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Generation of PK-15 cell lines highly permissive to porcine circovirus 2 infection by transposon-mediated interferon-gamma gene transfer

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ABSTRACT

Porcine circovirus 2 (PCV2)-associated diseases affect the swine industry worldwide. Vaccination is the major tool for the disease control, but the vaccine production is hindered by lower propagation rate of PCV2 *in vitro*. Previous studies showed that interferons (IFNs) can increase PCV2 yield in PK-15 cells. In the present study, we constructed a Sleepy Beauty (SB) transposon vector expressing porcine IFNg gene fused with the coding sequence for immunoglobulin G Fc domain. After dilution cloning, the transposon and transposase vectors were co-transfected into PK-15 cell clones with higher permissivity to PCV2 infection. Two transgenic PK-15 cell lines, namely PK15-IFNg^{Ran} and PK15-IFNg^{SB} which contained randomly integrated transfer vector or SB cassette without selection marker, were screened by PCR analysis. The characterization results demonstrated that the two transgenic cell lines can stably express IFNg-Fc fusion protein with potent antiviral activities. Both viral titration and quantitative PCR analyses showed that the two transgenic cell lines are highly permissive to PCV2 infection with significantly increased viral yields. These results indicate that the two transgenic PK-15 cell lines, PK15-IFNg^{SB} in particular, can be used for PCV2 vaccine development.

1. Introduction

Porcine circovirus type 2 (PCV2) is the primary agent of porcine circovirus-associated disease (PCVAD), one of the most economically important diseases affecting global swine industry (Allan et al., 1998). Since the vaccine was introduced into the world market in 2006, vaccination is the major tool for PCVAD control. Although porcine kidney PK-15 cell line is commonly used for PCV2 propagation, only about 20% of the cell population is susceptible to the virus infection (Tischer et al., 1987; Meerts et al., 2005). Different methods have been developed to increase PCV2 yield *in vitro*, including the treatment of infected cells with cytokines (Yang et al., 2013) or Tween-20 (Hua et al., 2018) and dilution cell cloning (Chen et al., 2013; Zhu et al., 2007). More recently, a PK-15 cell line highly permissive to PCV2 infection has been generated by interleukin-2 (IL-2) gene transfer (Ma et al., 2017). However, the plasmid vector-mediated gene transfer suffers from low efficiency and long time drug selection. More importantly, the cell line contains antibiotic resistance marker which is required to be eliminated by regulatory agencies for safety considerations (Carnes et al., 2010).

Transposons can change their positions within the genome *via* a cut-and-paste mechanism. In the transposition process, the transposase enzyme excises the element from its donor vector and integrates the transposon into a chromosomal locus. Therefore, transposons have been considered as the natural, non-viral gene delivery vehicles capable of efficient genomic insertion (Izsvák et al., 2009). Sleeping Beauty (SB) is a synthetic transposon that was constructed on the basis of transpositionally inactive elements isolated from fish genomes. The *SB* transposon-based gene delivery combines the favorable features of viral and non-viral vectors, and thus has become an important molecular tool for animal transgenesis, insertional mutagenesis and gene therapy (Ivics and Izsvák, 2011).

Interferons (IFNs) are a group of cytokines with antiviral and immune regulatory activities. PCV2 genome contains an IFN-stimulated response element (ISRE)-like sequence in the Rep promoter region, which plays an important role in the viral response to IFNs (Misinzo et al., 2008; Ramamoorthy et al., 2009). Therefore, recombinant IFNs

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have been used to enhance PCV2 propagation in PK-15 cells (Meerts et al., 2005). This indicates that IFN gene transfer may become an alternative strategy for the generation of PK-15 cell lines highly permissive to PCV2 infection. Like other cytokines, however, IFNs have short half-lives due to their small molecular size and rapid enzymatic degradation (Tian et al., 2013). Therefore, the expression of recombinant cytokines as fusion proteins with serum albumin or immunoglobulin G (IgG) Fc domain has been used to prolong their half-lives and therapeutic efficiencies (Guo et al., 2012). In the present study, we investigated the feasibility to generate PK-15 cell lines highly permissive to PCV2 infection by transposon-mediated IFN-gamma (IFNg) gene transfer. The cell lines generated may be useful for PCV2 vaccine production.

2. Materials and methods

2.1. Vectors, virus and cells

The recombinant adenoviral vector pShuttle-Fc used in this study was constructed by cloning the coding sequence for pig IgG Fc domain (Accession number: LOC396781; nt733-1425) into pShuttle-CMV vector (Chen et al., 2014). The modified Sleeping Beauty (SB) vector pTEG was constructed by cloning pig elongation factor-1α gene promoter (Accession number: FM995601; nt943-2215) and bovine growth hormone gene poly (A) signal (Accession number: M57764; nt2326-2551) into SB transposon vector pT2/HB (He et al., 2016). The transposase vector pSB16 was from Addgene (Beijing Zhongyuan, Ltd. China). PK-15 cell line (ATCC CCL-33, passage 13) was cultivated in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) containing 10% fetal bovine serum (FBS) at CO_2 at 37 ℃. PCV2b strain 0233 was passed for three passages and titrated on PK-15 cells as previously described (Li et al., 2015).

2.2. Vector construction

The cDNA without stop codon for porcine IFNg (Accession number: NM213948; nt 25-522) was synthesized by RT-PCR as previously described (Kirthika et al., 2017). Briefly, the freshly isolated peripheral blood lymphocytes were cultured in RPMI 1640 medium supplemented with 10% FBS. After stimulation with Con A (10 μg/mL) (Xia et al., 2005) for 48 h, the total RNA was extracted using MiniBEST Viral RNA/DNA Extraction Kit (TaKaRa). The reverse transcription (RT) was performed using PrimeScript™ 1 st Strand cDNA Synthesis Kit (TaKaRa). The double stand cDNA for porcine IFNg was amplified by PCR using the primer pair listed in Table 1 and cloned into pTEG vector after *Not*I and *Eco*RI digestion. The coding sequence for pig IgG Fc domain was amplified from pShuttle-Fc vector using the primer pair listed in Table 1 and cloned into pTEG vector after *Eco*RI and *Xba*I digestion. The resultant SB transfer vector was called pTEG-IFNg-Fc (Fig. 1).

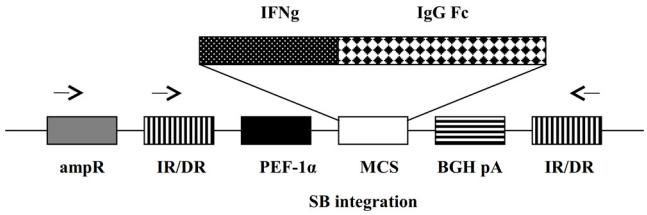

Fig. 1. The schematic structure of SB transposon vector used in this study. ampR, IR/DR, PEF-1α, MCS and BGH pA represent ampicillin resistance gene, left and right IR/DRs of SB transposon, porcine elongation factor-1a promoter, multiple cloning sites and the poly(A) signal of bovine growth hormone gene, respectively. The coding sequences for porcine IFNg and IgG Fc domain were cloned into the transposon vector after *Not*I/*Eco*RI and *Eco*RI/*Xba*I digestion. Arrows indicate the PCR primers for identification of randomly or SB-integrated transgenic cell clones.

2.3. Cell dilution cloning and identification

The parental PK-15 cells were cloned using limited dilution cloning as previously described (Zhu et al., 2007). Briefly, the cells were grown to complete confluence in T25 flasks and detached by trypsinization. The detached cells were seeded in duplicates on 96-well plates (˜1 cell/well) and grown to complete confluence for second round of dilution cloning. The cell clones were grown to 80% confluence and infected with PCV2 (10^4 $TCID_{50}$/well). At 24 h post infection (hpi), the cells were stained first with home-made mouse anti-PCV2 antibody (1: 500) and then with FITC-labeled goat anti-mouse IgG (Sigma) (1: 1000) for 1 h at 37 ℃. The cell clones with higher permissivity (PK-15^{higher}) or lower permissivity (PK-15^{lower}) to PCV2 infection were selected according to PCV2-positive numbers. At 72 hpi, the cellular DNA was extracted from PK-15^{higher} and lower PK-15^{lower} cell clones, and quantitative PCR was performed using PCV2 *cap*-targeted primer pair (Table 1) and SYBR Premix Ex Taq™ II (TaKaRa) by following the manufacturer's instruction. The standard curve was generated using pMD18-T vector containing 112-bp PCV2 Cap gene segment.

2.4. Generation of transgenic PK-15 cell lines

Transgenic PK-15 cell lines were generated using previously described protocol (He et al., 2016). Briefly, PK-15^{higher} cell clones (passage 5) were seeded on 24-well plates (10^5 cells/well) and grown for 12 h in DMEM containing 10% FBS. Cell transfection was performed using Lipofectamine™ 2000 (Introvigen, USA) by following the manufacturer's instruction. Briefly, the mixture of transposon vector pTEG-IFNg-Fc (0.5 μg) and transposase vector pSB16 (0.1 μg) or transfecting agent (2 μL) was added to OPTI-MEM I medium (50 μL). After 15-min incubation at room temperature, the two solutions were combined and added to each well after incubation for additional 20 min. Following 4-h incubation at 37 ℃, DMEM containing 2% FBS was added and the incubation was continued for additional 24 or 48 h. The transfected cells were cloned by two rounds of dilution cloning without drug

Table 1
PCR primers used in this study.

Gene	Primer pair	Sequence (5'→3')	Amplicon (bp)	Reference
IFNg	Sense	ATGAGTTATACAACTTATTTCTT	498	This study
	Anti-sense	TTTTGATGCTCTCTGGCCTT		
IgG Fc	Sense	GGAACAAAGACCAAACCA	693	This study
	Anti-sense	TCATTTACCCTGAGTCTTGGA		
SB IR/DR	Sense	CAGTTGAAGTCGGAAGTTTA	290	This study
	Anti-sense	AAGCTTCTAAAGCCATGAC		
AmpR	Sense	TCCGTGTCGCCCTTATTC	411	He et al., 2016
	Anti-sense	AAGCGGTTAGCTCCTTCG		
PCV2 Cap	Sense	AAGGGCTGGGTTATGGTATG	112	Cruz and Araujo, 2014
	Anti-sense	GAGTGGGCTCCAGTGCTGTTA		

selection and the cell clones were grown in T25 flasks for further characterization.

2.5. Screening of transgenic PK-15 cell clones

The genomic DNA was extracted from transgenic cell clones (passage 5) using TIANamp Genomic DNA Kit (Tiangen Biotech, China). The SB cassette-containing cell clones (PK15-IFNg^{SB}) were screened by PCR using the IR/DR-specific primer pair listed in Table 1. The cell clones with randomly integrated pTEG-IFNg-Fc vector (PK15-IFNg^{Ran}) were identified by PCR using the forward primer against ampicillin resistance (amp^{R}) gene and the reverse primer against SB right IR/DR (Table 1). The locations of PCR primers for identification of the two transgenic cell lines are shown in Fig. 1.

2.6. Detection of transgene expression in PK-15 cell clones

To detect the transgene transcription, PK15-IFNg^{Ran} and PK15-IFNg^{SB} cell clones (passage 5), together with PK-15^{Higher} control cells, were seeded (10^5 cells/mL) on 24-well plates and grown to complete confluence. The total RNA was extracted using MiniBEST Viral RNA/DNA Extraction Kit and the transcription of IFNg-Fc fusion gene was detected by RT-PCR using the IFNg-specific primer pair as described.

To detect the fusion protein expression, PK15-IFNg^{Ran} and PK15-IFNg^{SB} cell lines, together with PK-15^{Higher} control cells, were seeded in triplicates on 24-well plates and the culture media were collected at 24, 48 and 72 h post seeding. After brief centrifugation to remove cell debris, IFNg-Fc fusion protein concentrations (pg/mL) were determined using Porcine IFNg ELISA Kit (Yuanmu Biotech, China) by following the manufacturer's instruction.

To detect the antiviral activities of IFNg-Fc fusion protein, the culture media of PK15-IFNg^{Ran}, PK15-IFNg^{SB} and PK-15^{Higher} cells were collected at 72 h post seeding. The antiviral activities against vesicular stomatitis virus (VSV) and porcine pseudorabies virus (PRV) were detected using cytopathic effect (CPE) inhibition assay on MDBK or PK-15 cells as previously described (Walker et al., 2010; Zong et al., 2019). Briefly, 90% confluent MDBK and PK-15 cells were treated in triplicates with PK15-IFNg^{Ran}, PK15-IFNg^{SB} or PK-15^{Higher} culture medium diluted (1: 2 to 1: 512) in DMEM containing 2% FBS for 24 h at 37 ℃. Optimal concentration (100 $TCID_{50}$) of VSV or PRV was added into each well and the incubation was continued for additional 24 h. The cells were stained with 1% crystal violet in 15% ethanol and extracted with 70% ethanol and 1% acetic acid. The absorbance values at 580 nm were determined on ELISA reader and the antiviral activities (IU/mg protein) were calculated according to the standard curve generated using swine leukocyte IFNg (Sichuan Shihong Biotechnology, China).

2.7. Detection of genetic stability of transgenic PK-15 cell lines

Both PK15-IFNg^{Ran} and PK15-IFNg^{SB} cell lines were serially passed for 5, 10, 15, 20 and 25 generations, and the genomic DNA was extracted in 5-generation intervals using TIANamp Genomic DNA Extraction Kit. The quantitative PCR was performed in triplicates using IFNg gene-targeted primer pair (Table 1) and SYBR Premix Ex Taq™ II (TaKaRa) by following the manufacturer's instruction. The standard curve was generated using pMD18-T vector (TaKaRa) containing IFNg gene.

2.8. IFA analysis of transgenic PK-15 cell lines after PCV2 infection

Both PK15-IFNg^{Ran} and PK15-IFNg^{SB} cell lines (passage 5), together with the parental PK-15 and PK-15^{higher} control cells, were grown to 80% confluence on 24-well plates and infected with PCV2 ($10^{6.6}$ $TCID_{50}$/well) as described. At 24 hpi, the cell monolayers were fixed with 80% ice-cold acetone for 15 min at 4 ℃ and counterstained with 4′, 6-diamidino-2-phenylindole (DAPI). The stained cells were reacted first with home-made mouse anti-PCV2 antibody (1: 500) and then with FITC-labeled goat anti-mouse IgG (Sigma) (1: 1000) for 1 h at 37 ℃.

2.9. PCV2 titration on transgenic PK-15 cell lines

PCV2 titration was performed using previously described protocol (Li et al., 2015; Ma et al., 2017). Briefly, PK15-IFNg^{Ran} and PK15-IFNg^{SB} cell lines (passage 5), together with the parental PK-15 and PK-15^{higher} control cells, were grown to 80% confluence on 96-well plates and infected with 10-fold serially diluted PCV2 (8 duplicates for each dilution). The cells were washed with PBS, maintained in fresh medium containing 2% FBS for 72 h and examined for virus titers by IFA.

2.10. Quantitative PCR analysis of PCV2 propagation in transgenic PK-15 cell lines

To detect PCV2 dynamic propagation, PK15-IFNg^{Ran} and PK15-IFNg^{SB} cell lines, together with the parental PK-15 and PK-15^{Higher} control cells, were seeded on 24-well plates (10^5 cells/well), grown to 80% confluence and infected with PCV2 ($10^{6.6}$ $TCID_{50}$/well). At 24, 48 and 72 hpi, the total DNA was extracted using MiniBEST Viral RNA/DNA Extraction Kit and quantitative PCR was performed in triplicates using PCV2 *cap*-specific primer pair (Table 1) and SYBR Premix Ex Taq™ II as previously described (Cruz and Araujo, 2014). The standard curve was generated using pMD18-T vector containing 112-bp PCV2 Cap gene segment.

To determine PCV2 DNA copy numbers after inoculation at different MOI, PK15-IFNg^{Ran} and PK15-IFNg^{SB} cell lines were infected with PCV2 at MOI of 0.1, 0.5, 1.0, 5.0 or 10, and the total DNA was extracted at 48 and 72 hpi for quantitative PCR analysis as described.

2.11. Statistical analysis

Statistical analysis was performed using SPSS Statistics 22. The results were considered to be statistically significant at $p < 0.01$ or extremely significant at $p < 0.001$. For each separate set of date, at least three independent assays were performed, and the results were represented as mean ± standard deviation (SD).

3. Results

3.1. Vector construction

The expected 498-bp cDNA was amplified from the peripheral blood lymphocytes by RT-PCR using porcine IFNg gene-targeted primer pair. Sequence analysis showed that the IFNg cDNA was identical to previously described (Kirthika et al., 2017). The cDNA was cloned into transposon vector pTEG as a *Not*I/*Eco*RI segment. The coding sequence for pig IgG Fc domain was amplified from pShuttle-Fc vector and cloned into pTEG vector as an *Eco*RI/*Xba*I segment. Both integrity and orientation of IFNg-Fc fusion gene was confirmed by sequencing analysis. The schematic structure of recombinant vector pTEG-IFNg-Fc is shown in Fig. 1.

3.2. Parental PK-15 cell cloning

The parental PK-15 cells were cloned by two rounds of dilution cloning and the resultant cell clones were identified by IFA after PCV2 infection. Among 96 cell clones identified, 14.6%, 77.1% or 8.3% had higher (PK-15^{higher}), lower (PK-15^{lower}) or no permissivity to PCV2 infection. Quantitative PCR analysis showed that PCV2 DNA copy number in PK-15^{higher} cells was significantly ($p < 0.01$) higher than that in the parental PK-15 cells. In contrast, PCV2 DNA copy number in PK-15^{lower} cells was slightly ($p < 0.05$) lower than that in the parental PK-15 cells (Fig. 2).

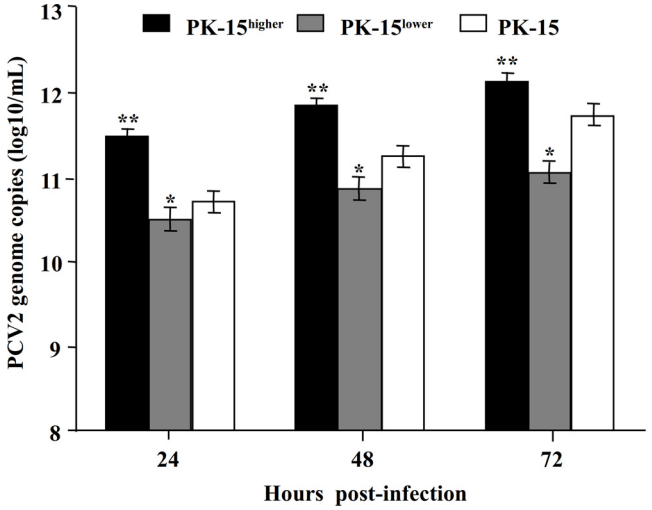

Fig. 2. Quantitative PCR analysis of PCV2 DNA copy numbers in different PK-15 cell clones. After two rounds of dilution cloning, the cell clones were infected with PCV2 and the cell clone with higher (PK-15^{higher}) or lower (PK-15^{lower}) PCV2 permissivity was screened by IFA. The two types of cell clones, together with the parental PK-15 cells, were infected with PCV2 and the total DNA was extracted at indicated times. Quantitative PCR was performed in triplicates using PCV2 *cap*-targeted primer pair. PCV2 DNA copy numbers in PK-15^{higher} and PK-15^{lower} cell clones were compared with that in the parental PK-15 cells. * $p < 0.05$, ** $p < 0.01$.

3.3. Generation of transgenic PK-15 cell lines

The PK-15^{higher} cells were co-transfected with transposon vector pTEG-IFNg-Fc and the tranposase vector pSB16. After two rounds of dilution cloning, the total DNA was extracted for cell clone screening by PCR. Among 24 cell clones screened, 58% contained SB cassette without antibiotic resistance marker and 42% contained intact pTEG-IFNg-Fc vector (Fig. 3A). The two types of cell clones were called PK15-IFNg^{SB} and PK15-IFNg^{Ran}, respectively.

Table 2
Antiviral activities of IFNg-Fc fusion protein expressed in transgenic PK-15 cell lines.

Culture medium	Antiviral activities (10^4 U/mL)		
	VSV/MDBK	VSV/PK15	PRV/PK15
PK15-IFNg^{Ran}	4.0 ± 0.0	3.5 ± 0.2	2.8 ± 1.1
PK15-IFNg^{SB}	3.8 ± 0.1	3.6 ± 0.3	3.1 ± 0.1

3.4. Characterization of transgenic PK-15 cell lines

The total RNA was extracted from PK15-IFNg^{SB}, PK15-IFNg^{Ran} or PK-15^{higher} cell lines and the transgene transcription was determined by RT-PCR. The expected 498-nt transcript was detected in PK15-IFNg^{SB} and PK15-IFNg^{Ran} cell lines, but not in PK-15^{higher} cells (Fig. 3B). The culture media of three PK-15 cell lines were collected at different time points and analyzed for IFNg-Fc fusion protein expression. Compared to that in PK-15^{higher} cell culture, a significant amount of IFNg-Fc fusion protein was detected in PK15-IFNg^{SB} (26.3 pg/mL) or PK15-IFNg^{Ran} culture medium (24.8 pg/mL) as early as 24 h after cultivation. The expression level of IFNg-Fc fusion protein increased slightly as the extension of cultivation time (Fig. 3C). The culture media of the two transgenic cell lines were harvested at 72 h post cultivation and analyzed for antiviral activity. On MDBK cells, a substantial anti-VSV activity was detected in PK15-IFNg^{SB} culture medium (3.8×10^4 IU/mL) or PK15-IFNg^{Ran} (4.0×10^4 IU/mL). On PK-15 cells, similar or slightly lower anti-VSV and anti-PRV activities were detected in the culture media of two transgenic PK-15 cell lines (Table 2). Finally, both PK15-IFNg^{Ran} and PK15-IFNg^{SB} cell lines were passed for 25 generations and the cellular DNA was analyzed by quantitative PCR. The transgene copy number in PK15-IFNg^{SB} cell line was almost 10-fold more than that in PK15-IFNg^{Ran} cell line, both of which increased slightly from passages 5 to 25 (Fig. 3D).

Taking together, we successfully established transgenic PK-15 cell lines stably expressing IFNg-Fc fusion gene.

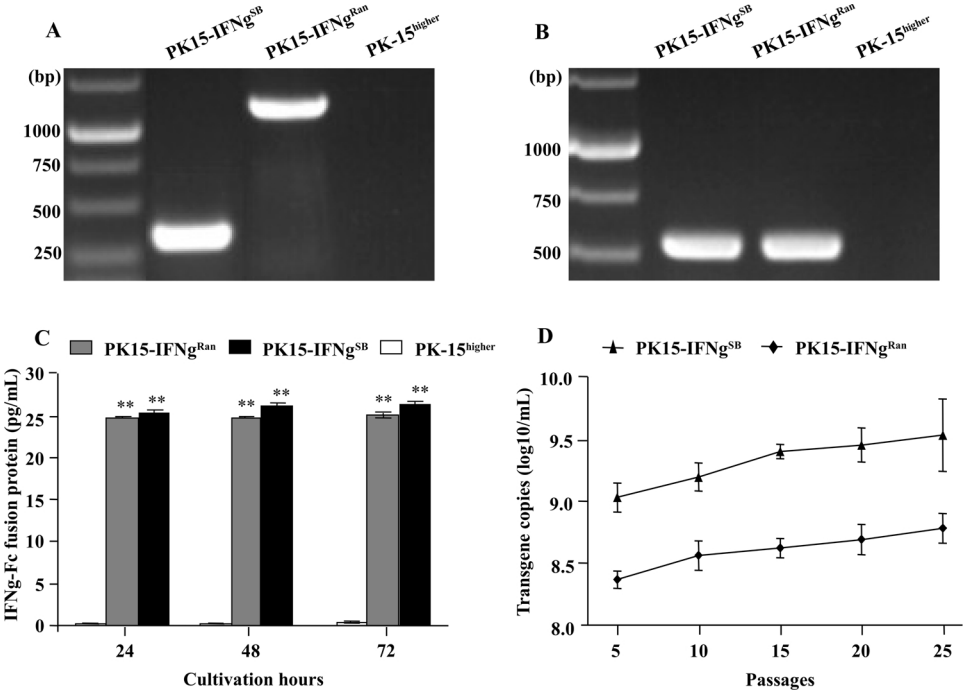

Fig. 3. Characterization of transgenic PK-15 cell lines. (A) Determination of transgene integration. The total DNA was extracted from the three PK-15 cell lines and analyzed by PCR. PK15-IFNg^{SB} cell line was identified using *SB* IR/DR-targeted primer pair. PK15-IFNg^{Ran} cell line was identified using ampR-targeted forward primer and *SB* right IR/DR-targeted reverse primer. (B) Analysis of transgene transcription. The total RNA was extracted from the three PK-15 cell lines at 48 h post cultivation and IFNg-Fc fusion gene transcription was detected by RT-PCR using porcine IFNg gene-targeted primer pair. (C) Analysis of fusion protein expression. The culture media of three PK-15 cell lines were collected at indicated times and IFNg-Fc fusion protein expression was detected using porcine IFNg ELISA Kit. Concentrations of IFNg-Fc fusion protein in the two transgenic cell lines were compared with that in PK-15^{higher} cells. ** $p < 0.01$. (D) Analysis of transgene stability. PK15-IFNg^{Ran} and PK15-IFNg^{SB} cell lines were serially passed for indicated passages and the transgene copy numbers were determined by quantitative PCR using porcine IFNg gene-targeted primer pair.

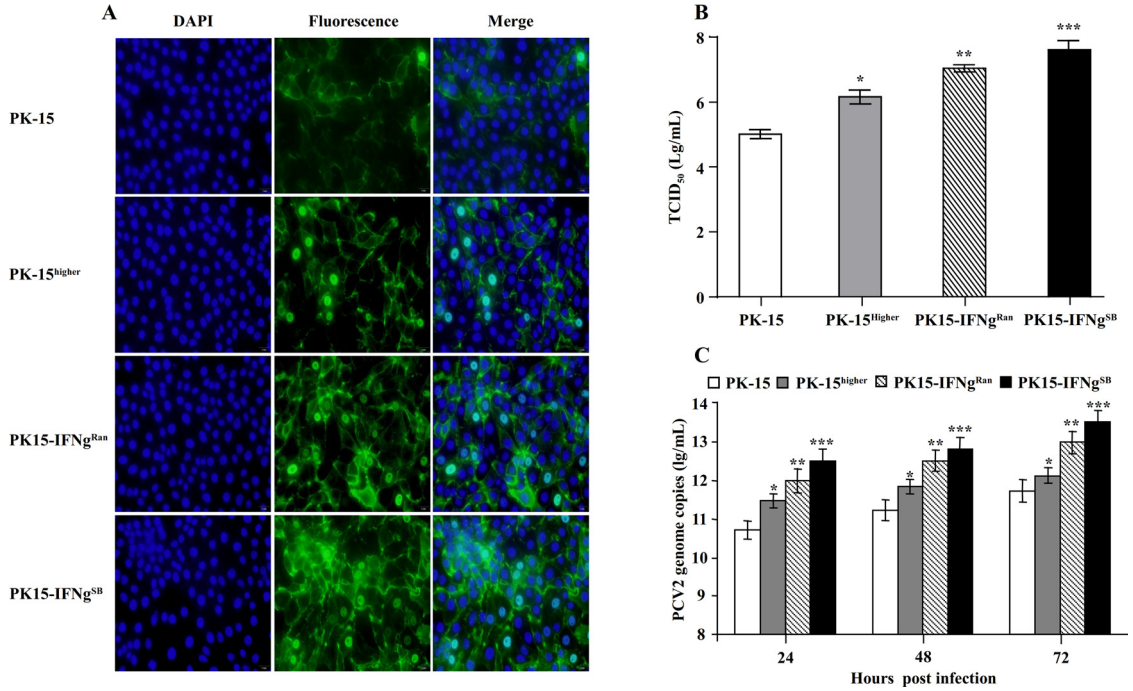

Fig. 4. Comparison of PCV2 permissivities of different PK-15 cell lines. (A) IFA analysis of different PK-15 cell lines after PCV2 infection. The four cell lines were infected with PCV2, counter-stained with DAPI and analyzed by IFA at 24 hpi. (B) Titration of PCV2 in different PK-15 cell lines. The four cell lines were infected with PCV2 and the viral titers ($TCID_{50}$) were titrated in triplicates on PK-15 cells by IFA at 72 hpi. The viral titers in PK-15^{higher}, PK15-$IFNg^{Ran}$ and PK15-$IFNg^{SB}$ cells were compared to that in the parental PK-15 cells. (C) Quantitative analysis of PCV2 proliferation in different PK-15 cell lines. The four cell lines were infected with PCV2 and the total DNA was extracted at 24, 48 and 72 hpi. The viral DNA copy numbers were determined in triplicates by quantitative PCR using PCV2 *cap*-targeted primer pair. PCV2 DNA copy numbers in the three cell lines were compared with that in the parental PK-15 cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.5. Comparison of PCV2 permissivities of different PK-15 cell lines

PK15-$IFNg^{SB}$ and PK15-$IFNg^{Ran}$ cell lines, as well as the parental PK-15 and PK-15^{higher} control cells, were infected with PCV2 and analyzed by IFA. The result showed that PK15-$IFNg^{SB}$ cell line had the highest PCV2-positive cell number, followed by PK15-$IFNg^{Ran}$, PK-15^{higher} and the parental PK-15 cell line (Fig. 4A). Virus titration assay showed that PCV2 titer in PK-15^{higher} (6.2 log), PK15-$IFNg^{Ran}$ (7.0 log) or PK15-$IFNg^{SB}$ (7.6 log) cell line was increased by 1.2 ($p < 0.05$), 2.0 ($p < 0.01$) or 2.6 log ($p < 0.001$) from that (5.0 log $TCID_{50}$) in the parental PK-15 cell line (Fig. 4B). Quantitative PCR analysis showed that PK15-$IFNg^{SB}$ cell line had significantly ($p < 0.001$) higher PCV2 DNA copy number, followed by PK15-$IFNg^{Ran}$ ($p < 0.01$), PK-15^{higher} ($p < 0.05$) cell lines as compared with that in the parental PK-15 cell line (Fig. 4C).

3.6. Determination of PCV2 DNA copy numbers after inoculation at different MOI

PK15-$IFNg^{SB}$ and PK15-$IFNg^{Ran}$ cell lines, together with the parental PK-15 and PK-15^{higher} control cells, were infected with PCV2 at different MOI and the total DNA was extracted for the viral genome detection. Quantitative PCR analysis showed that PCV2 DNA copy numbers in all of the four cell lines increased gradually in dose-dependent manner. Compared to that in the parental PK-15 cell line, the PCV2 DNA copy number increased slightly in PK-15^{higher} ($p < 0.5$) or significantly in PK15-$IFNg^{Ran}$ and PK15-$IFNg^{SB}$ ($p < 0.001$) cell lines from MOI of 0.5 (Fig. 5).

4. Discussion

The development of PCV2 vaccines requires efficient and reliable production of PCV2 in substantial quantities. Although PK-15 cell line is commonly used for the vaccine production, only about 20% of the cell population is susceptible to PCV2 infection (Meerts et al., 2005; Tischer et al., 1987; Zhu et al., 2007). This was confirmed by our PK-15 cell cloning study. Among 96 PK-15 cell clones screened, for example, only 14.6% showed higher permissivity to PCV2 infection, while 77.1% or 8.3% exhibited lower or no permissivity to the viral infection. Furthermore, our quantitative PCR analysis confirmed the presence of different PK-15 subpopulations with a significant difference in the viral genome copies after PCV2 infection. In this study, however, the PCV2 genome copy numbers detected in different PK-15 cell clones were much higher than the previously reported (Zhu et al., 2007). This may be explained by use of different PCV2 strain, PK-15 cell passage and/or quantitative PCR for the viral genome detection since similar PCV2 DNA copy number has also been reported (Ma et al., 2017).

PCV2 genome contains an ISRE-like sequence in the Rep promoter region (Misinzo et al., 2008; Ramamoorthy et al., 2009) and thus recombinant IFNs have been used to enhance PCV2 propagation in PK-15 cells (Meerts et al., 2005). However, recombinant IFNs are expensive with short half-lives due to their small molecular weights and quick enzymatic degradation (Zong et al., 2019). In this study, we evaluated the feasibility to generate PK-15 cell lines highly permissive to PCV2 infection by IFNg-Fc fusion gene transfer. By using the efficient SB transposon-mediated gene transfer, the antibiotic selection marker-free cell lines could be easily screened without time-consuming drug selection. After two rounds of dilution cloning, for example, up to 58% selection marker-free PK15-$IFNg^{SB}$ cell clones were identified by PCR analysis. The remaining PK15-$IFNg^{Ran}$ cell clones contained the intact pTEG-IFNg-Fc vector which was hopped into the cell genome by random gene integration in the absence of transposase vector.

Our quantitative PCR analysis showed that the copy number of IFNg-Fc fusion gene in PK15-$IFNg^{SB}$ cell line was almost 10-fold more than that in PK15-$IFNg^{Ran}$ cell line, which could be explained by more copies of transgene integrated into PK-15 cells after SB-mediated gene transfer. From passages 5–25, the transgene copy numbers in the two transgenic cell lines increased slightly, indicating their good genetic

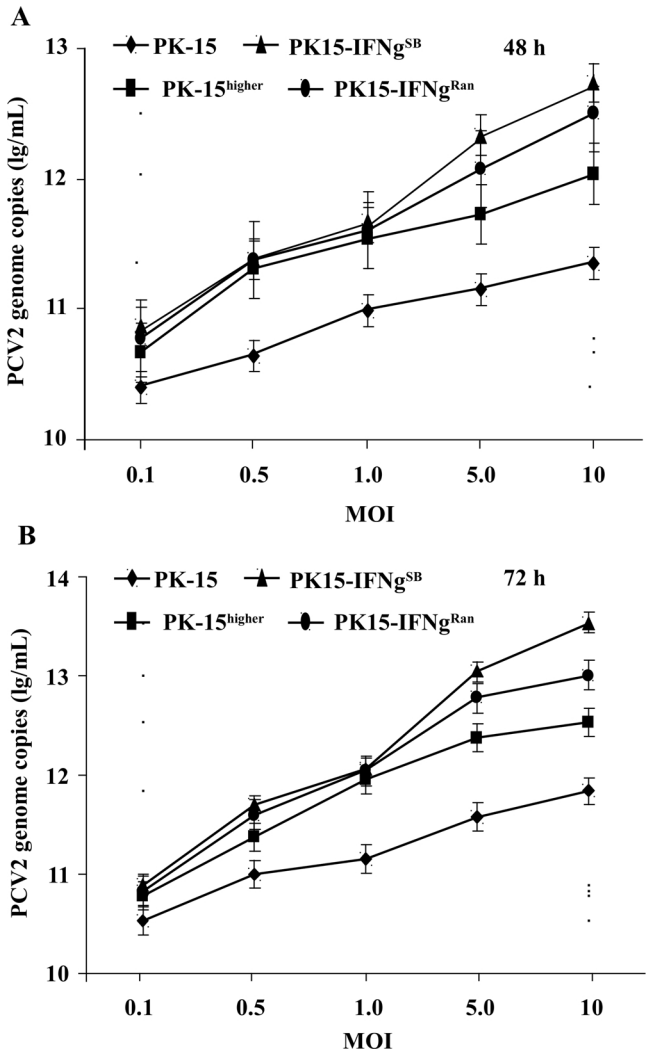

Fig. 5. Determination of PCV2 DNA copy numbers after inoculation at different MOI. The four PK-15 cell lines were infected with PCV2 at indicated MOI and the total DNA was extracted at 48 hpi (A) and 72 hpi (B). The viral DNA copy numbers were determined in triplicates by quantitative PCR using PCV2 *cap*-targeted primer pair.

stability. Moreover, the IFNg-Fc fusion gene was efficiently expressed in both PK15-IFNg^{SB} and PK15-IFNg^{Ran} cell lines, and the expressed fusion protein had potent anti-viral activities. These data suggest that the two transgenic PK-15 cell lines could be used as the permanent cells for recombinant IFNg production.

Both virus titration and quantitative PCR analyses showed that PK15-IFNg^{SB} and PK15-IFNg^{Ran} cell lines were highly permissive to PCV2 infection. After PCV2 infection, for example, the viral titer ($TCID_{50}$) in PK15-IFNg^{Ran} or PK15-IFNg^{SB} cell line was increased by 2.0 or 2.6 log10 from that in the parental PK-15 cell line. In addition, PCV2 genome copy numbers in the two transgenic PK-15 cell lines were also significantly increased in dose-dependent manner. Among the two transgenic PK-15 cell lines, PK15-IFNg^{SB} was more suitable for PCV2 vaccine production since its lack of antibiotic resistance gene.

In conclusion, we have generated PK15-IFNg^{Ran} and PK15-IFNg^{SB} cell lines by transposon-mediated IFNg-Fc fusion gene transfer. The selection marker-free PK15-IFNg^{SB} cell line is highly permissive to PCV2 infection and thus can be used for the vaccine production.

Ethics approval and consent to participate

All the experiments were carried out according to the recommendations in the Guide for the Care and Use of Laboratory Animals of the Yangzhou University. The protocol was approved by Medical Experimental Animal Center of Jiangsu Province (Permit Number: 2140880).

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