Construction of a peacock immortalized fibroblast cell line for avian virus production

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ABSTRACT The mammalian-derived *MDCK* cells are the most widely used for avian virus vaccine production at present. The use of heterologous cell systems for avian virus preparation may cause security risks. An avian cell line is available for avian virus vaccines urgently needed. In this study, a peacock immortalized fibroblast cell line that is suitable for avian virus vaccine production was generated. The primary peacock fibroblast cells were prepared, and the immortal cells PEF-1 were obtained by transferring hTERT into the primary cells and screening with G418. The PEF-1 has high cell viability and expresses exogenous TERT protein. More importantly, the virus replication ability was stronger in PEF-1 than in MDCK cells as evaluated by virus fluorescence and $TCID_{50}$, after being infected with NDV-GFP, VSV-GFP, and AIV. In conclusion, the peacock immortalized PEF cells are expected to be used for the production of peacock and other avian virus vaccines.

Key words: peacocks, fibroblast cells, immortalized, hTERT, vaccine production

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INTRODUCTION

The highly pathogenic avian influenza virus (**HPAIV**) is a zoonotic disease and one of the most concerning infectious diseases in the world (Taubenberger and Morens, 2008). HPAIV infection usually causes a highly contagious systemic disease with high lethality in avian species, resulting in severe economic losses. It also has high morbidity and mortality in the susceptible population (Bevins et al., 2016). Wild birds, especially migratory wild waterfowls, are natural reservoirs of avian influenza viruses. At present, highly pathogenic avian influenza viruses such as H9N2, H5N6, H6N6, H6N2, and H5N1 have been isolated from many avians such as chickens, ducks, and peacocks (Ismail et al., 2010; Fan et al., 2014; Li et al., 2017; Guo et al., 2021). Peacocks are ornamental animals often kept as exhibits in parks and zoos. There are many opportunities for human contact. Once peacocks spread these HPAIV to humans or other species, it will have serious consequences. *Chlamydophila psittaci* infects peacocks have been reported to successfully infect humans and cause a severe acute respiratory syndrome characterized by pneumonia, high fever, and difficulty breathing (Yang et al., 2011). This suggests that when peacocks are infected with viruses, which are likely to spread to humans.

Newcastle disease virus (**NDV**) is an economically important viral disease with high pathogenicity and lethality to birds and is classified as a Class A infectious disease by the World Health Organization. NDV belongs to the genus Avianvirus, the family Paramyxoviridae, and the order Monograviviruses (Desingu et al., 2017). According to the severity of the disease in birds, NDV is divided into 5 main pathogenic types: Vescerotropic velogenic, neurotropic velogenic, mesogenic, lentogenic, and asymptomatic (Cross, 1991). It affects the lives of more than 250 species of birds around the world. Researchers isolated NDV from dead peacocks in a 2012 outbreak that caused high mortality in Pakistani peacocks (Lee et al., 2004; Munir et al., 2012). The NDV strains currently isolated from peacocks belong to NDV genotypes VII and XII of class II (Kumar et al., 2013; Khulape et al., 2014; Chumbe et al., 2015; Desingu et al., 2017). This suggests that peacocks are not only capable of infecting birds susceptible to highly pathogenic viruses, but also potentially transmitting them to humans as well as other birds. Therefore, it is very important to produce avian-derived vaccines with good protection effects and high safety against these viruses.

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At present, chicken embryos (**ECE**) or animal cells are commonly used for the production of avian influenza virus or Newcastle disease virus vaccines (Bissinger et al., 2021). However, chicken embryos are considered less suitable for pandemic influenza due to common drawbacks such as some virus strains cannot be propagated efficiently in eggs, poor scalability and limited ECE supply, as well as possible poor protection against some influenza strains by embryonated chicken-derived vaccines production (Genzel and Reichl, 2009; Raymond et al., 2016). Besides, chicken embryos need to break the shell during virus inoculation, resulting in a high risk of contamination (Li et al., 2021). In contrast, animal cell culture platforms are highly flexible, versatile, easily scalable, and can be very efficient (Milian and Kamen, 2015). Especially with the use of single-use equipment, small manufacturing facilities can rapidly produce pandemic vaccines when they are needed (Coronel et al., 2019). Researchers have evaluated several adherent and suspension cell lines for influenza vaccine production. Of these, adherent Madin-Darby Canine Kidney (**MDCK**) cells were considered the most productive cell line. MDCK cells are readily available, widely used in influenza research, and have been successfully licensed for vaccine manufacturing (Doroshenko and Halperin, 2009). However, heterologous species' genomic DNA and protein may remain in the vaccine. Birds using the vaccine may integrate the gDNA into their genomes. In addition, due to the presence of heterologous proteins in the vaccine, it can cause an immune response in birds and even be life-threatening (Song et al., 2016). But there is still no suitable avian-derived cell line for the production of avian-derived virus vaccines. Therefore, the preparation of avian-derived cell lines for the production of viral vaccines is crucial.

Chickens and peacocks belong to the order Galliformes, and phylogenetic tree analysis found that in the process of avian evolution, Indian peacocks, chickens, and turkeys belonged to the same branch, with the closest relationship (Dhar et al., 2019). It is found that peacocks not only live longer, but also have strong immunity and anti-stress ability. Researchers analyzed the genome of Indian peacocks and found that 97 genes were positively selected for peacocks compared to chickens. These genes are widely involved in the regulation of immunity, energy metabolism, cell growth, and differentiation (Liu et al., 2022). This means that peacocks seem to be more suitable for preparing avian-derived virus vaccines. However, the low yield of peacock embryos severely limits the production of viral vaccines. Therefore, the generation of a peacock cell line for virus vaccine production may be a good option.

Although isolating and culturing primary cells of avian origin for the production of viral vaccines appear feasible, there are many problems with primary cells for producing viral vaccines such as cumbersome preparation, easy contamination, low passage times, and difficulty in infection, which are not conducive to experimental operations (Shittu et al., 2016; Shukla and Shah, 2018). Therefore, the construction of cell lines is crucial to solving these problems. Human telomerase reverse transcriptase (**hTERT**), encoding the catalytic subunit of telomerase, prevents age-induced shortening of telomeres, thereby preventing replicative senescence, and has been used as a strategy for immortalizing various primary cells. Unlike approaches that use viral oncogenes such as the SV40 large T antigen or HPV 16 E6/E7 genes to immortalize cells, cell immortalization with hTERT results in cell lines with minimal genetic alterations and stable non-transformed phenotype (Kim et al., 2011; Lagosz-Cwik et al., 2021). In this study, we isolated and cultured the blue peacock fibroblast cells, and constructed the immortalized peacock fibroblast cell line by introducing the hTERT gene, which has good viability. In addition, immortalized PEF-1 cells are susceptible to RNA viruses such as NDV-GFP, VSV-GFP, and AIV, and with higher virus titers than in MDCK cells.

MATERIALS AND METHODS

Cell Cultures and Viruses

Chicken embryonic fibroblast cell line DF1 cells and MDCK cells were obtained from ATCC, cultured in DMEM (Gibco, Carlsbad, CA) supplemented with 10% FBS (Nulen, Shanghai, China), and incubated at 37°C in a 5% CO_2 incubator. The details are as described in our previous study (Cheng et al., 2019). Newcastle disease virus (NDV-GFP) was the low virulent strain LaSota, named NDV-GFP (Lin et al., 2021). Avian influenza virus (**AIV**), the A/Chicken/Shanghai/010/2008 (H9N2) virus (SH010) isolated from chickens in shanghai, China, in 2008 and identified as H9N2 avian influenza A virus (Cheng et al., 2015). The GFP-tagged vesicular stomatitis virus (**VSV**) VSV-GFP, was stored in our Laboratory (Wang et al., 2022).

Isolation and Culture of Primary Peacock Fibroblasts

Take out the 10-day-old blue peacock embryos (Ruyi Ecological Agriculture Co., Ltd., Anhui, China) under a sterile operating table, remove the heads, the limbs, and the bones, wash the remaining tissue with PBS 3 times, cut into 1 cm^3 tissue, and digest with 0.25% Trypsin-EDTA (25200072, Gibco, Carlsbad, CA) at 37°C under constant temperature shaker. After 15 min, terminate digestion and centrifugation at 1,000 g for 5 min; the supernatant was discarded, the DMEM supplemented with 10% FBS was pipetted to precipitate, and the peacock primary fibroblasts were inoculated into T25 culture flasks (NEST Biotechnology, Wuxi, China) and cells were incubated at 37°C in a 5% CO_2 incubator.

Cell Transfection and Drug Screening

Primary peacock fibroblasts were seeded in 12-well (NEST Biotechnology, Wuxi, China) at 5×10^5 /mL,

where 6 wells were transfected with 500 ng /well of pCI-neo-hTRET plasmid obtained from the addgene (#1781) and the remaining 6 wells served as controls. Twenty four hours after transfection, added G418 to 6 wells of the transfection group and the control group at the concentrations of 250 μg/mL, 500 μg/mL, 750 μg/mL, 1,000 μg/mL, 1,500 μg/mL, and 2,000 μg/mL, respectively. After 1 week of G418 selection, the cells in the control group with the addition of more than 1,000 μg/mL of G418 were all died, while there were still many cells in the transfection group. We continued to screen the cells in the transfected group with 500 μg/mL G418. One week later, the drug screening was stopped and the positive clones were passaged to 24-well plates and cultured to more than 60 passaged.

Viral Infection

PEF-1 cell lines and MDCK cells were seeded into 12-well plates at 5×10^5 /mL. After the cell contact inhibition, the serum-free medium was replaced, and NDV-GFP, VSV-GFP and AIV were inoculated into PEF cell lines and MDCK cells at MOI = 1.0, respectively. Virus fluorescence was observed 12 h and 24 h after infection.

Cell Counting Kit-8

PEF-1 cell lines proliferation was measured using the commercial cell counting kit-8 (**CCK-8**) (40203ES60, Yeasen, Shanghai, China) as the manufacturer's protocol described. Cells were seeded in 96-well plates at a density of 1×10^3/mL and divided into 4 groups, each group has 6 wells. In addition, 24 wells with only add 100 μL culture medium were also divided into 4 groups as a control group. After cultured in the incubator for 12, 24, 36, and 48 h. 10 μL CCK-8 solution was added to incubate with cells for 2 h at 37 °C. The absorbance at 450 nm was analyzed with the standard microplate reader. Calculation of cell viability: The OD (X h) = the OD value of the cells (X h) − the OD value of the control group (X h).

Titration of Viral Infectivity

DF1 cells were inoculated in 96-well plates, and the supernatants of 12 h and 24 h infected cultures were collected, and the virus titer was determined and expressed by the tissue culture infectious dose ($TCID_{50}$). The virus supernatant was diluted 10 times in a gradient, from 10^{-1} to 10^{-10}, and each dilution was inoculated into 8 wells. At the same time, a negative control was set, and the culture was continued in a 37°C CO_2 incubator. After 4 to 5 days' of inoculation, the number of wells with cytopathic effect (**CEP**) at each dilution was recorded, and the virus titer was calculated by the Reed-Muench method.

Western Blot Analysis

The total cell proteins were extracted by radio immunoprecipitation assay (Beyotime, Shanghai, China) using a protease cocktail and phenylmethylsulfonyl fluoride (**PMSF**; Yeasen). The lysate was centrifuged at 13,000 rpm for 10 min to obtain the supernatant, and a 5 × SDS loading buffer was added before the lysates were boiled for 10 min. The proteins isolated from the cell lysates were separated via SDS-PAGE and analyzed using Western blotting. The antibody included anti-TERT rabbit polyclonal antibody (D160502; Sangon Biotech (Shanghai) Co., Ltd, Shanghai, China), and β-tubulin overnight at 4°C. The membrane was washed with tris buffered saline and Tween−20 (**TBST**) 3 times. Then, the secondary antibody was added for 1 h incubation at 4°C on a shaker Images were obtained using the Tanon 5200 imaging system (Tanon, Shanghai, China).

Statistical Analysis

Results are expressed as the mean ± SD. GraphPad Prism 8.0 was utilized to graph the results. Data were analyzed by Student's t test. $P < 0.05$ was considered statistically significant, and $P < 0.01$ was considered highly statistically significant ($^*P < 0.05$; $^{**}P < 0.01$).

RESULTS

Primary Peacock Fibroblast Cell Isolation and Culture

To produce the peacock fibroblast cell line (**PEF**), we aseptically removed the blue peacock embryos incubated for 10 d (Figure 1A). Then, the peacock embryo was decapitated, the limbs, the internal organs and the bones were removed and the remaining tissue was cut into 1 cm^3 size. After trypsinization, it was inoculated into a T25 culture flask. The seeded fibroblast cells were found to have a typical elongated spindle shape (Figure 1B). Next, we explored the transfection efficiency of primary PEF. The pcDNA4.0-EGFP plasmid was mixed with different concentrations of liposomes and then transfected into primary PEF. It was found that 500 ng of plasmid was transfected most efficiently with 3.5 μL of liposome (Figures 1C and 1D).

Construction of Immortal Peacock Fibroblast Cell Line

Human telomerase reverse transcriptase (hTERT) can maintain telomere activity and keep cells proliferating, so the pCI-neo-hTERT plasmid that encodes the hTERT protein and neo marker gene is widely used in the construction of primary cell lines. In order to construct peacock fibroblast cell lines, we transfected the pCI-neo-hTERT plasmid into the PEF cell. The 24 h post-transfection cells were screened with G418 and the

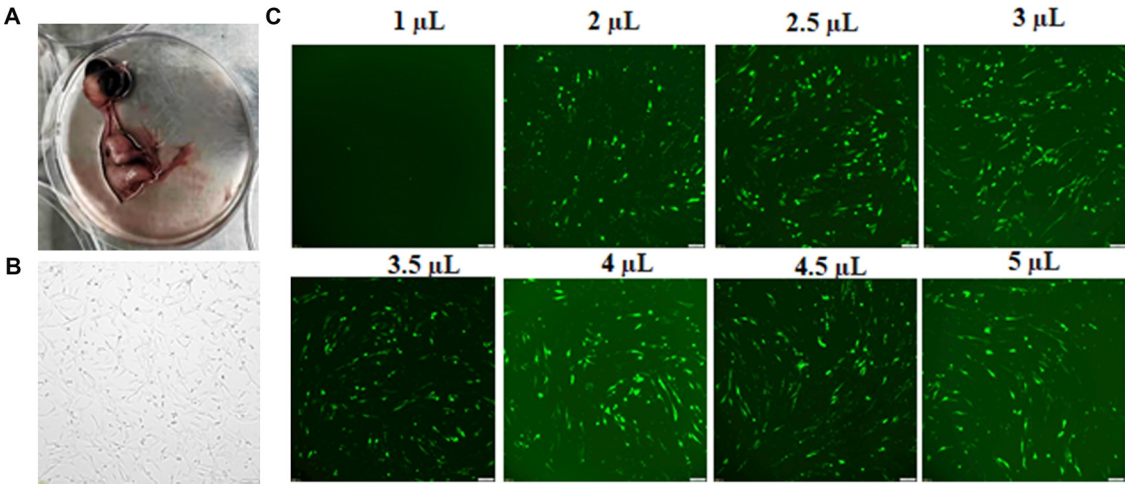

Figure 1. Primary peacock fibroblast cell (PEF) isolation and culture. (A) 10 days hatching blue peacock embryo. (B) Peacock primary fibroblast cells isolated and cultured from blue peacock embryo. (C) Detection of transfection efficiency of primary peacock fibroblast cells. (D) The efficiency of transfection quantified by means fluorescent intensity. Data are expressed as the means ± SD of three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$, Scale bar = 100 μm.

positive clones were seeded into a 24-well plate for continued cultivation. After the serial passage to 60 passages, the PEF cells still have typical fibroblast morphology (Figure 2A), and the F60 PEF cells have good cell viability detected by CCK-8 (Figure 2B). To confirm that the immortal PEF cell line was successfully obtained, we detected the expression of exogenous hTERT by Western blotting and found that the primary cultured PEF cells did not express exogenous TERT, while the immortal PEF cells were expressed exogenous TERT (Figure 2C). Next, we cryopreserved the PEF cell line that has been passaged to F60, and then revived the culture. It was found that the cryopreserved PEF cell line could proliferate rapidly, and the cell shape was complete, the cells were long spindle, and the nucleus was obvious (Figure 2D). The above results showed that we have successfully obtained an immortal peacock fibroblast cell line (PEF-1), that has good cell activity.

Comparison of Viral Replication Ability of PEF-1 and MDCK Cells

The purpose of constructing immortal PEF-1 in this study is to produce avian virus vaccines, so the constructed cell lines should be susceptible to virus infection. Therefore, we infected the PEF-1 cell line with three RNA viruses, NDV-GFP, VSV-GFP, and AIV. The fluorescence intensity represents the replication of NDV-GFP and VSV-GFP. We found that 12 h after infection with NDV-GFP and VSV-GFP, the fluorescence of the two viruses could be clearly seen in PEF-1 cell layers (Figure 3A). In addition, by observing the PEF-1 cells infected with AIV, after 12 h of infection, it was found that the dead cells could be observed under the microscope, and there were fragments of dead cells on the cell surface; 24 h after infection, the long-spindle PEF-1 cell line shrinks. It became smaller, the intercellular space became larger, and the cell debris increased (Figure 3C). This suggests that PEF-1 cell lines are susceptible to RNA viruses. At present, MDCK cell lines are widely used in viral vaccine production. To explore the differences in virus replication of NDV-GFP, VSV-GFP, and AIV in PEF-1 and MDCK cells, we seeded the same number of PEF-1 cells and MDCK cells, respectively. The same titers of NDV-GFP, VSV-GFP, and AIV were used to infect the above cell cultures. After 12 h and 24 h, the virus replication was observed. We found that NDV-GFP and VSV-GFP fluorescence could be observed in both PEF-1 and MDCK cells

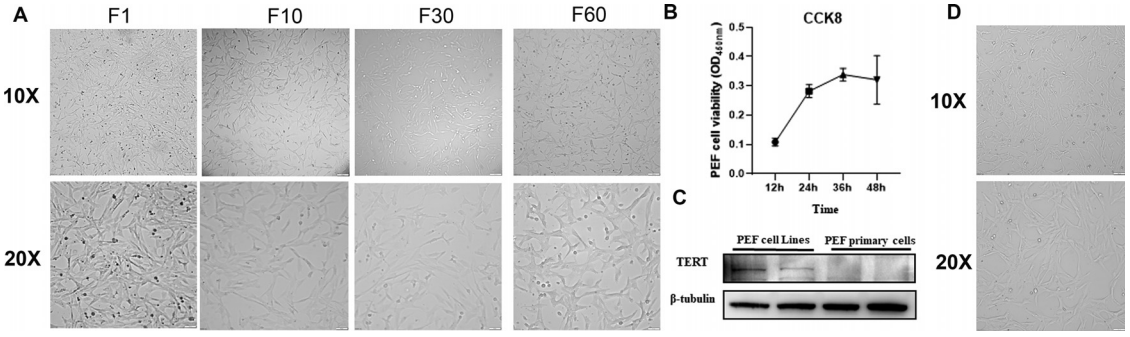

Figure. 2. Construction of immortal peacock fibroblast cell line. (A) Cell morphology of positive PEF cells after G418 screening F1, F10, F30, F50, F60. (B) The cell viability of F60 of PEF-1 was detected by CCK-8. (C) Western Blot detected the expression of exogenous hTERT in primary PEF cells and PEF-1 cell lines. (D) Morphology of cultured PEF cell lines after cryopreservation. Scale bar = 100 μm and 50 μm.

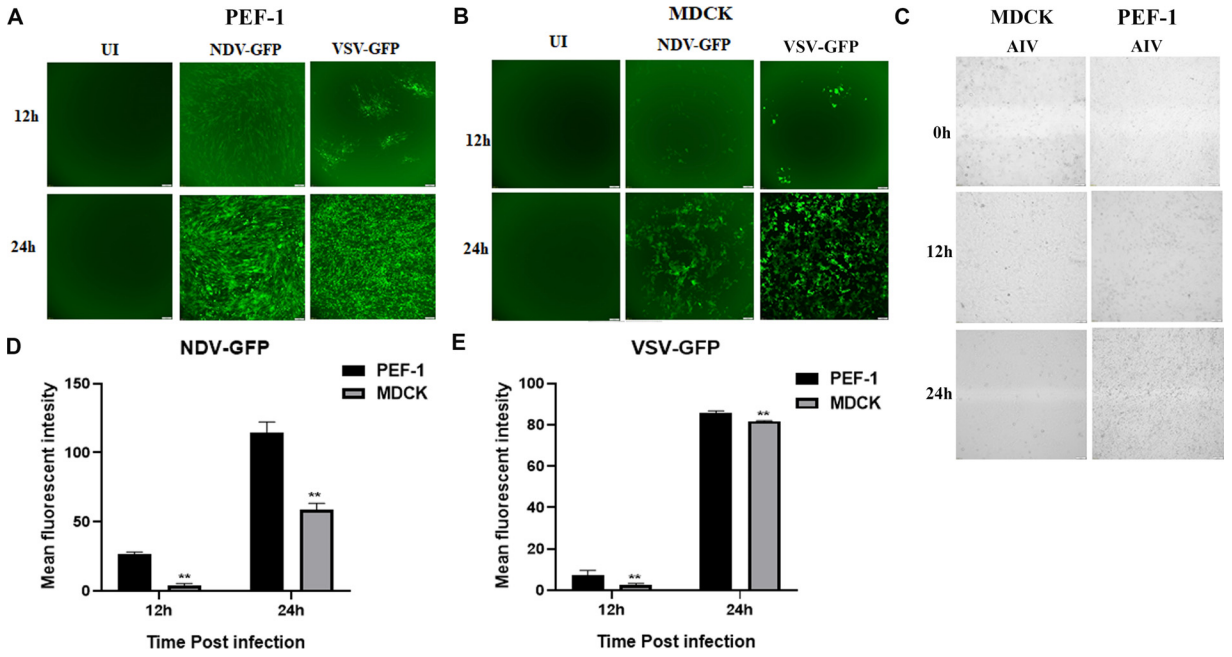

Figure 3. Comparison of viral replication ability of PEF-1 and MDCK cell lines. (A) Viral fluorescence in PEF cells infected with NDV-GFP and VSV-GFP at 1.0 MOI. (B) Viral fluorescence in MDCK cells infected with NDV-GFP and VSV-GFP at 1.0 MOI. (C) PEF-1 MDCK cells infected with AIV at 1.0 MOI. (D) The NDV-GFP mean fluorescent intensity in PEF-1 and MDCK cells. (E) The VSV-GFP mean fluorescent intensity in PEF-1 and MDCK cells. Data are expressed as the means ± SD of three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$, scale bar = 100μm.

(Figures 3A and 3B). But the mean fluorescence intensity in PEF-1 cells was stronger than in MDCK cells after NDV-GFP, and VSV-GFP infection during 12 h and 24 h (Figures 3D and 3E). In addition, cytopathic effect was observed in both cell lines after AIV infection for 24 h (Figure 3C). This indicates that the PEF-1 cell line is susceptible to virus infection and that the PEF-1 cell line also favors viral replication as compared to the MDCK cell line.

PEF-1 Cells Have Stronger Viral Replication Capacity Compared to MDCK Cells

To further demonstrate that PEF-1 can be used as a potential cell line for the production of avian virus vaccines, we collected the supernatant of the medium infected with the NDV-GFP, VSV-GFP, and AIV at 12 h and 24 h, respectively. The virus titers in PEF-1 and MDCK cells were detected by $TCID_{50}$. It was found that the viral titers of NDV-GFP, VSV-GFP, and AIV viruses in PEF cell lines were significantly higher than those in MDCK cells 24 h after infection (Figures 4 A −4C). This indicates that the PEF cell line can be used as a vector for the preparation of peacock-derived virus vaccines.

DISCUSSION

Highly pathogenic avian influenza and Newcastle disease viruses have brought huge economic losses to the global breeding industry. Wild birds have long been considered as natural hosts for these viruses (Lee et al., 2017). In recent years, researchers have continuously isolated highly pathogenic avian influenza, Newcastle disease, coronavirus, and other viruses from domestic and wild peacocks (Cavanagh, 2005; Desingu et al., 2016; Kouam et al., 2019). Once these viruses infect peacocks, they will not only challenge the survival of peacocks but also threaten the safety of humans and other avians. At present, the vaccines used for poultry are derived from cultured MDCK cells (Bertelsen et al., 2007; Wang et al., 2020). The use of heterologous cell systems for avian virus preparation may cause security risks. Multiple doses of non-live vaccines are associated with adverse health outcomes, including autism, immune system overload, a possible association between vaccines and autoimmune syndromes, and increased mortality in vaccine recipients, which are possible non-specific effects of immunization (**NSE**) (Conklin et al., 2021). The heterologous protein present in the non-avian-derived vaccine will act as the immune source of the birds, which may cause a strong immune response and affect the health of the poultry. In addition, heterologous genomic DNA may be present in non-avian vaccines. Under a series of accidental factors, these DNAs may integrate into the host genome. In severe cases, it will lead to the occurrence of cancer, or some new biological traits will appear (Song et al., 2016; Doerfler, 2021). Therefore, heterologous vaccines have great potential for biosafety.

Therefore, chicken embryos are still the main avian vectors for the study of avian influenza viruses and the production of vaccines. However, their use has significant limitations, such as high cost, security of supply (e.g., susceptible to avian influenza outbreaks in poultry), batch-to-batch reproducibility, risk of accidental infection, and lengthy production processes (Giotis

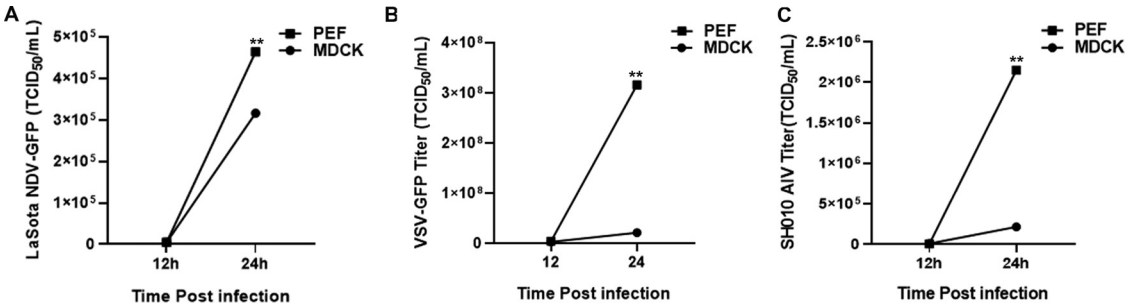

Figure 4. PEF-1 cells has stronger viral replication capacity compared to MDCK cells. (A) The supernatants NDV-GFP viral titers were analyzed by a standard TCID50 method, after NDV-GFP infection PEF-1 cells and MDCK cells 12 h and 24 h. (B) The supernatants VSV-GFP viral titers were analyzed by a standard TCID50 method, after VSV-GFP infection PEF-1 cells and MDCK cells 12 h and 24 h. (C) The supernatants AIV (SH010) viral titers were analyzed by a standard TCID50 method, after AIV infection PEF-1 cells and MDCK cells 12 h and 24 h. Data are expressed as the means ± SD of three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$.

et al., 2019). An avian cell line that is available for avian virus vaccines is urgently needed. Thence, primary chicken embryonic fibroblasts (**CEFs**) have been used for avian vaccine production. However, CEF is intolerant to the virus, easy to die after infection with the virus, and its vaccine yield is very low (Kang et al., 2016). Compared with chickens, blue peacocks show strong immunity. Moreover, peacocks are closely related to chickens in evolution (Dhar et al., 2019). The use of peacock embryonic fibroblasts (PEF) to produce avian-derived virus vaccines appears to be effective.

The replication of isolated and cultured primary cells is tightly controlled by several proteins that regulate cell cycle progression, and a "Hayflick limit" occurs when they replicate to a certain number of generations, at which point they stop dividing and enter a "senescent" state. To efficiently produce viral vaccines, it is necessary to continuously isolate and culture PEFs. However, the yield of peacock embryos is low, which obviously cannot meet the actual needs. Immortalized cell lines can overcome the "Hayflick limit" and proliferate indefinitely (Maqsood et al., 2013). Human telomerase reverse transcriptase (hTERT) is transferred into primary cells, and the activated telomerase uses itself as a template to continuously synthesize telomere DNA, which is used to maintain the length of telomeres, maintain the stability of chromosomes, and make cells transcend the M2 stage and acquires the ability to divide and proliferate indefinitely, resulting in immortality (Maqsood et al., 2013; Song, et al., 2020; Masnikov et al., 2021). In this study, we isolated and cultured a primary blue peacock fibroblast cell line, and successfully introduced the plasmid pCI-neo-hTERT encoding hTERT protein into the primary cultured peacock fibroblasts. Positive clones were obtained after G418 screening. After the continuous passage to more than 60 generations, we obtained a highly active immortalized peacock fibroblast cell line PEF-1, that expressed the exogenous TERT protein.

Another purpose of this study was to use the immortalized peacock fibroblast cell line for the production of avian-derived virus vaccines. Cells used to produce viral vaccines must be susceptible to virus infection (Kiesslich and Kamen, 2020). To prove that PEF-1 cells can be used to produce viral vaccines, we infected PEF-1 cells with three RNA viruses, NDV-GFP, VSV-GFP, and AIV. It was found that the fluorescence of NDV-GFP and VSV-GFP could be observed after 12 h of infection. After 24 h, the cells infected with NDV-GFP and VSV-GFP fluorescence intensity spread over the entire field. In contrast, PEF-1 infected with VSV-GFP and AIV began to shrink and the intercellular space increased. This indicates that the immortalized PEF-1 cells are susceptible to virus infection and have the potential for the production of viral vaccines

At present, MDCK cells are widely used to produce virus vaccines (Kim et al., 2018; Tzeng et al., 2020; Bissinger, et al., 2021). Although the safety of vaccines produced by MDCK has been evaluated in mice and other species, the efficacy, and safety of virus vaccines for the prevention and treatment of avian species are still unclear (Garcia and Zavala, 2019; Ganguly et al., 2020; Wang et al., 2020; Wang et al., 2021). Therefore, it is crucial to develop avian-derived cell lines for the production of avian virus vaccines. Vesicular stomatitis virus (**VSV**) is often used as a model for assessing cellular viral productivity (Shen et al., 2019). For further confirmation, PEF-1 cells can be used to produce viral vaccines. We infected the same number of PEF-1 and MDCK cells with three RNA viruses, NDV-GFP, VSV-GFP, and AIV, respectively. Virus fluorescence of NDV-GFP and VSV-GFP could be observed in both cells 12 h after infection. Besides, 24 h after infection, it was found that NDV-GFP and VSV-GFP virus fluorescence was stronger in PEF-1 cells. In addition, the $TCID_{50}$ titration also showed that the titers of NDV-GFP, VSV-GFP, and AIV in PEF-1 cells were higher than those in MDCK cells. This suggests that PEF-1 can be used to produce viral vaccines, and its ability to amplify the virus is superior to that of MDCK cells.

In conclusion, we obtain an immortalized peacock PEF-1, and it can efficiently propagate the virus. The cell line will provide a vector for the production of avian-derived viral vaccines.

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DISCLOSURES

The authors declare no conflict of interest.

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