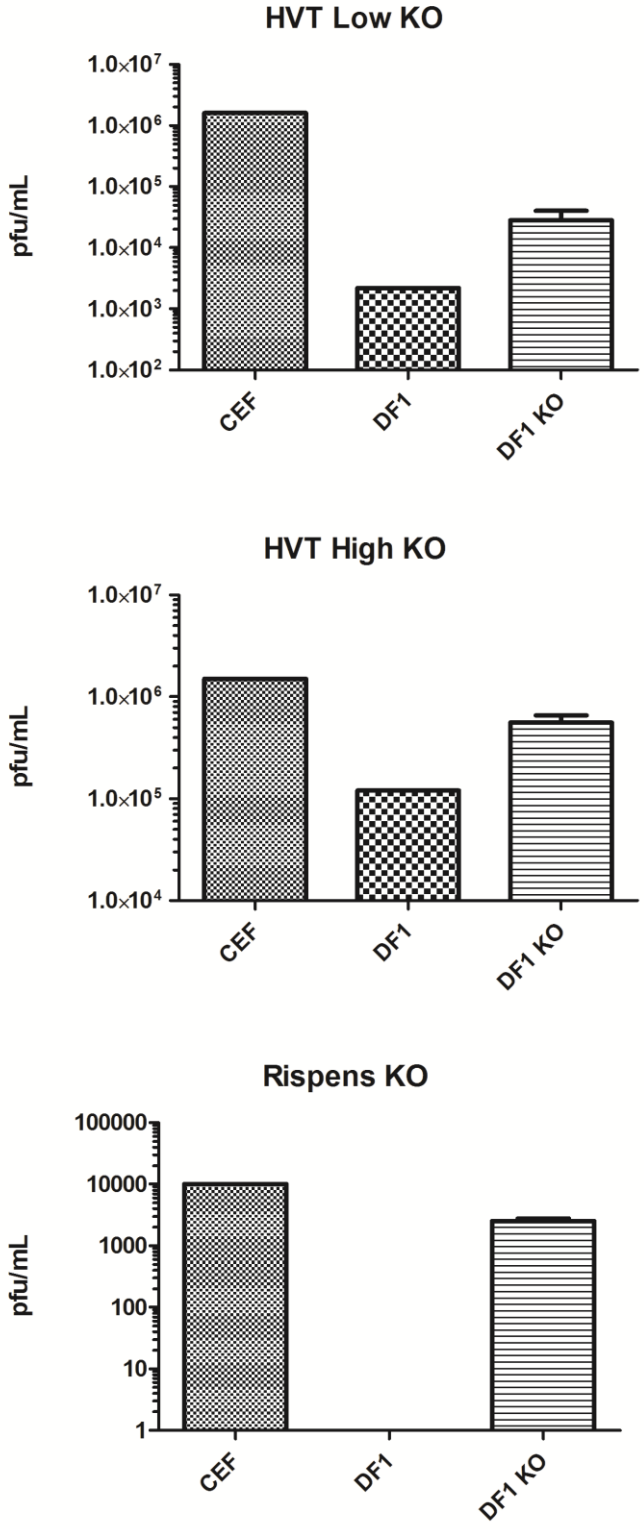


Figure 12: Plaque assays for Low dose HVT

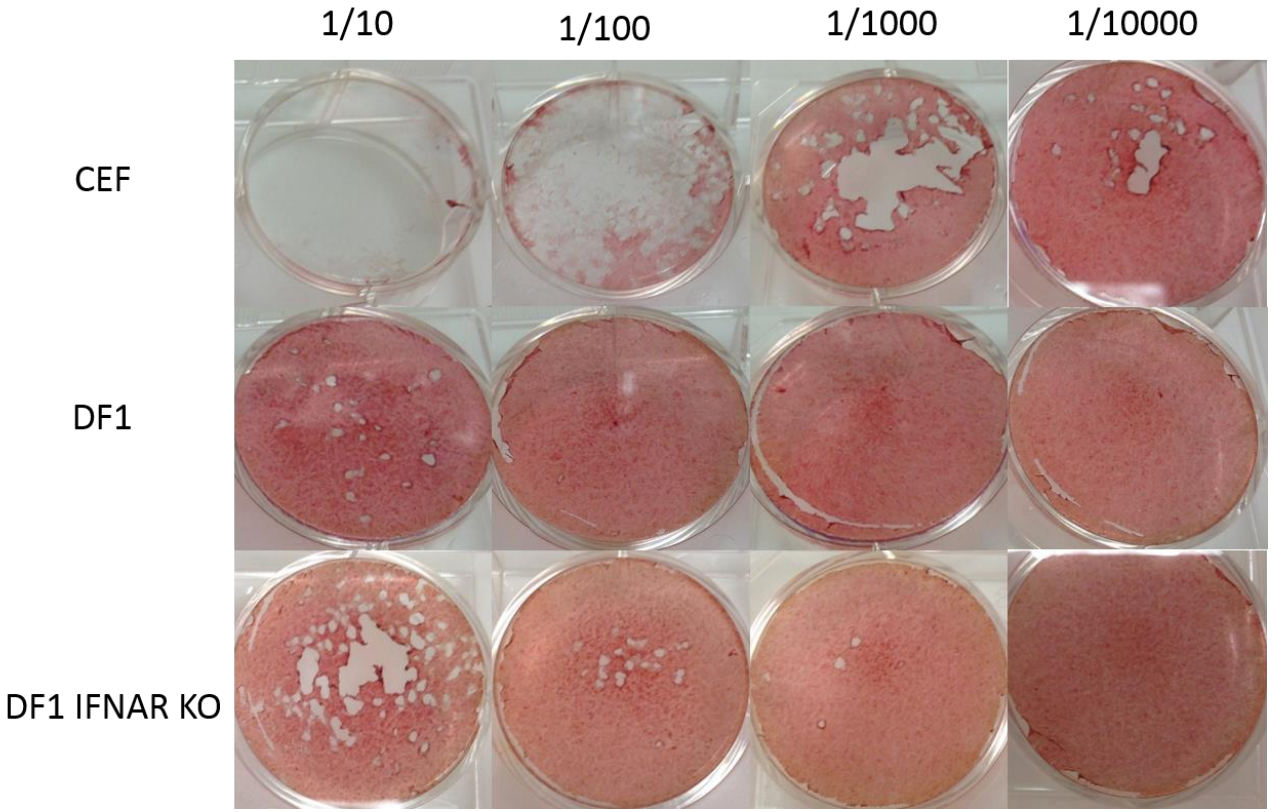
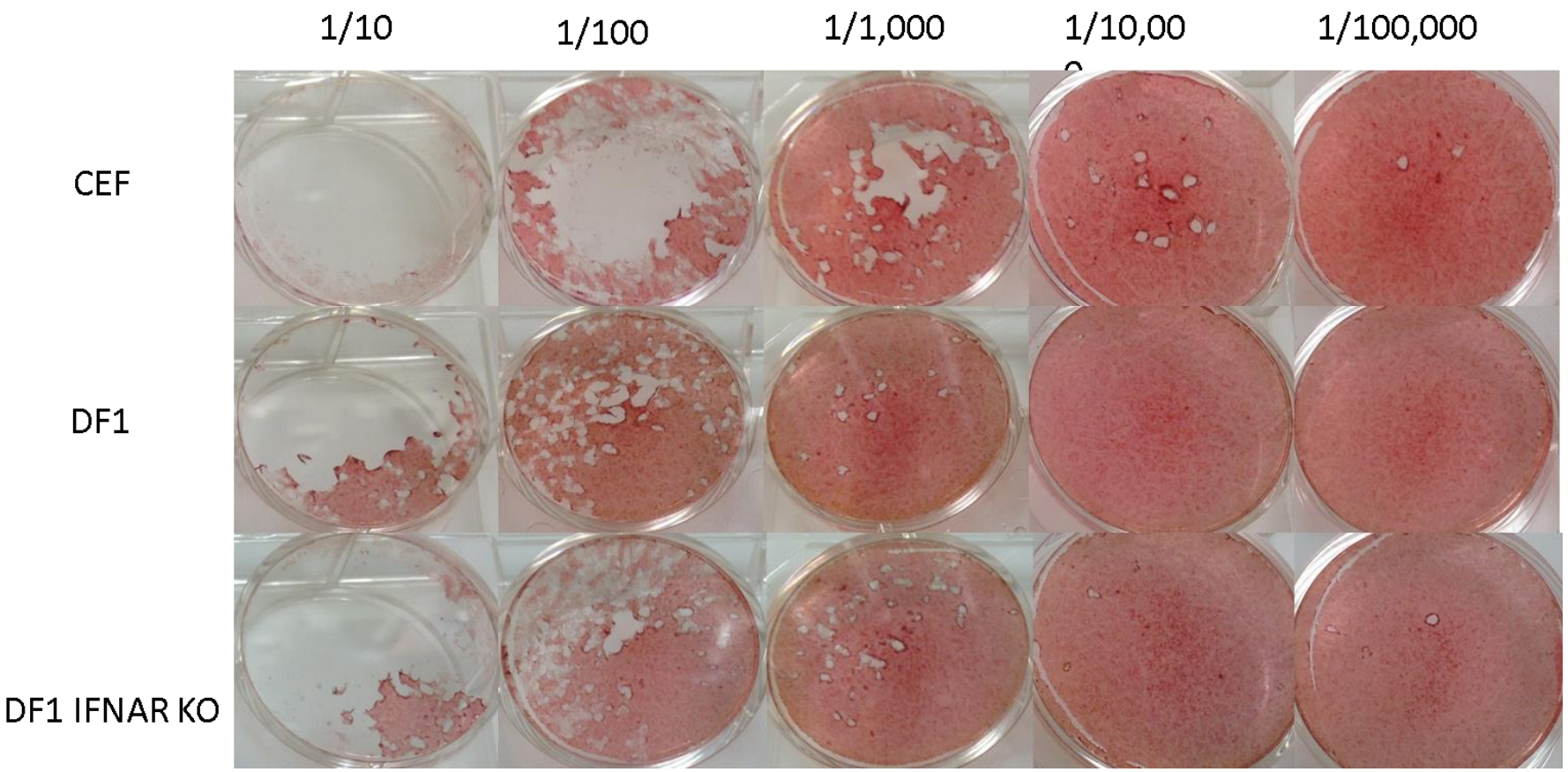


Figure 13: Plaque assays for high dose HVT



Concluding remarks

MDV is an economically important disease of poultry and control of these infections critically relies on the use of vaccines. Currently, vaccines are derived from several vaccine serotypes which are propagated in primary embryonic fibroblast cell cultures. From an industry perspective it would be preferable to have a continuous cell line for the production of the vaccine. Nevertheless, attempts to find continuous cell lines suitable for growing these viruses have had limited success. In this project we have identified a highly promising substrate candidate for MDV vaccine. This newly developed cell line highlights the potential for MDV vaccine to be produced in cell lines. Importantly, the development of cell lines for MDV vaccine production would support more flexible production with potentially shorter scale-up times and more consistent manufacturing.

Implications

The replacement of primary CEFs as a substrate to grow MDV vaccines on is of high importance to vaccine producers. We have demonstrated here that the removal of antiviral defence from a continuous cell line (DF1) has the ability to increase their MDV growth by >12 fold compared to WT cells and has almost bridged the gap between DF1 KO and CEFs with only a 2.5 fold difference now existing between the two as opposed to a 12.5 fold difference. Therefore, we have demonstrated the concept of removing antiviral genes is valid and if we were to target a different gene or multiple genes, we may generate an immortal cell that can outperform CEFs in MDV production. This would reduce the cost of producing the MDV vaccine, increase batch to batch consistency and provide a more stable substrate in times of outbreak.

Recommendations

From our data we would not recommend using either DF1 WT or HD11 cells for MDV growth. Neither of these cell lines had suitable performance in our culture systems. Similarly, the removal of genes to generate a new cell line did not provide evidence of success. If this line of research were to be continued we would

recommend generating pure populations of KO cells to fully evaluate if gene removal could lead to uncontrolled cell growth.

Based on our promising proof of concept results with DFI KO cells, we would strongly recommend further investment in the development of cell lines deficient in antiviral mechanisms. We have a number of other gene targets which have the potential to increase the MDV growth and when combined may provide a more efficient system than CEFs for MDV growth.

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POULTRY CRC

Plain English Compendium Summary

Sub-Project Title:	**Increased vaccine production in cell lines**
Poultry CRC Sub-Project No.:	1.1.11
Researcher:	Andrew Bean
Organisation:	CSIRO
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Sub-Project Overview	
Background	A major problem faced by Australian poultry industries is reduced productivity due to disease. Over the past several decades the main mechanisms used to control viral disease has been the use of vaccines. Outbreaks of viruses have exemplified the need to maintain vaccination strategies. The availability of cost-effective means of producing and supplying vaccines is critical to the control disease in poultry.
Research	We have established a state-of-the-art technique for the development of poultry cell lines for vaccine related research that will service the Australian poultry industry. We have employed new technologies, such as CRISPR, to understand how chicken cells respond to MDV vaccine growth. This provides crucial insights into the identification and assessment of new cell lines that can be used more widely by the poultry industry for vaccine production.
Sub-Project Progress	• Establishment of the techniques and a new cell line with the potential to enhance vaccine growth. • Training and development of early career scientists to meet the future needs of the Australian Poultry Industry. • Strong support from Zoetis in converting proof-of-concept research into commercial applications.
Implications	This project has identified proof-of-concept for several new approaches that can be used to develop and commercialise vaccines and health products for the Australian poultry industry. These new technologies can be used in the future to further develop these cell lines for enhanced vaccine production. This approach was aimed at enhancing the health and productivity of the poultry industry and ensure that it operates in a sustainable manner to meet growing consumer demands. The outcomes of this project provides a strong springboard to providing innovative solutions to address significant issues associated with the cost effective production of veterinary vaccines.
Publications	In this 1 year project, the research team has been highly successful in generating world class research. To date there have not been any published manuscripts, nevertheless, it is expected that in the future a publication that includes some of these initial findings will be produced.